The Regulation of Bovine ATP citrate Lyase Promoter

By

Yuliana Yosaatmadja

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science in Biochemistry

Massey University

2006

Acknowledgements

First of all I would like to express my deepest gratitude to Associate Professor John Tweedie and Dr Kathryn Stowell for all your support, encouragement, enthusiasm and understanding. Thank you so much for giving me this wonderful opportunity to work as a part of an amazing lab. Both of you have been the best supervisors any one could have asked for.

Special thanks to Alice Clark, Natisha Magan and Claire Mawson for all the help in the world and the kindness that you have given me in the past few years. Thanks heaps for making my days in the lab fun and exciting, you guys are the best.

I would also like to thank everyone in the Twilight Zone you have been the best lab mates in the world; it has been a real privilege to work with such a kind, warm and talented group of people. With out all of you guys none of this would have been possible. Robyn Marston, Dr Amram Williams, Dr Mark Patchet, Dr Henning Kohen, Hilbert Grievink and Andy Hollings, thank you so much for all your kind words, advice and encouragement. To Carole Flyger, Kelly Senior, and Keisaku Sato thank you for all your help.

I would like to extend many thanks to Dad, Mum and Francisca. I am blessed to have such a wonderful and supportive family who have been there when I needed you most.

To my dance buddies, Nilangani Nadyeshda Pathirana and Hennya Puspawati, thank you so much for keeping me on my toes and for understanding what it feels like to be in my shoes. Many warm thanks to Endang Savitri who has kindly adopted me for the last few months. Tiara Parahita, Irma Wiryawan, Thomas and Maya Ariyawan, Dave and Carmen Wills, Trie and Erna Priantoro, Chandra Hartono, Yoana Hidayat, Tatiana Tumenggung, Calvine Sie and Logan Brown thank you for all of your support and kindness. Finally I would like to extend my gratitude to my best friend Dede for believing in me and for being there for me for the past 19 years.

Abstract

The synthesis of fatty acids is important for many house keeping functions such as the formation of cell membranes and as energy storage. This process occurs mostly in the adipose tissues and liver of monogastric animals.

The regulation of fatty acid biosynthesis in monogastric animals such as human and rat have been studied intensively. Several lines of experimental evidences have shown that fatty acid biosynthesis is dependent on the nutritional state of the animal and other hormonal influences, such as insulin and glucagon. However the molecular regulation of fatty acid biosynthesis is relatively unknown in ruminants.

Ruminants are large mammals that have a predominantly herbivorous diet and therefore have a very different metabolism to monogastric animals. Although a large percentage of ruminant feed is carbohydrate, very little of these dietary carbohydrates are available for *de novo* fatty acid biosynthesis and therefore many of the enzymes involved in the conversion of glucose to fat such as ATP citrate lyase may be down-regulated as a mean of physiological adaptation for glucose conservation.

ATP citrate lyase (ACLY) is a lipogenic enzyme that catalyses the cleavage of cytosolic citrate into acetyl CoA and oxaloacetate and it is unique to the fatty acid biosynthesis pathway. The molecular regulation of the bovine ACLY gene is unknown, however approximately 10 Kb of bovine ACLY gene has been sequenced and characterised. To investigate the molecular regulation of the bovine ATP citrate lyase gene, several experimental methods were used in this study such as reporter gene assays and electrophoretic mobility shift assays.

Abbreviations

A	Adenine
ACC	Acetyl CoA Carboxylase
ACLY	ATP citrate lyase
Amp	Ampicillin
ATP	Adenine triphosphate
BLAST	Basic local alignment search tool
bp	Base pair (DNA)
BSA	Bovine serum albumin
C	Cytosine
cAMP	Cylic Adenine monophosphate
ChREBP	Carbohydrate response element binding protein
CLUSTAL W	General purpose multiple sequence alignment program
Cpm	Count per minutes
CRE	cAMP response element
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
DTT	Dithiothrethiol
EDTA	Ethylene diamine tetra-acetic acid
EMSA	Electrophoretic mobility shift assay
FAD^-	Flavin adenine dinucleotide (oxidised)
FADH	Flavin adenine dinucleotide (reduced)
FAS	Fatty acid synthase
FCS	Foetal calf serum
G	Guanine
GRE	Glucocorticoid response element
HCL	Hydrochloric acid
HeLa	Human cervical carcinoma cells
HNF	Hepatocyte nuclear factor
IPTG	Isopropyl thiogalactosidase
Kb	Kilo base (DNA)

LB	Luria Bertani
L-PK	Liver pyruvate kinase
luc	Luciferase
MCS	Multiple cloning site
MFPK	Multifunctional protein kinase
MgCl	Magnesium chloride
mt	Mutant or mutated
MWT	Molecular Weight
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide (oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidised)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NFY	Nuclear factor Y
OAA	Oxaloacetate
ONPG	O-Nitrophenol β-D-galactosidase-pyranoside
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBSE	Phosphate buffered saline plus EDTA
PCR	Polymerase chain reaction
PEG	Polyethelyne glycol
pGL3B	pGL3 basic plasmid
PKA	Protein kinase A
PKB	Protein kinase B
RNA	Ribose nucleic acid
RNase	Ribonuclease
Rpm	Revolution per minute
RT	Reverse transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
SP1	Specificity protein 1
SP3	Specificity protein 3
SRE	Sterol regulatory element

SREBP	Sterol regulatory element binding protein
STET	Sucrose, tris base, EDTA and triton X buffer
T	Thymine
TAE	Tris base, Acetic acid and EDTA
Taq	*Thermus aquaticus* DNA polymerase enzyme
TBE	Tris borate EDTA buffer
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-Tetramethylethylenediamine
TEN	Tris-EDTA buffer with sodium
Tet	Tetracycline
TRE	Thyroid hormone receptor response element
UV	Ultra violet light
wt	Wild type
X-gal	5-bromo-4-chromo-3-indolyl-β-D-galactosidase
β Gal	β galactosidase

Table of Contents

List of Figures

List of Tables

1.0 Introduction

The molecular regulation of fatty acid biosynthesis in monogastric animals such as humans, rats and mice has been studied intensively for many years. However, the regulation of this process in ruminants such as in cattle is relatively unknown. Due to their large size and predominantly herbivorous diet, ruminants have a very different metabolism to monogastric animals (Allen *et al.*, 2005). The low glucose uptake from ruminant feed has a huge impact to the metabolism of glucose and fatty acid biosynthesis in these animals (Hanson and Ballard, 1967). It is speculated that these differences may forge a means to enable a different transcriptional regulation for particular genes that maybe involve in the fatty acid biosynthesis pathway in ruminant.

1.1 Fatty Acids

Fatty acids are a class of organic compounds that contains long hydrophobic chains of acetyl groups. They play many house keeping functions in an organism, such as energy storage, formation of cell membranes, lipidation of proteins, signal transduction (Khan & Heuvel, 2003) and gene expression (Pergorier, May, & Girard, 2004). Therefore fatty acids are essential for the general metabolism of many organisms. Two of the most important functions of fatty acids are as an energy source and their involvement in cell signalling and signal transduction.

1.1.1 Fatty acids as energy source

In many organisms, fatty acids are stored in the form of triaclyglycerol which is highly reduced and anhydrous. Fatty acids provide long term storage of energy for many animals, where the break down of a gram of triaclyglycerols will give a higher yield of ATP in comparison to a gram of glycogen which is a principal storage form of glucose (short term energy storage) in the muscle (Goodwin *et al.*, 1998). In mammals, triacylglycerols accumulate in the cytoplasm of adipose tissue and are degraded in the mitochondria (Eaton *et al.*, 1996).

Monogastric animals store excess carbohydrates in the form of fat. Fatty acids that are used for energy storage are synthesised mainly in the liver and in adipose tissues. There are two classes of adipose tissue; brown adipose tissues which are rich in

mitochondria; where fatty acids and triglyceride are oxidised to provide energy fuel for other tissues and white adipose tissue which are useful for storage of triglycerides which can also be used as energy source (Fliers *et al.*, 2003; Forest *et al.*, 2003).

1.1.2 Role of fatty acids in cell signalling and gene expression

The regulation of eukaryotic gene expression can occur at several different levels including transcription mediated by specific transcription factors. Transcription factors are proteins that help in recruiting RNA polymerase to a specific promoter region of a gene which in turn enables transcription initiation (Roeder, 1991). Recently several lines of experimental evidence have shown that there are many fatty acid activated nuclear receptors which are involved in the regulation of genes involved in fatty acid biosynthesis pathways. Nuclear receptors are a family of proteins that are usually regulated by the binding of ligands that are able to initiate a series of signal transduction cascade which eventually will lead to transcription activation of a particular gene (Horowitz *et al.*, 1996).

One such study has shown that lipophilic molecules induce a signal transduction pathway that can regulate gene expression in conjunction with nuclear receptors (Khan and Heuvel, 2003).

1.2 Fatty acid metabolism and biosynthesis

Fatty acids are degraded by sequential removal of two carbon units to generate acetyl CoA, NADH and $FADH_2$; these processes occur in the mitochondria by oxidation at the β-carbon within the fatty acid. These fatty acid molecules are then transported into the mitochondrial matrix, but not until they get activated in the cytosol (Sul and Wang, 1998). The long chain fatty acids are activated by the addition of coenzyme A, catalysed by Acyl CoA synthase. The activated fatty acid molecules are then transported across the inner mitochondrial membrane by conjugating them to carnitine molecules. The acyl carnitine compounds are then shuttled across the inner mitochondrial membrane by a translocase and the acyl group is transferred back to the CoA on the mitochondrial matrix. Fatty acids are synthesised and degraded by different pathways, meaning that both pathways are not reversible and are regulated by different enzymes catalysing different reactions.

The main precursor for fatty acid synthesis in the monogastric animal is glucose. In an animal kept in a well fed state, glucose is broken down into pyruvate via the

glycolytic pathway in the cytosol; pyruvate is converted into acetyl CoA and fed into the citric acid cycle in mitochondria forming citrate. The citrate is then transfered to the cytosol where the cytosolic citrate is cleaved by ATP Citrate Lyase (ACLY) to give acetyl CoA and oxaloacetate (OAA). The cytosolic acetyl CoA is used in fatty acid biosynthesis (refer to figure 1.1).

Both NADH and $FADH_2$ are produced from the citric acid cycle and eventually give rise to ATP by oxidative phosphorylation. Therefore in the liver of monogastric animals, one of the major functions of the glycolytic pathway is not just to provide pyruvate for ATP production, but also acetyl CoA for fatty acid biosynthesis (Sul and Wang, 1998).

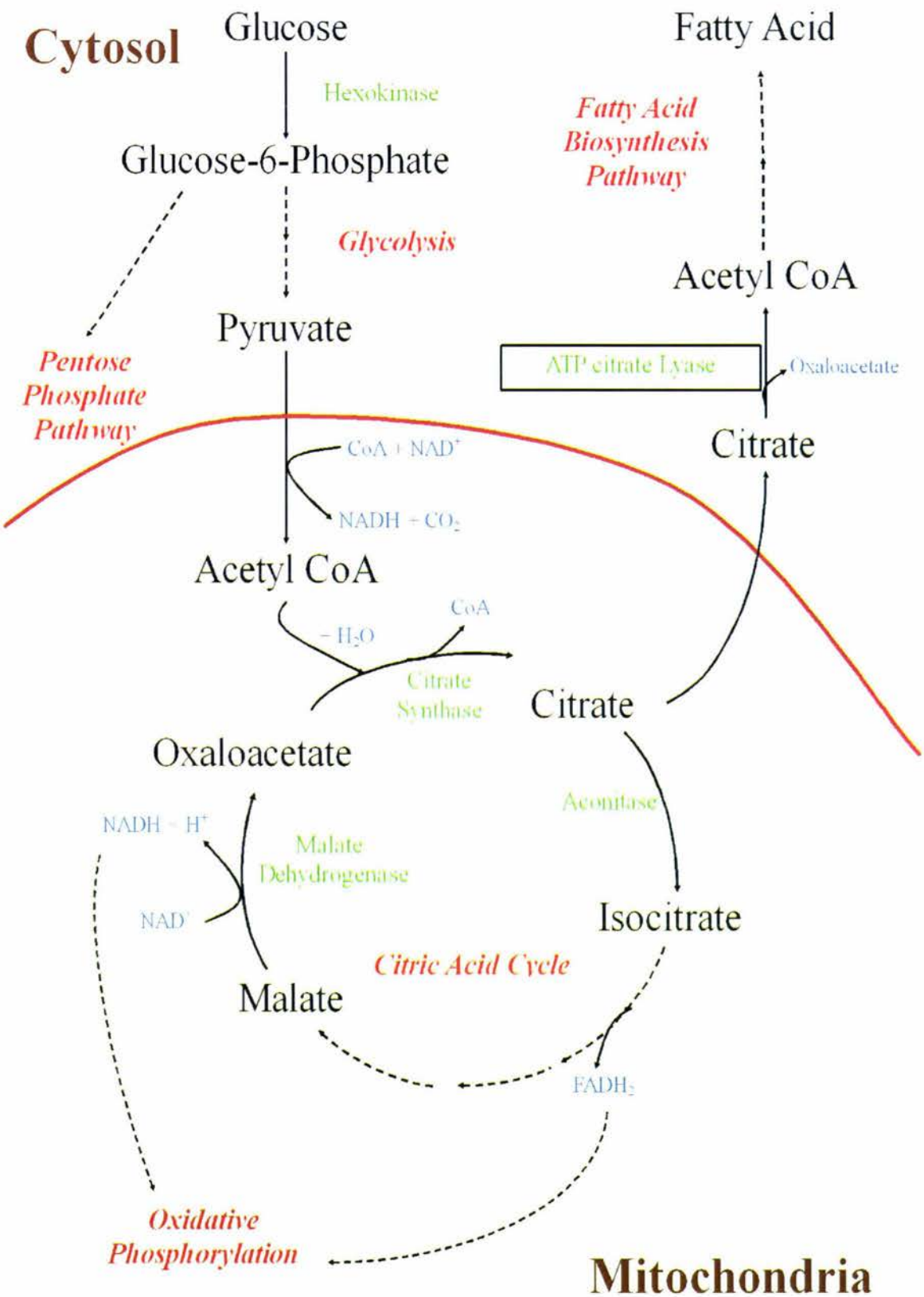


Figure 1.1: A general overview of glucose metabolism and Fatty acid biosynthesis in monogastric animal. In a high glucose concentration, glucose is converted into pyruvate in the cytosol through the glycolysis pathway. Pyruvate is then feed into the citric acid cycle (TCA cycle) in the form of acetyl CoA in the mitochondria. Citrate is produce as one of the intermediates in the TCA cycle and is transported across the membrane from the mitochondria into the cytosol. Citrate is then cleaved by the ATP citrate lyase ezyme to give acetyl CoA and oxaloacetate (Sul and Wang, 1998).

1.3 Nutritional regulation of fatty acid biosynthesis

The regulation of fatty acid biosynthesis is highly dependent on the nutritional state of the animal and is mediated by other hormonal factors such as insulin and glucagon. The relative amount of fat and carbohydrate in the diet appear to regulate the rate of fatty acid synthesis. When an animal is fed a low fat or high carbohydrate diet, the rate of fatty acid synthesis increases, conversely when an animal is fed with a diet that is low in carbohydrates or high in fat content, the rate of fatty acid synthesis is reduced (Towle *et al.*, 1997).

The precise molecular mechanisms mediating the influence of dietary carbohydrate and fat on fatty acid biosynthesis is unknown, however it is speculated that each of the nutritional components plays a different role in regulating the expression of genes involved in adipogenesis. For example, the intake of glucose up-regulates the expression of lipogenic enzymes (enzymes involved in fatty acid biosynthesis in the liver), in adipocytes and also in pancreatic β cells in monogastric animals (Girard *et al.*, 1997; Towle *et al.*, 1997). Some of these lipogenic enzymes are acetyl CoA carboxylase (ACC), fatty acid synthase (FAS) and ACLY. Significantly, all three of these enzymes are induced by high levels of carbohydrate or glucose in the diet, leading to fatty acid biosynthesis (Towle *et al.*, 1997).

Previous data have shown that glucose is the key to lipogenic gene regulation. There are three lines of evidence that support this statement: (1) ACC mRNA and protein in pancreatic β cells has been shown to increase over a period of time after exposure to high concentrations of glucose (Brun *et al.*, 1993); (2) FAS mRNA levels increase in adipose tissue of rats after a high carbohydrate diet (Foufelle *et al.*, 1992). This increase is eliminated in the presence of actinomycin D, an inhibitor of RNA synthesis. These results support the hypothesis that glucose dose dependent increase in FAS mRNA is due to transcriptional activation. And finally (3) in the absence of glucose, insulin is unable to up-regulate the expression of FAS and ACC in adipose tissue, indicating that glucose metabolism is required for this process (Foufelle *et al.*, 1994).

1.4 Transcriptional regulation of fatty acid biosynthesis

Fatty acid biosynthesis involves many enzymes whose genes are regulated at the transcriptional level, through transcription factors binding to the respective promoter regions.

To further understand the mechanism of fatty acid biosynthesis, it is essential to appreciate the importance of transcription factors that are responsible for the regulation of genes that are involved in the fatty acid biosynthetic pathway.

1.4.1 Sterol regulatory element-binding proteins

Sterol regulatory element-binding proteins, also known as SREBPs are a family of transcription factors that are involved in the regulation of fatty acid biosynthesis (Figure 1.2). The promoter region in the genes that are regulated by SREBPs contain a specific sequence of 5'-TCAGGCTAG-3' which is the cognate DNA sequence for SREBP (Sato *et al.*, 2000). These proteins belong to the basic-helix-loop-helix-leucine zipper (bHLH-Zip) family of transcription factors. There are three isoforms of SREBPs; SREBP-1a, SREBP-1c and SREBP-2. SREBP-1a and SREBP-1c are derived from a single gene through an alternative transcription start site, whilst the SREBP-2 gene is located on a different chromosome (Brown and Goldstein, 1997). SREBP-1c is ten fold more abundant in most animal tissues than SREBP-1a and approximately twice as abundant as SREPB-2 (Shimomura *et al.*, 1997). The differences in the expression and localisation of each of these SREBPs suggest that these proteins contribute to different regulatory functions.

SREBP-1a is thought to be responsible for maintaining the basal levels of cholesterol and fatty acid synthesis *in vivo*, due to the fact that it is constitutively expressed at very low levels in most animal tissues (Horton, 2002). SREBP-1c appears to preferentially regulate the expression of lipogenic genes. This was shown in Horton *et al.*, 2002, using transgenic mice that over-expressed SREBP-1c, showing that there was a six-fold increase in hepatic fatty acid synthesis in conjunction with minimal changes in cholesterol biosynthesis. The expression of SREBP-1c in the liver has been shown to be positively regulated by insulin (Kim *et al.*, 2004). Insulin causes an increase in the precursor form of SREBP-1c and was thought to aid in the proteolytic

cleavage of these SREBP precursors which in turn activates them into the fully functional and active SREBP (Ferre *et al.*, 2001).

SREBP-2 preferentially regulates the expression of genes that are involved in cholesterol biosynthesis pathways. Therefore SREBPs are thought to mediate the expression of insulin-dependent lipogenic genes (Horton, 2002; Kim *et al.*, 2004; Nadeau *et al.*, 2004).

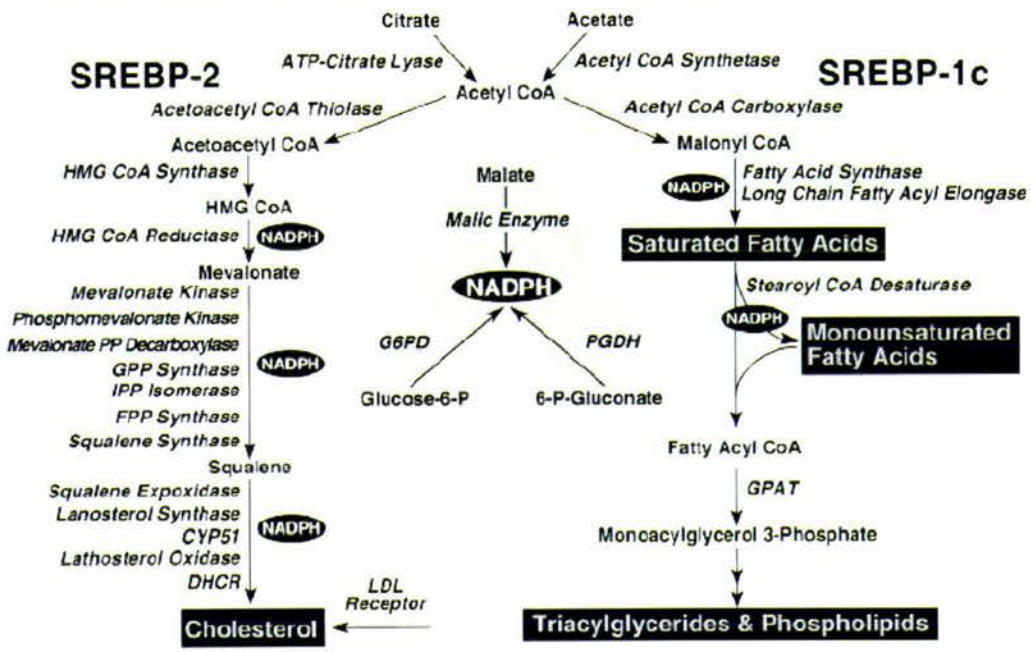


Figure 1.2: Roles of SREBP in fatty acid metabolism and biosynthesis.
Describes the roles of SREBP in the cholesterol, triacylglycerides and fatty acids biosynthesis pathways (Hornton *et al.*, 2002).

Apart from its role in the direct regulation of gene encodings the enzymes involved in fatty acid synthesis, SREBPs also regulate the gene encoding enzymes that are responsible for the production of NADPH (a major electron donor) which is a crucial co-factor in fatty acid synthesis (Horton, 2002).

1.4.2 Carbohydrate response element binding protein (ChREBP)

Certain *cis*-acting DNA sequences are thought to be involved in the glucose responsiveness of genes for many lipogenic enzymes. These DNA sequences are

often referred to E boxes. E boxes sequence are sequence that consist of two 5'-CACGTG-3' motif separarted by a few base pair (Stoeckman *et al.*, 2004). E boxes or E box like sequence were found to be responsive to carbohydrate. These elements were first discovered in the gene encoding the glycolytic enzyme liver pyruvate kinase (L-PK). This sequence elements are usually conserved throughout different genes such as; fatty acid synthase that catalyses the conversion of acetyl CoA to fatty acids (Towle *et al.*, 1997), the S14 or Spot 14 gene that are expressed specifically in tissues that produces lipids such as white and brown adipose tissues and liver (Moncur *et al.*, 1998), and ATP citrate lyase that catalyses the cleavage of citrate into acetyl CoA and oxaloacetate in the cytosol (Kim *et al.*, 1996) (Figure 1.3). The presence of the complete E box sequence elements are important for the binding of several transcription factors that are regulated by the presence of high glucose concentration (Towle *et al.*, 1997).

FAS	GGCCGCTGTCACGTGGGCGCC
S14	TCTGTTTGCCAGTTCTCACGTGGTGGCCAC
ACLY	TCTGCTTTCCT.TTCACACGTG.GCGCCTC

Figure 1.3: Comparison of E boxes sequence elements from Fatty acid synthase S14 and ATP Citrate Lyase.
The aligment of E box sequence from fatty acid synthase (FAS), S14 gene that encodes for a spot 14 proteins, and the ATP citrate lyase gene (ACLY). The underlined region shows the core sequence of E boxes. These sequences are shown to be perfectly aligned and that they contain the complete E box sequence (Kim *et al.*, 1996).

One of the transcription factors that has been found to bind to E boxes or E box like sequence is the carbohydrate response element binding protein (ChREBP/ChoREBP). ChREBP is a transcription factor that is activated by high glucose concentrations in the liver and is known to bind to carbohydrate response elements within the L-PK promoter region. ChREBP is a liver specific member of the basic-helix-loop-helix-leucine zipper (bHLH-Zip) transcription factor family (Yamashita *et al.*, 2001). ChREBP is constitutively expressed in the liver and is phosphorylated under conditions when glucose concentrations are low. ChREBP contain three protein kinase A (PKA) phosphorylation sites including one located in the putative DNA binding domain of the protein. When the blood glucose is low,

glucagon stimulates the production of cAMP which in turn activates PKA phosphorylating ChREBP. Upon phosphorylation ChREBP is no longer bound to DNA and is rendered inactive. In response to an increase in glucose concentration, ChREBP is dephosphorylated which allows it to be transported to the nucleus and to bind to its specific DNA binding sequence (Kawaguchi *et al.*, 2001).

ChREBP was found to be an inefficient DNA binding protein in the absence of Mlx (Max like protein X). Mlx is a bHLH/LZ protein in the liver that interact with the bHLH/LZ domain in the ChREBP (Stoeckman *et al.*, 2004). Not only that the binding of the ChREBP/Mlx heterometic complexes to the E box are able to activate transcription of lipogenic enzymes and they are also able to distinguish the E boxes that are responsive to glucose and those that are not. Because when an experiment was carried out by Stoekman *et al.* (2004) using single E box element, they found that the ChREBP/Mlx could bind to the DNA sequence, however instead of acting as a transcription activator, the ChREBP/Mlx complex was acting as a transcription repressor.

1.4.3 Other transcription factors: Stimulatory proteins (SP) and nuclear factor Y (NF-Y)

The stimulatory protein family (Sp) of transcription factors were first identified by their ability to bind to GC-rich regions of promoters (Suske, 1999). All four human (Sp1, Sp2, Sp3 and Sp4) Sp-proteins have similar features which contain three zinc fingers close to the C-terminus and glutamine-rich domains adjacent to serine/threonine stretches within their N-terminal region. Functionally the Sp-proteins are closely related however, some have the ability to up regulate transcription whilst other can down regulate. The distribution of each of the Sp proteins is different to each other. Sp1 and Sp3 proteins are the most abundant and found in many different tissue (Hagen *et al.*, 1994; Saffer *et al.*, 1991), Sp2 proteins are found in various cell line whilst Sp4 is generally expressed in tissue specific manner, found predominantly in neuronal cells and in certain epithelial cells (Suske, 1999)

Nuclear factor Y (NF-Y) is a transcription factor which is known to bind to a CCAAT sequence commonly found within the promoter of a gene (Mantovani, 1998). The CCAAT sequence is present in many housekeeping genes, including those that are involved in cholesterol metabolism (Dooley *et al.*, 1998), and fatty acid biosynthesis such as ATP citrate lyase (Elshourbagy *et al.*, 1990; Kim *et al.*, 1994). Most CCAAT boxes are extremely conserved in terms of position, orientation and sequence within the same gene of the different species (Mantovani, 1998).

1.5 Fatty acid biosynthesis in ruminants

Ruminants are large mammals that have a very different metabolic role for fatty acid biosynthesis in comparison to monogastric animals. Although a large percentage of ruminant feed is actually carbohydrate, almost all of the ingested carbohydrates are converted into volatile fatty acids in the rumen, and only a small amount of glucose from the feed is available to the animal. Consequently very little dietary carbohydrate is available for *de novo* fatty acid biosynthesis (Houtert, 1993). The metabolic profile of the adult ruminant is one of quite severe glucose limitation, with gluconeogensis from the volatile fatty acid propionate being the major source of glucose (Walt and Linington, 1989). This places severe restrictions on the adult ruminant which responds by restricting the use of glucose to only those roles for which no other metabolite can serve. One of these glucose-sparing mechanisms is the restriction of *de novo* synthesis of fatty acids from glucose. Initially, this restriction on *de novo* fatty acid synthesis from glucose was thought due to lack of ATP citrate lyase enzyme activity (Walt and Linington, 1989), but several lines of experimental evidence to suggests that the absence of ATP citrate lyase activity is actually part of ruminants physiological adaptation for glucose conservation since only a small amount of glucose is absorbed by the rumen wall. Most of the precursors for triacylglycerol synthesis come from volatile fatty acid (acetate) produced in the rumen fermentation of dietary carbohydrate (Houtert, 1993), where they are absorbed from the rumen in the form of free acids.

Once the volatile fatty acids have been absorbed, they can be used by the animal. Acetate is converted into acetyl CoA, which is the precursor for fatty acids and ketone bodies (refer to figure 4) (Walt and Linington, 1989). Butyrate is partially

metabolised in the epithelium of the rumen and the omassum to become ketone bodies acetoacetate and β-hydroxybutyrate (Houtert, 1993). Propionate, being the only glucogenic is a major source of gluconeogensis precursor. (Walt and Linington, 1989).

About 90% of the adipose tissue fatty acids are synthesised through a *de novo* fatty acid biosynthesis pathway in ruminant tissue. Rather than using glucose for generating the essential NADPH for fatty acids synthesis through the pentose phosphate pathway, NADPH is generated through NADP-isocitrate dehydrogenase (Crabtree *et al.*, 1981) Whilst in non ruminants the liver plays a major role in *de novo* fatty acid biosynthesis. The liver of ruminants does not play a major role in this process. The liver however, does provide an environment for gluconeogenesis and the liver has adapted to generate glucose efficiently which is a great advantage to the animal (Young, 1977).

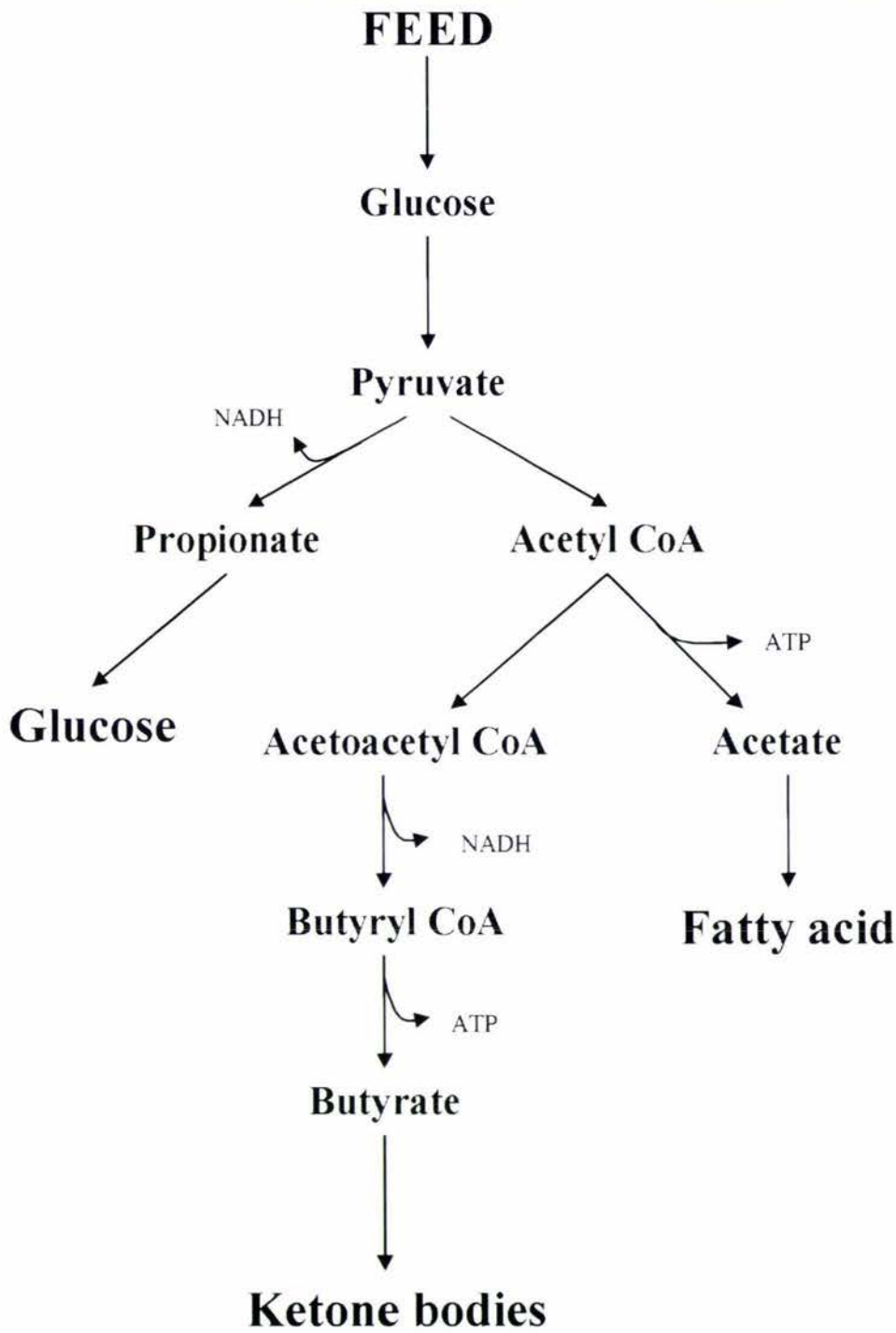


Figure 1.4: The different fates of volatile fatty acids absorbed in the rumen wall.
The volatile fatty acids are the by-products of fermentation in the rumen. These volatile fatty acids are easily absorbed by the rumen wall and are used as precursors to generate glucose, fatty acids or ketone bodies which are all different energy source for the ruminant (Walt and Linington, 1989).

1.5.1 ATP Citrate Lyase in Ruminants

The rumen is the largest and the most important compartment of the ruminant digestive tract in terms of metabolism. Carbohydrates from feed are fermented here and converted into volatile fatty acids. These volatile fatty acids are readily absorbed from the rumen, but the amount of glucose that escapes fermentation and is available to the animal is insufficient for its requirements (Walt and Linington, 1989). Glucose is required as an energy source for the brain and the erythrocytes. These tissues prefer glucose to any other energy source, therefore the role of gluconeogenesis to provide glucose in the liver is essential for ruminants. Gluconeogenesis is required to provide glucose as fuel for the brain and the erythrocytes. In addition, glucose is required in many tissues as a substrate for the pentose phosphate pathway, a major source of NADPH for biosynthetic reactions. Ruminants rely heavily on gluconeogenesis from propionate to provide approximately 90% of the glucose requirements. The rate of gluconeogenesis is elevated in ruminant after feeding (Young, 1977)

Because of insufficient glucose uptake from their feed, ruminants have evolved mechanisms for glucose conservation by down-regulate the conversion of glucose to fatty acids and by using other precursors for fatty acids biosynthesis. These mechanisms might involve the down-regulation of the lipogenic enzymes that play a role in the conversion of glucose into fatty acids. One of these enzymes is ATP citrate lyase.

Normally the level of ATP citrate lyase is relatively low in the adipose tissue of adult ruminants when compared to other mammals such as rats and humans. However acetyl CoA synthetase, the enzyme required for acetyl CoA production from acetate is present at high levels in the cytosol of ruminant adipocytes. The elevated level of acetyl CoA synthase is seen as an advantage for the ruminant, because it bypasses the need for citrate to transport acetyl CoA across the mitochondrial membrane and provides a precursor other than glucose (Hanson and Ballard, 1967).

1.6 ATP Citrate Lyase

ATP citrate lyase is a lipogenic enzyme and catalyses the only reaction in the pathway from glucose to fatty acids which is unique to this pathway. Like many other enzymes involved in fatty acid biosynthesis it is regulated by the nutritional state of the animal (Sul and Wang, 1998). In non ruminants, ATP citrate lyase is abundant in lipogenic tissues such as the liver and adipose tissue. ATP citrate lyase is up-regulated on high carbohydrate diet and down-regulated by diets low in carbohydrate. It is a cytosolic enzyme that catalyses the cleavage of citrate into acetyl CoA and oxaloacetate coupled to the hydrolysis of ATP to ADP. Cytosolic acetyl CoA is important for several biosynthetic pathways including lipogenesis and cholesterol biosynthesis (Towle *et al.*, 1997).

The gene for ATP citrate lyase (ACLY) has been cloned, characterised and sequenced. It is located on chromosome 17 in humans (Park *et al.*, 1997), chromosome 11 in mouse (Remmers *et al.*, 1992), and chromosome 15 in rats (Moon *et al.*, 1996).As suggested by ACLY is regulated by glucose, the gene contains elements that are commonly seen within other glucose regulated genes, elements such as multiple Sp1 and Sp3 binding sites, CCAAT boxes, SREs, and E-boxes. Furthermore this gene appears to lack a regular TATA box in its promoter sequence (Elshourbagy *et al.*, 1990; Tong, 2000).

1.6.1 Rat ATP Citrate Lyase gene

The cloned sequence of rat ACLY gene has been shown to extend over 55 kb, containing a total of 29 exons. The translation start site (ATG) was mapped within exon 2 and the termination codon TAA was mapped within exon 29 which also contains the 3' untranslated region and poly (A) signal. In addition exon 14 was found to be the target of alternative splicing, and interestingly it is the shortest exon, only 30 nucleotides in length (Moon *et al.*, 1996).

No TATA box was found within rat ACLY promoter sequence, but it contains multiple binding sites for Sp1 and Sp3, an E box and insulin response elements (figure 1.5). The presence of these elements supports the speculation that transcriptional activation of ACLY gene is regulated by the nutritional state of the

animal (Fukuda and Iritani, 1999; Fukuda *et al.*, 1996; Moon *et al.*, 1996; Moon *et al.*, 2000; Moon *et al.*, 2002a).

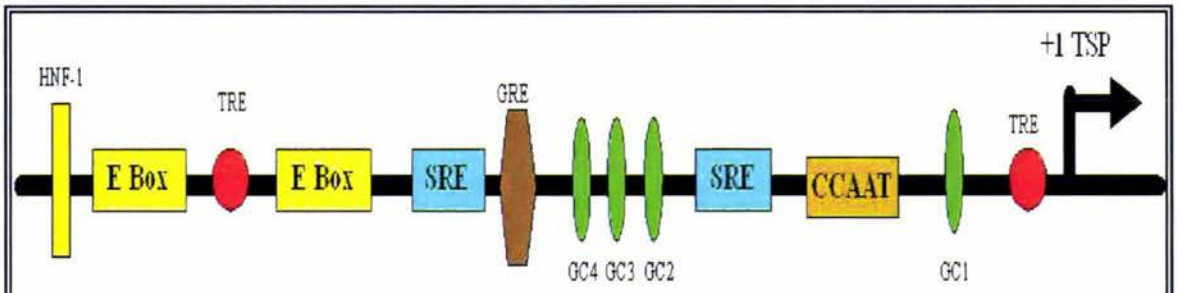


Figure 1.5: Rat ATP citrate lyase gene promoter.
A schematic diagram of rat ACLY gene promoter region. The important transcriptional elements are shown (Thyroid hormone receptor response element (TRE), the inverted CCAAT box, the sterol regulatory elements (SRE) the E box, the glucocorticoid response element (GRE) and the GC boxes). The black arrow is indicating the transcriptional start site (Kim *et al.*, 1994; Moon *et al.*, 2002).

1.6.2 Human ATP Citrate Lyase gene

The human ACLY gene was mapped to chromosome 17q21.1. The 5' flanking region contains binding sites for hepatocyte nuclear factor 1 (HNF1), E boxes, a glucocorticoid response element, sterol regulatory elements and GC boxes (Figure 1.6). The promoter is TATA less, however it contains a CCAAT box which may be important for the binding of a transcription initiator protein (Park *et al.*, 1997). The presence of the CCAAT box is also thought to be important for the binding of SREBP to initiate transcription of the human ACLY gene (Moon *et al.*, 1996).

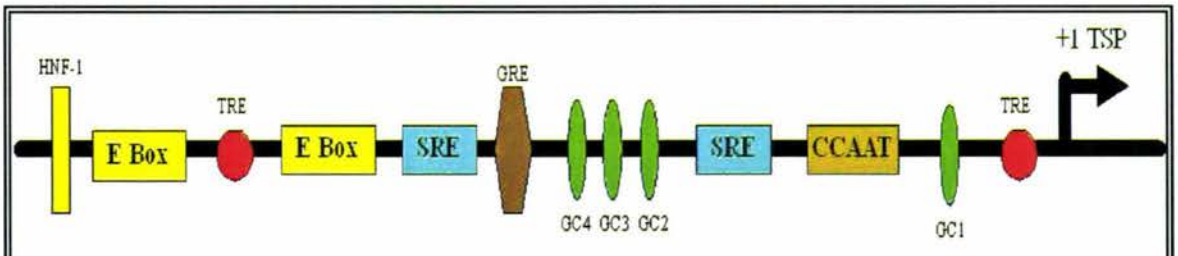


Figure 1.6: Human ATP citrate lyase gene promoter.
A schematic diagram of human's ACLY gene promoter region. The important transcriptional elements are shown (Thyroid hormone receptor response element (TRE), the inverted CCAAT box, the sterol regulatory elements (SRE) the E box, the glucocorticoid response element (GRE) and the GC boxes). The black arrow is indicating the transcriptional start site (Park *et al.*, 1997).

1.6.3 Bovine ATP Citrate Lyase gene

Aproximately 10 kb of the bovine ACLY gene was isolated by a student at Massey University (Tong, 2000). The region includes exon 1, exon 2 and exon 3, in addition to the intervening introns and about 4 Kb of the sequence upstream of the putative transcription start site. The transcription start site was determined using 5' RACE and the promoter contains six GC boxes, two SREs, two CCAAT boxes, a thyroid hormone binding site and an E box. The bovine gene also appears to lack a TATA box (Figure 1.7). The transcription start site was predicted to be in exon 1, but the translation start site was predicted to be in exon 2 (Tong, 2000).

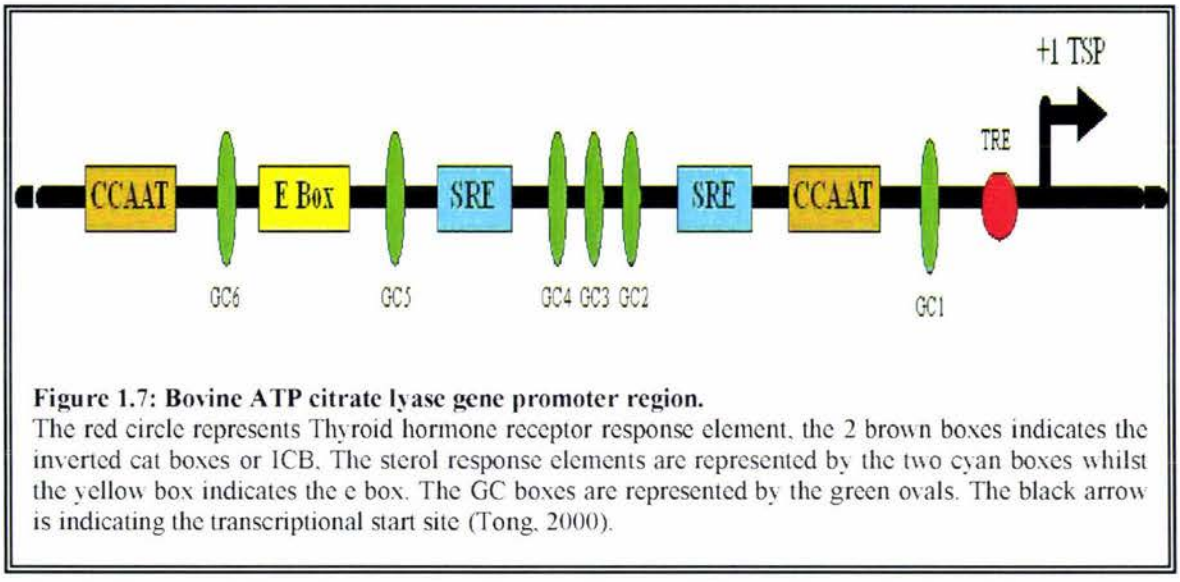


Figure 1.7: Bovine ATP citrate lyase gene promoter region.
The red circle represents Thyroid hormone receptor response element, the 2 brown boxes indicates the inverted cat boxes or ICB. The sterol response elements are represented by the two cyan boxes whilst the yellow box indicates the e box. The GC boxes are represented by the green ovals. The black arrow is indicating the transcriptional start site (Tong, 2000).

1.6.4 The regulation of ATP citrate lyase gene expression

Like many other genes, ATP citrate lyase is regulated at both the transcriptional post transcriptional level.

1.6.4.1 Transcriptional regulation of ATP citrate lyase

The presence of multiple GC box sequence elements within the 5' regulatory region of the ACLY promoter sequence suggests that Sp1 is one of the most important transcription factors in the transcriptional regulation of this gene (Fukuda *et al.*, 1999; Moon *et al.*, 2000). Sp1 is a transcription factor that binds to GC boxes and plays a role in recruiting TATA binding protein in the transcription activation complex (Suske, 1999).

Previous work has shown that Sp1 protein can act as a strong transcriptional activator through binding to promoters with multiple CG boxes. Sp1 is known to be phosphorylated reducing DNA binding ability (Armstrong *et al.*, 1997) and is glycosylated under low glucose concentrations in the cells. The glycosylated Sp1 was found to be more susceptible to proteolytic degradation (Han and Kudlow, 1997). Sp1 is also capable of forming homotypic and heterotypic multimeric complexes (Suske, 1999). Some of the proteins that are capable of binding to Sp1 are: TATA-box binding protein (TBP), TBP-Associated factors, and some cell cycle regulatory proteins (Suske, 1999). This may be important in the transcription regulation of ACLY, as the binding of these proteins helps in recruiting RNA polymerase (Roeder, 1991).

There are three isoforms of the Sp3 protein; one 110-115 kDa in size, and two other the 60-70 kDa proteins. Sp3 is a bifunctional transcriptional regulator; it can either activate or repress gene expression by binding to its sequence within a promoter. It has been shown that Sp3 can be a transcriptional activator when it is acetylated (Ammanamanchi *et al.*, 2003) but others have shown that Sp3 can act as a repressor (Lania *et al.*, 1997; Suske, 1999). It also have been suggested that the full length Sp3 protein (110-115 kDa) does act as an activator whilst the two smaller Sp3 proteins can act as repressors (Kennett *et al.*, 1997)

Fukuda *et al.* (1999) examined the binding of Sp1 and Sp3 to the rat ACLY 5' regulatory region. It was found that the two proteins bind to the GC elements upstream of the ACLY promoter region. Using immunodetection , they showed that the band intensity of these two proteins are similar, suggesting that they are present at similar levels. They also found that the activities of the two proteins are different, because Sp1 was shown to down regulate the expression of fatty acid synthase (FAS), ATP citrate lyase (ACLY) and acetyl CoA carboxylase (ACC); whilst Sp3 was shown to up regulate FAS and ACC. The effect of Sp3 on ACLY gene expression is still unclear. They also suggested that the binding of Sp1 and Sp3 proteins to the promoter region of ACLY gene is dependent to the nutritional state of the animal (Fukuda *et al.*, 1999).

SREBP is thought to be one of the most important transcription factors for lipogenic genes. SREBP has been shown to bind and activate ACLY gene in studies using transgenic mice and reporter gene assays. When SREBP-1a is over expressed in the transactivation using ACLY promoter constructs, the ACLY promoter is highly stimulated (Moon *et al.*, 2000). The binding of Nuclear Factor Y (NF-Y) appears to be important in the regulation of ACLY by SREBP, because mutation of NF-Y binding sites reduces the transactivation by SREBP-1.

1.6.4.2 Post translational regulation of ATP Citrate Lyase

ATP citrate lyase is a homotetrameric cytosolic enzyme (Ramakrishna and Benjamin, 1979). Like many other proteins, it undergoes phosphorylation at specific serine and threonine residues. ATP citrate lyase was found to be phosphorylated in two distinct peptides designated peptide A and B (Ramakrishna *et al.*, 1989). When the experiments were carried out *in vitro*, the phosphorylation of ATP citrate lyase was shown to occur on peptide A by cAMP-dependent protein kinase and on peptide B by Multifunctional protein kinase (MFPK) (Ramakrishna *et al.*, 1990).

The hormone insulin is not only involved in the transcriptional regulation of ACLY gene, but it also appears to play an important role in the phosphorylation of ATP citrate lyase protein. Insulin appears to stimulate the phosphorylation of ATP citrate lyase in peptide A which is mediated by protein kinase B (PKB) (Berwick *et al.*, 2002).

The phosphorylation of ATP-CL does not appear to have any effect on ATP citrate lyase enzyme activities (Ramakrishna *et al.*, 1989). However it cannot be ruled out that the phosphorylation of ATP citrate lyase might be important in other aspects of post-trasncriptional regulation such as enzyme stability, subcellular localization or for regulation involving protein-protein interactions (Berwick *et al.*, 2002).

1.7 Aims of this research

The human and rat ACLY gene have been studied extensively over the years, however the bovine ACLY gene has only been recently cloned and characterised. There are significant differences in the metabolism of glucose between cattle and other mammals. These differences would be an interesting topic to study, especially in relation to the gene regulation of ACLY. As mentioned previously, ATP citrate lyase is the only enzyme that is unique in the *de novo* fatty acid synthesis pathway, and that the down-regulation of the ATP citrate lyase in ruminants is thought to be at the transcriptional level. In order to do so, firstly the promoter region of bovine ACLY needs to be characterised and the sequence needs to be identified. In addition to this the promoter sequence needs to be examined for putative transcription factor binding sites.

1.7.1 Project objectives

- To align and characterise the bovine ACLY 5' regulatory region.
- To clone and characterise minimal 5' regulatory region of bovine ACLY in conjunction with functional assays utilizing transient transfection.
- To confirm putative transcription factor binding sites and the proteins that associate with these elements using electrophoretic mobility shift assays (EMSA).

2.0 Materials and Methods

2.1 Materials

The restriction endonucleases used were *Sal* I from New England Biolabs, *Kpn* I and *Hind* III from Roche Diagnostic Mt Wellington, Auckland. Expand™ and *Taq* DNA polymerase enzymes were purchased from Roche Diagnostic Mt Wellington, Auckland. All other chemicals were analytical grade or better.

pGL3 Basic and pGEM®- T vector system were purchased from Promega Corporation, WI, USA. pCMV-Sport-βgal vector was purchased from Invitrogen NZ Limited, Penrose, Auckland, New Zealand.

XL-1 Blue strain which has the genotype of F'::Tn*10* *pro* A^-B^- *lacI*^{q} Δ(*lacZ*)*M15 recA1 endA1 gyrA96* (Nal^r) *thi hsdR17* (r_K^- m_K^-) *gln V44 relA1 lac* (NEB catalogue), was obtained from Stratagene, Torrey Pines Road La Jolla, CA, USA.

PCR purification kit QIAquick, MinElute™ Gel extraction kit, Maxi prep and Midi prep used to purify DNA using silica column beads were purchased from QIAGEN, New Zealand distributors: Biolab Scientific LTD, Albany, Auckland, New Zealand. The Quantum® Prep Plasmid Miniprep kit was purchased from BioRad Laboratories, CA, USA.

Alexander Cells (PLC/ PRF/ 5) were purchased from ATCC, VA, USA. Sterile tissue culture flasks, plates, cell scrapers and tubes were purchased from Nunc Inc, Naperville, IL, USA. Foetal calf serum, Opti MEM media, trypsin and Pen/Strep were purchased from Life Technologies GIBCO BRL, Auckland.

FuGENE™6 transient transfection reagent and ONPG for β Galactosidase assays were purchased from Roche Diagnostic Mt Wellington, Auckland.

Luciferase reagent was purchased from Promega Corporation, WI, USA.

Protein quantification of nuclear extract was by Bradford protein assay kit was purchased from BioRad Laboratories, CA, USA. 40% (w/v) solution of acrylamide was from Merck, Germany. Primary antibodies of Sp1 and Sp3 were purchase from

Santa Cruz Biotechnology CA, USA; whilst the ChREBP antibody was purchased from Novus Biologicals, Inc USA.

γ^{32} P-[ATP] were purchased from Perkin Elmer Life Science Inc, Boston, MA, USA. The Poly (dIdC) was purchased from Amersham Parmacia Biotech AB, Uppsala, Sweden.
T4 DNA ligase was purchased from Invitrogen NZ Limited, Penrose, Auckland, New Zealand. Lysozyme and RNase were purchased from Sigma Chemical Company, MO, USA. Thermosensitive alkaline phosphatase (TsAP) was obtained from Life Technologies GIBCO BRL, Auckland.

Agarose and 1kb Plus DNA Ladder were purchased from Invitrogen Corporation, Auckland. All DNA primers were purchased from Sigma Genosys Australia Pty. LTD, NSW, Australia. Luria Bertani (LB) base and DMSO were purchased from Sigma Chemical Company, MO, USA.

Acrocap Filter Unit (0.2 μm) sterilization filters for cell culture media and Acrodisk Syringe Filters (0.45 μm and 0.2 μm) for general solutions were purchased from Pall Corporation, MI, USA.

dNTPs was obtained from Amersham Biosciences, Auckland. Bacteriological agar was purchased from Oxoid LTD, Hampshire, England.

2.2 Methods

2.2.1 Rapid boil DNA preparation

DNA templates for PCR and other plasmid DNA were prepared by a rapid boil method (Holmes and Quigley, 1981). 1.5 mL of an overnight *E. coli* culture was pelleted and resuspended in 350 μL STET buffer (0.1 M NaCl. 10 mM Tris-HCl pH 8.0, 1mM EDTA and 5% Triton X-100). The addition of 25 μL of 10 mg/mL Lysozyme and boiling for 40 seconds lysed the resuspended cells. The lysate was centrifuged immediately for 10 minutes at 12000 rpm to pellet cellular debris. The pellet was removed and 375 μL of isopropanol was added to the supernatant to precipitate DNA. The sample was incubated for 30 minutes at -20°C and the DNA

was pelleted by centrifugation at 12000 rpm for 5 minutes. The pellet was washed in ice cold 95% ethanol and centrifuged for 1 minute at 12000 rpm. The DNA pellet was air dried and resuspended in either TE buffer (10 mM Tris-HCl and 1 mM EDTA pH 8.0) or distilled water.

2.2.2 Polymerase Chain Reactions (PCR)

All PCR reactions were carried out in the GeneAmp 2700 PCR system (Applied Biosystems) using 0.2 mL tubes. The general steps of PCR are: denaturation of double-stranded DNA, annealing of primers and extension of DNA (30x Cycles) by thermostable DNA polymerase.

Each PCR reaction contained:

Buffer	5 µL
Primer forward (10 µg/µL)	2 µL
Primer Reversed (10 µg/µL)	2 µL
dNTPs (3mM)	5 µL
DNA polymerase	0.5 µL
DNA template (1ng/µL)	1 µL
Distiled watter	34.5 µL
Total	50.0 µL

A negative control (no DNA template) was included with each PCR reaction to monitor potential contamination in the sample.

2.2.3 Agarose Gel Electrophoresis

Agarose Gel Electrophoresis was carried out using 1% agarose dissolved in 1x TAE Buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.0) containing ethidium bromide (1 µg/mL agarose). The ethidium bromide forms a complex with DNA enabling DNA to be visualised by exposure to UV light. DNA Loading dye (40% (w/v) sucrose, 0.25% (w/v) bromophenol blue) was added to each sample prior to loading to ensure that the sample will sink to the bottom of the well due to the density of the dye.

Current (approximately 80V) was applied to the gel causing the DNA to migrate toward the positive end.

2.2.4 PCR product purification

The PCR products were purified using QIAquick PCR purification kit (Qiagen). The essential feature of this kit is that the DNA binds to the silica beads at high salt concentration allowing the contaminants to be eluted. The purified DNA is eluted from the beads by reducing the salt concentration.

2.2.5 Restriction endonuclease digests

All restriction endonuclease digests were carried out with the appropriate buffer as recommended by manufacturer (25 µL total volume). The samples were incubated at 37°C for 2 hours to ensure complete digestion.

2.2.6 Gel extraction

Appropriate bands were excised from agarose gels under UV light. The DNA was purified using the MinElute™ Gel extraction kit (Qiagen), following the manufacturers' protocol.

2.2.7 Maxi, Midi and Mini plasmid preparation

The Maxi and the Midi plasmid preparation kits were used to extract large quantity and high quality plasmid, used for transient transfection assays. The Mini plasmid preparation however was used when a small quantity of plasmid DNA was required, for cloning and sequencing. All plasmid preparations were done according to the manufacturer's protocols.

2.2.8 Quantification of DNA

DNA was quantified using an Ultrospec 300 Spectrophotometer (Pharmacia biotech). DNA was diluted 100 fold and 50 fold in TE buffer in a final volume of 100 µL. The absorbance of the samples was measure using 75 µL quartz curvettes at 260 nm and 280 nm.

The concentration was calculated using the formula:

Concentration of DNA = A_{260nm} X 50 µg/mL X Dilution factor

The ratio of A_{260nm} / A_{280nm} indicates the purity of the sample DNA. The ratio of A_{260nm} / A_{280nm} = 1.8, means that the sample is pure DNA. If the ratio is lower than 1.8, it indicates that there is protein contamination, and a ratio higher than 1.8 suggest that there is RNA present in the sample.

2.2.9 DNA sequencing

The templates (PCR products or plasmid DNA) for sequencing were premixed with 3.2 pmol of primers in a total volume of 15 µL. For sequencing plasmid DNA, 300 ng of plasmid is required in the premixed sample. Whilst for sequencing PCR products, 1 ng/100 bp is required. All sequencing reactions were carried out in the DNA sequencing facility of the Allan Wilson Centre for molecular evolution and ecology, Massey University.

2.2.10 Removing phosphate groups from vector DNA ends

The phosphate groups on digested DNA vectors were removed by addition of 1 µL of Thermosensitive alkaline phosphatase (TsAP) and 4 µL of 10x TsAP buffer to 1 pmol of 5′ DNA end and after incubation for 15 minutes at 65 °C, the reaction was stopped by the addition of 4 µL of stop buffer (200 mM EDTA pH 8.0) and incubation at 65 °C for 15 minutes.

2.2.11 DNA ligation

Ligation is the process of joining two compatible ends of DNA fragments. Therefore as the general rule the insert and the vector must be digested using the same restriction enzyme to generate compatible ends. The insert to vector ratio will determine the efficiency of ligation.

The ratio of insert to vector ratio used was 1:1, 1:3 and 3:1. The amount of insert that was added to the ligation reaction was calculated using the following formula:

$$\text{Amount of insert DNA (ng)} = \frac{\text{Amount of vector DNA (ng)} \times \text{Size of insert (bp)}}{\text{Size of vector (bp)}} \times \frac{\text{Insert}}{\text{Vector}}$$

Ligations were carried out in DNA ligase buffer (5x) and 1 unit of T4 Ligase with total volume of 20 µL. The ligation mix was then left on the bench to incubate at room temperature for 1 hour. After incubation, 5 µL of the ligation mix was used for transformation.

2.2.12 pGEM®- T vector DNA ligation

The pGEM®- T vector is a linear vector that contains T overhangs at each end to facilitate direct ligation of PCR products that contain A extensions at their 5′ end as a result of PCR using Expand™ and *Taq* DNA polymerase enzymes. The pGEM-T kit contains 2x rapid ligation buffer (60 mM Tris-HCl pH 7.8, 20 mM $MgCl_2$, 20 mM dithiothrethiol (DTT), 2 mM adenosine triphosphate (ATP) and 10% PEG) and control insert DNA.

The ligations were carried out using half the volume recommended by the protocol, 25 ng control vector with 1:1 vector to insert molar ration of control DNA, and a 3:1 insert to vector molar ratio of PCR product in total volume of 10 µL. The ligation mix was left at room temperature to incubate for 1 hour before it was used to transform XL-1 Blue strain for blue/ white selection.

2.2.13 *E. coli* transformation

Frozen glycerol stocks of *E. coli* cultures were streaked on to an LB (Luria-Bertani) agar plate containing a specific antibiotic (100 mg/mL) such as tetracycline or ampicillin as a selection method for plasmid DNA containing an antibiotic resistance gene. The plate was incubated at 37°C overnight, a single colony was picked and the process was repeated. Several colonies from the second plate were then picked and transferred in to 5 mL LB broth with addition of 5 µL of ampicillin (100 mg/mL). These cultures were incubated overnight in a shaker (200 rpm) at 37°C overnight.

E.coli XL-1 Blue was streaked onto an LB plate containing tetracycline and incubated overnight at 37°C. A single colony was picked and put into a 5 mL LB broth with 5 µL of tetracycline added and left to shake at 37°C until the OD reach 0.4-0.5 at 600 nm. Alternatively 100 µL-200 µL of an overnight broth may be used to inoculate fresh LB for more rapid growth.

Once the OD has reached between 0.4-0.5 at 600 nm, 1-2 mL of the *E.coli* culture was transferred into a microcentrifuge tube and centrifuged for 1 minute at 12000 rpm. The supernatant was removed and the cell pellet was resuspended in 1/10 volume ice cold TSS buffer (10 g/L tryptone, 5 g/L yeast extract, 100 g/L PEG-4000, 0.085 M NaCl, 5% DMSO, 0.05 M $MgCl_2$ at pH 6.5). The resuspended cells were placed on ice and used within 2-3 hours.

Competent cells were incubated for 10 minutes after addition of plasmid DNA (10-100 ng). After the incubation the cells were heat shocked at 42 °C for 90 seconds. 400 µL of SOC medium was added before the cells were incubated in the shaker (200 rpm) at 37 °C for 60 minutes for recovery. The cells were then plated in several fraction on appropriate selective media (Hanahan *et al.*, 1991).

Glycerol stocks were prepared by the addition of 200 µL 100% glycerol and 800 µL of overnight *E.coli* culture containing plasmid DNA into a microcentrifuge tube. The sample was mixed and stored at -70 °C.

2.2.14 Mammalian Cell Culture

All mammalian cell culture was carried out in the laminar flow hood under sterile condition. The cells were kept in a 37 °C oven at 5% CO_2 under humid conditions.

2.2.14.1 Preparation of OPTI MEM media

OPTI MEM media was prepared by dissolving the powder in milli-Q water and with addition of 2.4 g of sodium bicarbonate per litre of media. The medium was titrated to reach pH 7.3 with HCL and was filter sterilised using Acrocap Filter Units 0.2 µm (Pall). The media was stored at 4 °C and the supplements (4% of foetal calf serum and 1% of pen/strep (5000 units/mL penicillin G. sodium and 5000 µg/mL streptomycin in 0.85% saline) antibiotic) were added into the media when ready for use.

2.2.14.2 Establishing cells from frozen stock

The cells were stored in the gaseous phase of liquid nitrogen, thawed quickly, transferred into 15 mL tubes containing 5 mL of media to be centrifuged for 1 minute at 750 rpm in a . The pelleted cells were resuspended in 2 mL of complete media,

and transferred into a T25 flask containing 8 mL of complete media covering the base of the flask.

2.2.14.3 Passaging cells

The cells were passaged once they were about 80% confluent. The cells were rinsed twice in 1x trypsin PBSE (1 mL trypsin and 9 mL of PBSE (0.14 M NaCl, 4.3 mM $NaHPO_4$ pH 7.2, 2.7 mM KCl, 0.5 mM EDTA) after the removal of media. Once the trypsin was removed, the flask was hit sharply to dislodge and separate the cells. The cells were resuspended in 10 mL of complete media and 5 mL of the resuspended cells were transferred into a new T75 flask containing 15 mL of completed media.

2.2.14.4 Freezing cell lines

The cells need to be about 80 % confluent in T75 flask before freezing. The cells were passaged and resuspended in 95 % FCS and 5 % DMSO. The resuspended cells were then transferred into a cryo-tube, wrapped in tissue paper and stored in -70 °C overnight before transferring into the gaseous phase of liquid nitrogen for long term storage.

2.2.14.5 Transient transfection

The cells were grown to 60-80 % confluence, and were transferred into 12 well plates containing 800 µL of complete media. The cells were grown overnight in the incubator.

In the Laminar flow hood, FUGENE ™ 6 was diluted in serum free media by adding FUGENE directly into the medium. The DNA to FUGENE ratio required for an efficient transfection is 2:3 (DNA : FUGENE) eg; 2 µg of DNA will require the addition of 3 µL of FUGENE with the serum free media. The appropriate amount of DNA was added into the tube, followed with addition of 100 µL of serum free media / FUGENE mix and the mixture was left to incubate for 15 minutes at room temperature to ensure the binding of FUGENE to the DNA.

After the incubation, 100 µL of DNA, serum free media and FUGENE mix was added into each well, and the cells were incubated in the 37 °C incubator for 24 -48 hr.

2.2.14.6 Harvesting Cells

The media was removed by using a vacuum pump, followed by rinsing of the cells twice with 1 mL PBS (0.14 M NaCl, 4.3 mM $NaHPO_4$ pH 7.2, 2.7 mM KCl). 80-200 µL of cell lysis buffer was added into each well and cells were incubated at room temperature for 15 minutes. The cells were scraped from the bottom of the well using a cell scraper and were transferred into microcentrifuge tubes.

2.2.14.7 Alexander cell extract preparation

OPTIMEM complete media was used to grow Alexander cells to 80-90 % confluence in a T75 flask. After the removal of the media, the cells were rinsed twice with 2 mL PBS (0.14M NaCl, 4.3 mM $NaHPO_4$ pH 7.2, 2.7 mM KCl). 1 mL of TEN buffer (40 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.15 M NaCl) was added to the flask before the cells were scraped off the bottom of the flask and transferred into 1.5 microcentrifuge tubes. After centrifugation of the cell suspension at 12000 g for 5 minutes, the supernatant was discarded and the pellet was resuspended in 300 µL of extraction buffer (containing complete Mini EDTA-Free protease inhibitor cocktail (Roche), 40 mM Hepes pH 7.9, 0.4 M KCl, 1 mM DTT and 10 % glycerol). The cells were disrupted by 3 freeze-thaw cycles in liquid nitrogen. The cell extracts were centrifuged at 12,000 g for 5 minutes (at 4 °C) and the supernatant was dispensed in 30 µL aliquots and frozen in liquid nitrogen for storage in -70 °C.

2.2.15 Bradford protein determination assay.

The concentration of the Alexander cell extract and the liver extract were determined by Bradford Protein assay in a 96-well microplates (Nunc). The concentrated dye reagent (BioRad) was diluted 1:5 in water which was then dispensed into 200 µL aliquot and added to each BSA (1 mg/µL) standard (diluted to a range of 0-2.5 µg) and Alexander cell extract and liver cell extract (undiluted, diluted 1:2, 1:5, 1:10, 1:20 and 1:40 in water). The samples were left to develop at room temperature for 20 minutes before reading the absorbance at 595 nm. A protein standard curve for BSA was constructed and the amount of protein for the Alexander cell and the liver extract were determined.

2.2.16 β-Galactosidase assay

The β-galactosidase assay was carried out in clear flat bottom 96 wells plate. 5 μL of cell lysate from transfected cells were added into each well containing 100 μL of β-galactosidase buffer (60 mM NaH_2PO_4, 40 mM Na_2HPO_4, 10 mM KCl, 1 mM $MgCl_2$) and 50 μL ONPG buffer (60 mM NaH_2PO_4, 40 mM Na_2HPO_4, and 2 mg/mL ONPG). The plate was placed at 37 °C to incubate until the yellow colour developed and the absorbances were read at 405 nm. The readings were used to normalise the luciferase values.

2.2.17 Luciferase assay

The luciferase assay was set up in opaque flat bottom 96 well plates. 5 μL of the cell lysate from transfected cells were added into each wells. The plate was put into the Fluostar Galaxy plate reader (BMG Technology) which was set up to inject 20 μL of luciferase reagent (Promega) and measure the level of luminescence every second. Maximum readings were taken from every assay.

2.2.18 Electrophoretic mobility shift assay (EMSA)

EMSA uses non denaturing polyacrylamide gel electrophoresis (PAGE) and labelled double-stranded oligonucleotide to display the mobility shift based on the observation that DNA/Protein complex travels more slowly through the gel than free DNA fragments. This technique was used to demonstrate the sequence specific binding of DNA to protein in bovine ATP citrate lyase gene promoter.

2.2.18.1 Preparation of γ^{32}P-[ATP] labelled double-stranded oligonucleotide

Labelling of oligonucleotide was carried out in 1.5 mL microcentrifuge tubes. The following components were mixed and incubated at 37°C for 45 minutes.

Oligonucleotide (100 ng/μL)	1 μL
10 x PNK buffer (0.5 M Tris-HCl pH 8.0, 0.1 M $MgCl_2$, 50mM DTT, 0.5 mg/ml BSA)	1 μL
Γ^{32}P-[ATP] (10μCi/μL, Perkin Elmer)	4 μL
T4 polynucleotide kinase (10u/μL, Roche)	1 μL
H_2O	3 μL
TOTAL	10 μL

6 μL (6x excess) of the unlabelled complement (100 ng/μL) was mixed with 2.5 μL of 1 M KCl and 31.5 μL of water. The unlabelled complement mixture was then added to its labelled counterpart, heated to 95 °C in boiling water bath for 5 minutes and was left to slowly cool to room temperature for 1 hour. This process is important to ensure that all of the labelled oligonucleotides were annealed to their unlabeled counterpart.

The labelled double-stranded oligonucleotides were gel purified to ensure the removal of any residual single stranded oligonucleotide, unincorporated label or other contaminant. Before loading the labelled double-stranded oligonucleotide on a 10 % polyacrylamide gel (37 cm long with 0.4 mm spacers), an equal volume of gel shift buffer (40 mM Tris pH 7.6, 16 % ficol, 100 mM KCl. 0.4 mM EDTA and 1 mM DTT) was added. The polyacrylamide gel electrophoresis was carried out at 30 W (~ 15000 V) for 1.5 hour. After the dismantling the gel apparatus, the gel was wrapped in saran wrap and exposed to X-ray film for approximately 1 minute, to display the location of the double-stranded oligonucleotide in the gel.

The appropriate band was excised from the gel, and the DNA was eluted by incubating the gel slice in 500 μL of 50 mM KCl at 37°C overnight. The gel suspensions were vortexed to mix and centrifuged at 12,000 g for 5 minutes before transferring the supernatant into fresh 1.5 mL microcentrifuge tubes the following day. 1 μL of each labelled oligonucleotide was analysed for radioactive label incorporation using Cerenkov (Beckman LS3801 Scintillation Counter) counting. The samples were stored in a thick walled Perspex container at 4°C until required.

2.2.18.2 Preparation of double-stranded unlabelled competitor

5 µL of each complimentary single stranded oligonucleotide (1 µg/µL) were added together in a microcentrifuge tube, heated to 95 °C in a boiling water bath for 5 minutes and left to cool down gradually to room temperature in the water bath. The double-stranded unlabelled competitors were stored at -20 °C until required.

2.2.18.3 Electrophoretic mobility shift assay (EMSA)

Alexander cell extract, bovine liver extract and the HeLa extract (approximately 5 µg of protein) were used in EMSA, to investigate the binding transcription factors to specific elements in the promoter region of ATP citrate lyase gene.

The following components were mixed in a 0.6 mL microcentrifuge tube: 12.5 µL of gel shift buffer, 1 µL poly (dI-dC) (1 mg/mL in $MgCl_2$ and water to make up to total volume of 25 µL. The competitor (5 ng and 50 ng) or the antibodies (5 µL) were added just before the addition of the labelled oligonucleotide (approximately 0.5 ng). The mixture was mixed and left to incubate at room temperature for 15 minutes. 12.5 µL of each reaction was loaded into a non denaturing 4 % polyacrylamide gel in 0.25 X TBE and gel electrophoresis was performed at 200 volts for 1 hour and 5 minutes. 2 µL of DNA loading dye was loaded into one of the wells to monitor the oligonucleotides movement through the gel. The gel was transferred onto DE-81 paper and dried for 15 minutes using a Bio-Rad Gel Dryer 583. The X-ray (Kodak) film was exposed to the dried gel for at least 15 hours at -70 °C using a radioactive safe cassette with intensifying screens. The X-ray film was developed the next day using the100 Plus™ Automatic X-ray film processor.

3.0 Characterisation of ATP Citrate Lyase Promoter Region

Approximately 10 Kb sequence of the bovine ATP citrate lyase (ACLY) gene was isolated and characterised by Xingzhang Tong (MSc thesis, Massey University, 2000). The region sequenced contained exon I, II and III in addition to the intervening intronic sequences. The transcription start site was mapped at a position within exon I, and the translation start codon (ATG) was located in exon II (Tong, 2000).

3.1 The promoter elements

The transcription start site was determined by 5' RACE and the putative *cis* elements were predicted using a computer program such as TSSW, TSSG or TESS program (Baylor College of Medicine, Houston US http://searchlauncher.bcm.tmc.edu/) (Tong, 2000). The promoter region of bovine ATP citrate lyase gene is predicted to extend approximately 2 Kb upstream of the transcription start site, and this sequence contains 6 GC boxes, 2 CCAAT boxes, 2 sterol regulatory elements (SRE), an E box (also known as carbohydrate response element) and a thyroid hormone response element (TRE) (Refer to figure 3.1). These elements are speculated to play an important role in the transcriptional regulation of other genes involved in the fatty acid biosynthesis pathway such as fatty acid synthase (FAS) and acetyl CoA carboxylase (ACC) (Towle *et al.*, 1997). For example, the E box has been shown to be able to regulate the expression of the human ACLY gene (Fukuda and Iritani, 1999), FAS gene (Kim *et al.*, 1996) and liver pyruvate kinase (L-PK) gene (Stoeckman *et al.*, 2004; Wang and Wollheim, 2002). Sterol regulatory element binding proteins (SREBPs) that binds to the SREs are known to regulated the expression of these genes (Horton *et al.*, 2002), together with Sp1 and Sp3 proteins that recognise the GC-rich region of the promoter (Fukuda *et al.*, 1999).

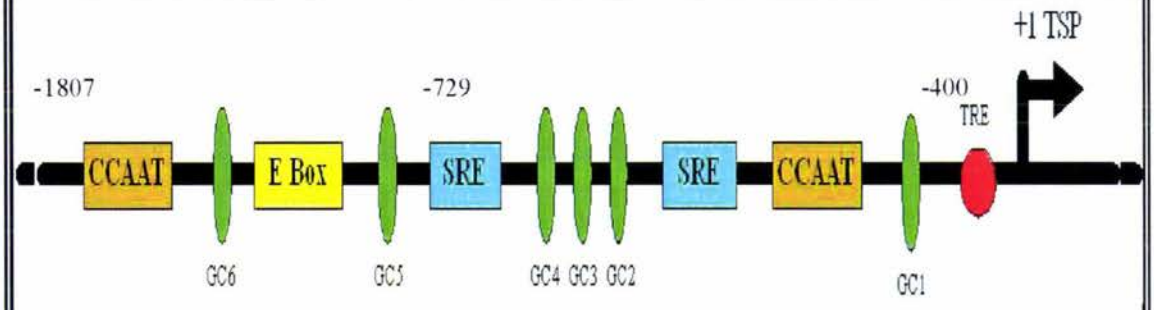


Figure 3.1: Bovine ATP citrate lyase gene 5' promoter region.
The red circle represents the thyroid hormone receptor response element (TRE), the 2 brown boxes indicate the CCAAT box, sterol regulatory elements (SRE) are represented by the 2 cyan boxes whilst the yellow box indicates the E box and GC boxes are represented by green ovals. All elements are depicted relative to the transcription start site (+1 TSP and arrow) (Tong, 2000).

3.2 Regulation of ATP citrate lyase gene

The *cis* elements that are located in the promoter region can play important roles in the regulation of a gene. These elements are spatially located differently in individual promoters, and therefore it is possible that each gene may be regulated in a different manner. The bovine ATP citrate lyase gene is thought to be down regulated as a mechanism for glucose conservation. The precise molecular regulation of the bovine ATP citrate lyase gene is unknown, how ever it is possible that the down regulation of the gene occurs at the transcriptional level. To investigate the differences between a ruminant and a monogastric gene promoter, the bovine ACLY gene promoter was aligned against the human ACLY promoter using the CLUSTAL W program (www.ebi.ac.uk/clustalw/).

3.2.1 Alignment of Bovine ACLY promoter

In order to align the bovine ACLY promoter to the human, rat and mouse promoter sequence, firstly these ACLY promoter sequences had to be identified. The human ACLY promoter region was found by searching the relevant region of the human genome (www.ncbi.nih.gov). The human ACLY gene is located on the long arm of chromosome 17q12-q21. Initially a search on chromosome 17 was carried out, the ACLY gene was located and that particular contig was selected and downloaded. The numbering of the contig was altered relative to the transcription start site of the gene, which was approximately 6 Kb upstream of the transcription start site in the human promoter. The sequence (ref|NT_010755.15|Hs17_10912) was downloaded in

FASTA format, and was aligned against the 5' upstream region of the bovine ACLY using the analysis program CLUSTAL W. The other promoter regions was identified in a similar manner.

The global alignment of a gene can be achieved using CLUSTAL W. This particular alignment is useful to identify the exact location of an element in comparison to another sequence. Unlike BLAST, CLUSTAL W is unable to guarantee an optimal alignment but it is suitable for the identification of similar motifs in two or more sequences.

The results from the CLUSTAL W alignment of the bovine promoter against other promoter regions are shown in appendix 1 and the alignments scores were tabulated in table 3.1.

%	Bovine	Human	Rat	Mouse
Bovine	100	86.67	72.39	43.20

Table 3.1: CLUSTAL W score for alignment between bovine, human rat and mouse ACLY 5' regulatory region.
This table shows the alignments score (in percent) generated from the CLUSTAL W program when the bovine ACLY promoter sequence was aligned against human, rat and mouse ACLY promoter region.

The alignments of bovine ACLY promoter region and the human ACLY promoter was shown to have the highest score of 86.67 %, however when the bovine ACLY promoter was aligned with mouse ACLY promoter, the alignment score was shown to be very low of 43.20 % similarity. These results suggest that the bovine ACLY promoter sequence is more closely related to the human ACLY promoter than the mouse ACLY promoter sequence.

A schematic representation of the sequence motif and alignments of the bovine promoter against the human promoter regions are shown in figure 3.2. Approximately 2 Kb of the 5' regulatory region of the bovine ACLY gene was used in this study is due to the repeat sequence region located at about – 2 Kb upstream of

the transcription start site. There are many similarities that can be observed in both promoters. They both contained a pair of sterol regulatory elements (SRE), several GC boxes, thyroid hormone receptor response elements (TRE) and CCAAT boxes. Both the human and bovine elements interestingly have high sequence identity. Due to this similarity, it is possible that these elements may share a common function within each promoter. Both the human and bovine promoters also have multiple GC boxes that have been found in other promoters to have multifunctional properties (Lania *et al.*, 1997) (appendix 1), for example one of the GC boxes may act as a transcriptional activator and the other as a repressor.

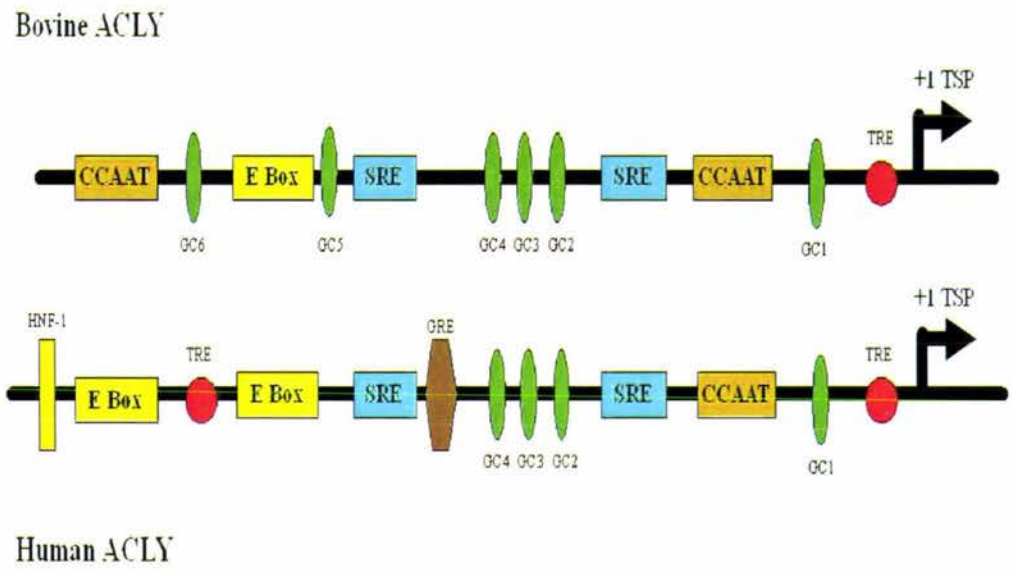


Figure 3.2: A representation of the bovine ACLY promoter region aligned with the human ACLY promoter showing the important transcriptional elements.
TRE represent the Thyroid hormone Response Element, SRE is the Sterol Response Element, GRE is the Glucocorticoid Response Element, while HNF1 is the hepatocyte Nuclear Factor 1. Transcriptional start site is represented by the black arrow (+1). All elements are shown and numbered relative to the +1.

Apart from the many similar features amongst the two promoters, there are also many differences that might be important in distinguishing differences in regulation. The human ACLY promoter is lacking the second CCAAT box and the 6th GC box, but it contains many additional elements such as hepatocyte nuclear factor 1 (HNF1), another thyroid hormone receptor response element (TRE) and a glucocorticoid response element (GRE) (Figure 3.2). One of the most interesting differences

between the two promoters is that bovine ACLY promoter has an incomplete E Box, suggesting that there is only one sequence element rather than having the two sequence elements that are separated by several base pairs, the differences in the E box sequence might be the key to the different regulation between the human and the bovine ACLY gene (Appendix 1.A)

When the bovine ACLY promoter was aligned using CLUSTAL W program against the rat ACLY gene promoter (ref|NW_047339.1|Rn10_WGA1859_3) (Appendix 1.B), the results was quite similar to the bovine/human alignment in term of similarity and differences of the putative *cis* elements found in the 5′ regulatory region of ACLY gene. The CLUSTAL W alignments showed a very interesting difference between the rat E box and the bovine E box. The rat E box sequence element was found to be similar to the bovine E box sequence element in term of it being a single element, however the rat E box sequence of 5′ - GGCCACGTG - 3′ rather than the 5′ - CACGTG - 3′ E box sequence element from the bovine ACLY promoter was found to be responsive to glucose (Moon *et al.*, 2002).

The CLUSTAL W alignment was performed on the bovine, human and rat ACLY promoter region, and the result showed that the three sequences were very similar in term of the first nine putative *cis* elements (figure 3.3).This alignment suggest that the three promoters may be regulated in a very similar manner.

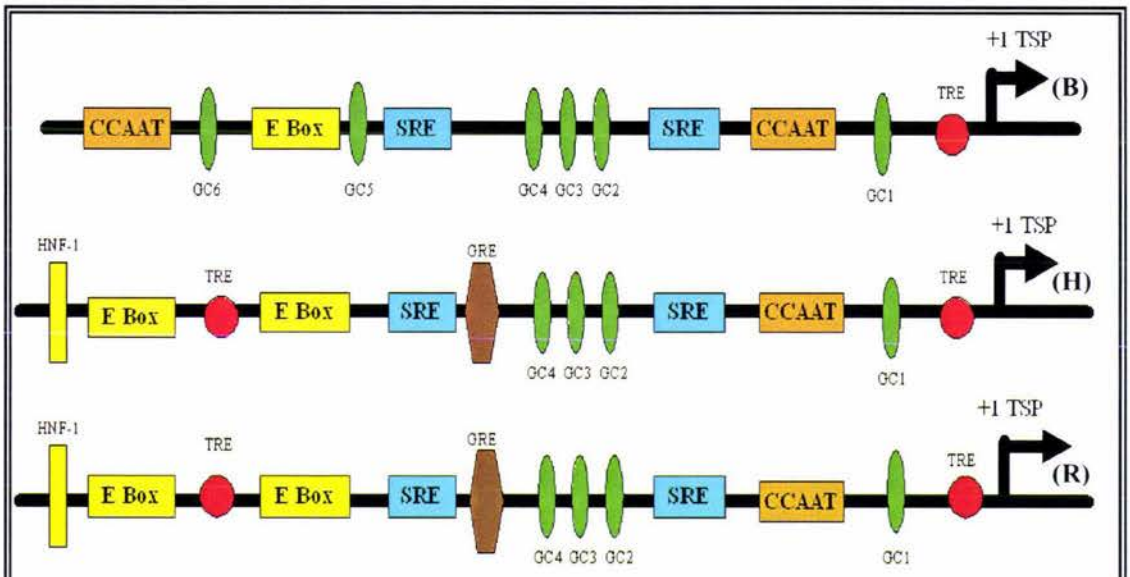


Figure 3.3: Schematic diagram of the bovine (B), human (H) and rat (R) alignment.
The alignment was carried out using CLUSTAL W program and the result shows that the alignment of the three promoters regions were found to be similar. Transcriptional start site is represented by the black arrow (+1). All elements are shown and numbered relative to the +1 (TSP).

The mouse ACLY promoter (ref|NT_039521.4|Mm11_39561_34) was aligned against bovine ACLY promoter and the alignments showed that the sequences are less similar (43% identical) in comparison to the bovine and the human promoter alignments (86% identical). However when both the mouse and rat promoter were aligned against each other, the sequences appear to not have a better alignment. This is surprising, as the mouse and the rat gene sequence are expected to be more similar as they are phylogenetically closer.

3.3 Summary

Approximately 1.5 Kb of the bovine ACLY promoter was aligned against the ACLY promoter of several monogastric mammals such as human, rat and mouse. The results showed that the bovine ACLY promoter has the highest similarity to the human promoter sequence (86%) in comparison to the rat (72%) or the mouse (43%). The mouse and the rat promoter sequences were found to be less similar when aligned against each other.

Apart from the similar features that the bovine promoter sequence has with the human and the rat promoter sequence, there are also some distinctive differences such as the E box sequence that might be the key to different gene regulation between the ruminant and monogastric animal.

Further examination of these elements are required to assign functional significance by using reporter gene assays or DNA/protein binding assays such as EMSA.

4.0 Transient Transfections and Luciferase Assays

4.1 Introduction

Transient transfection is a means of DNA delivery into eukaryotic cells. Unlike stable transfection, where the target DNA taken up by the cells is incorporated into its genomic DNA, transient transfection does not involve the incorporation of the transfected DNA into the genome of the cell.

Plasmid DNA containing a specific cloned gene, can be introduced into mammalian cells for different reasons such as: characterisation and mutational analysis of a particular gene, investigation of regulatory elements in a specific promoter region of a gene, investigation of cell growth and behaviour upon introduction of a new gene, or it could be for purification a specific protein (http://www1.qiagen.com/literature). In this particular project, transient transfection was used to investigate the activity of the bovine ATP Citrate Lyase gene promoter region through a series of deletion constructs.

The DNA to be transfected is treated to facilitate its uptake into cells. In this study the DNA was treated with the cationic lipid FUGENE 6^{TM}. Cells were transfected with two different plasmids. The pCMV-SPORT-β-gal reporter vector was used to normalise the luciferase assay (Figure 4.1). Normalising the luciferase assay is important as it monitors the efficiency of DNA uptake into the cells. The plasmid contains a β-galactosidase gene driven by the cytomegalovirus (CMV) promoter to transcribe the β-galactosidase protein that can be detected through a colour change in the β-galactosidase assays. The second plasmid used was the pGL3Basic reporter plasmid fused to various promoter regions of the ACLY gene, which were generated through PCR deletion methods. The pGL3Basic plasmid has a cDNA region coding for luciferase (*luc*$^{+}$) cloned from the North American firefly *Phontius pyralis*, but lacks a eukaryotic promoter. Therefore the expression of the *luc*$^{+}$ gene is dependent on the promoter region fused upstream of the gene (www.promega.com).

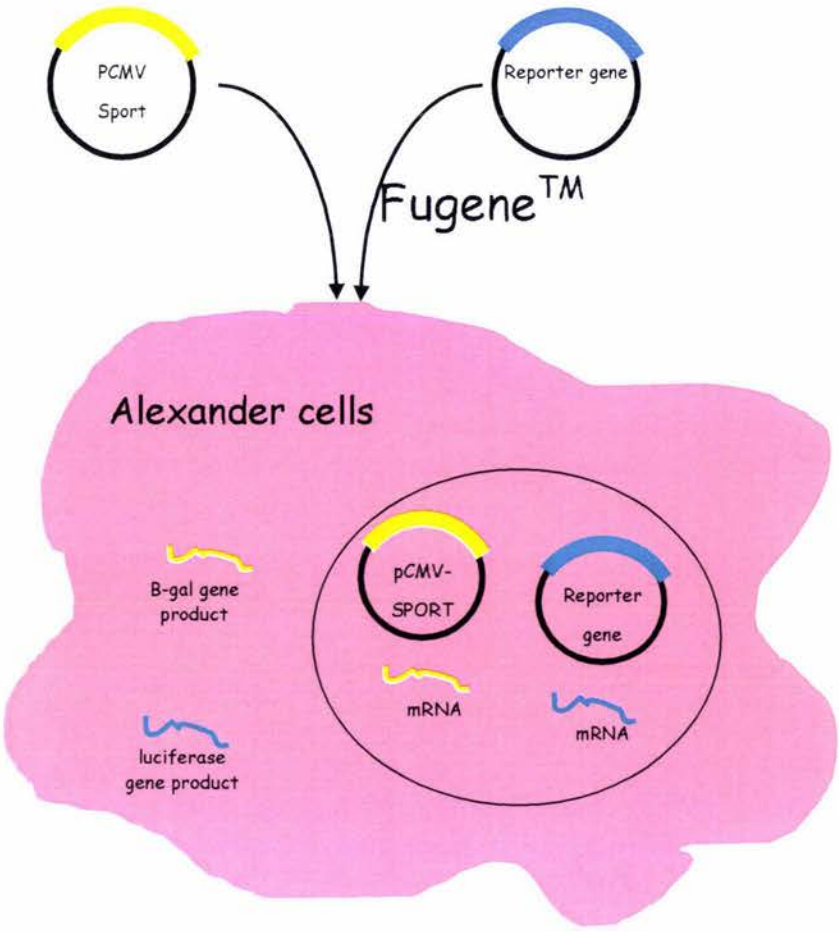
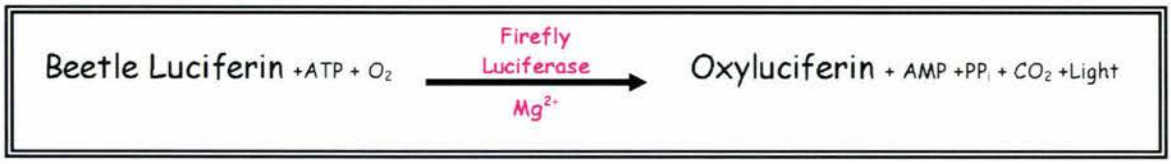


Figure 4.1: Transient transfection.
Two plasmids were transfected into the cells with the help of Fugene 6™, which is a lipid base molecule that forms a stable complex with DNA and is able to transport DNA across the cell membrane. The transfected DNA was not incorporated into the genome of the host cells. However the cells provide the appropriate transcriptional and translational machinery, to produce the reporter protein luciferase which enables gene expression to be studied.

Luciferase assays were used to investigate the promoter activity of the ACLY gene in the transfected cells. The firefly luciferase gene products are able to produce light by catalysing the conversion of the chemical energy of luciferin oxidation through an electron transition, forming oxyluciferin. This reaction requires ATP-Mg^{2+} as a co-substrate. The light emission is detected using FLUOstar Galaxy (BMG).



4.2 Deletion constructs of ACLY promoter

Polymerase Chain Reaction (PCR) is a method that is used to amplify small amounts of DNA by using a thermostable DNA polymerase enzyme. Like other PCR reactions two primers were required to make these deletion constructs. One reverse primer was used for all three constructs, in conjunction with three specific forward primers to give the desired length (Figure 4.2).

To enable directional cloning into pGL3B, the primers were designed to contain *Kpn* I and *Hin*d III restriction sites at the 5' and the 3' end respectively. The primers were tested using the amplify programme to ensure no dimers or secondary structures formed (http://engels.genetics.wisc.edu/amplify/index.html).

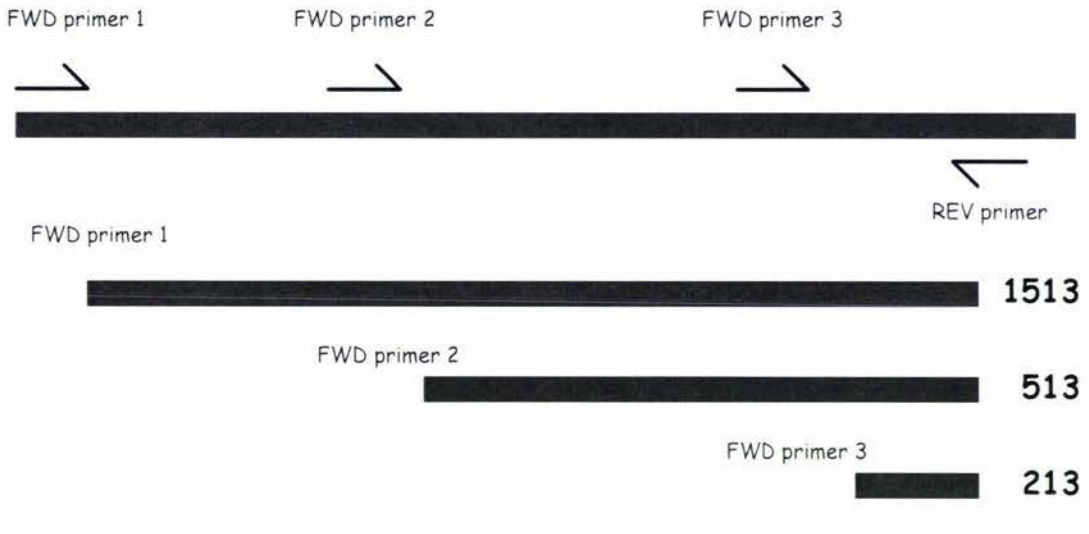


Figure 4.2: PCR deletion of bovine ACLY promoter region.
Polymerase chain reaction (PCR) was carried out using 3 different forward (FWD) primers and 1 reverse (REV) primer to generate 3 different length PCR products.

Each product contains different elements that may be important in the transcriptional regulation of the gene and therefore the effect of these elements when removed can be demonstrated by the reporter gene assay.

4.2.1 PCR Template Preparation

The plasmid pGEM 3Zf(-) used as the template contains 8.2 Kb of the bovine ACLY gene (Tong, 2000). The identity of the plasmid was checked using a *Sal* I restriction digest which gave a 8.2 Kb insert and a 3.2 Kb fragments as expected (Figure 4.3).

Digestion reaction	
DNA	5 µL
Buffer H	2.5 µL
Sal I	1 µL
H_2O	16.5 µL
Total	25 µL

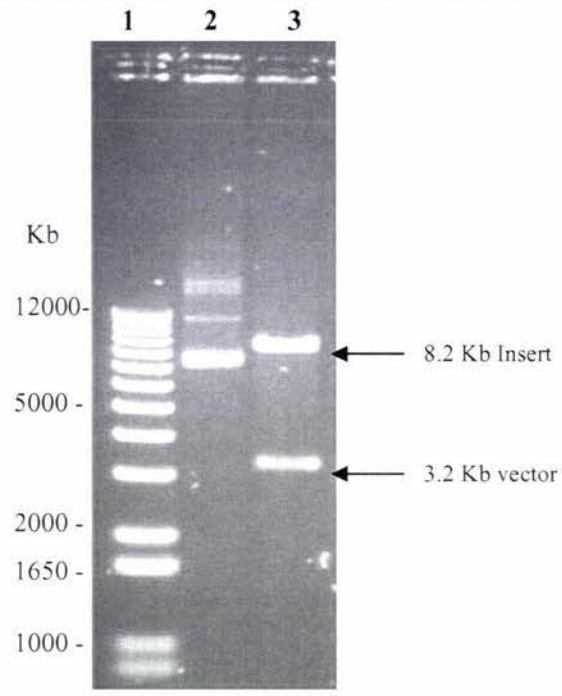


Figure 4.3: Diagnostic digest to confirm the identity of the 8.2 Kb of bovine ATP citrate lyase gene.
A digestion reaction using the *Sal* I restriction enzyme. 5 µL of each sample was loaded with the addition of 2 µL of DNA loading dye and gel electrophoresis was carried out in a 6% agarose gel run in 1x TAE buffer for 1 hour at 80 V. Lane 1 of the gel contains the 1Kb DNA ladders, lane 2 has the negative control, which is the uncut plasmid DNA and lane 3 contains the plasmid cut with *Sal* I enzymes as indicated.

4.2.2 Polymerase Chain Reaction (PCR)

Each PCR was carried out as stated in the method section 2.2.1.2. The PCR reaction for each construct was carried out in different conditions (Table 4.1). *Taq* DNA polymerase was used for amplification of the 1513 construct, whilst the 513 and the 213 constructs were amplified using the Expand™ DNA polymerase. Both polymerases give an A overhang for cloning into the pGEM®-T vector. Expand™ DNA polymerase also possesses the *Pwo* DNA polymerase activity for proof reading activity.

Construct	Cycles	Anealing temperature	Polymerase	MgCl (25 mM)
-1513	30	60 °C	*Taq*	*
-513	30	55 °C	Expand™	7.5 µL
-213	30	60 °C	Expand™	*

Table 4.1: Table of PCR reaction for the three constructs.
The PCR reactions for the -1513 and -213 constructs were carried out in 10X reaction buffer containing Mg^{2+}, whilst the PCR reaction for -513 constructs were carried out using the 10X reaction buffer with out the Mg^{2+}. The * means that the 10 X buffer used contained Mg^{2+} ions.

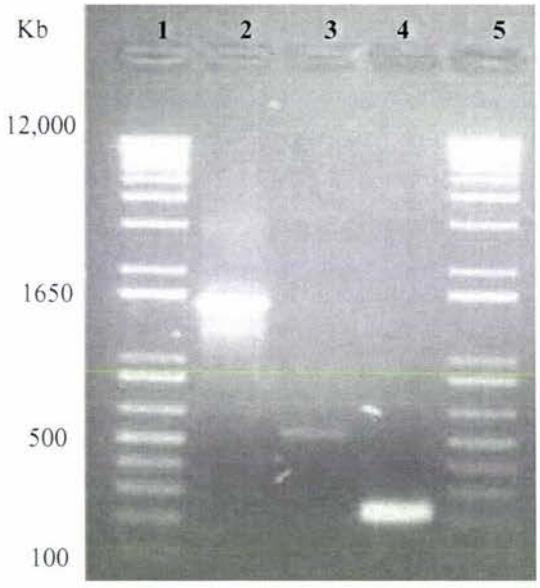


Figure 4.4: PCR products from the 8.2 Kb of bovine ATP citrate lyase gene template.
The PCR products and the ladder were loaded into a 1 % agarose gel and electrophoresis was carried out in 1 x TAE at 80V for 1 hour.

Lanes 1 and 5 contain the 1 Kb DNA ladder. Lane 2 contains the -1513 construct, lane 3 the -513 construct and lane 4 the -213 construct.

All products were purified using the QIAquick PCR purification kit (QIAGEN) according to the manufactures' protocol and were quantified using the Ultrospec 300 Spectrophotometer (Pharmacia biotech).

4.2.3 pGEM®- T vector DNA ligation

The pGEM®-T vector contains T7 and Sp6 RNA polymerase transcription initiation sites that facilitate DNA sequencing, and a multiple cloning site flanking T overhangs. The open reading frame (ORF) for the lac Z gene within the vector allows blue white selection when cloning.

The PCR deletion construct products were ligated into the pGEM®-T vector through the A overhang that was created during the PCR by DNA polymerase (appendix 4.A). PCR products may contain a mutation generated by DNA polymerase. Therefore the PCR product was ligated into the pGEM®-T vector and DNA sequencing was performed. This verifies that each vector contains the correct DNA sequence. It is critical that the sequence is correct for the reporter gene assay experiments (refer to section 4.3).

The ligation experiment contains 8 separate reactions including several controls to ensure the accuracy of the ligation reaction (table 4.2).

Each of the ligation reactions were mixed by vortex and pulse centrifuge at 12000 g, then left on the bench to incubate at room temperature for 2 hours. 5 µL of the ligation mixture was used to transform 50 µL of XL-1 blue *E. coli* competent cells (refer to section 2.2.2.3). The transformed *E. coli* were plated onto LB_{amp}/ IPTG (16.7µL of 24 mg/ml)/ X gal (50 µL of 20 mg/ml) plates and incubated overnight at 37 °C.

Reaction (µl)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
2X T4 Ligase buffer	5	5	5	5	-	-	-	-
pGEM®-T vector	1	1	1	1	1	-	-	-
T4 Ligase	1	1	1	1	-	-	-	-
PCR product (25 ng/µL)	3	1	0.3	-	-	-	-	-
TE Buffer	-	-	-	-	5	5	-	-
Uncut vector (pGL3Basic (0.25 µg/µl))	-	-	-	-	-	1	-	-
H_2O	0	2	3.7	3	4	4	10	10
Total volume (µL)	10	10	10	10	10	10	10	10

Table 4.2: Ligation reactions for cloning into pGEM®-T vector .
A typical ligation reaction with the appropriate controls.
The reactions are :
Reaction 1: Insert : Vector (3:1)
Reaction 2: Insert : Vector (1:1)
Reaction 3: Insert : Vector (1:3)
Reaction 4: Background control (cut vector + ligase)
Reaction 5: Cut vector without ligase Negative control
Reaction 6: Uncut vector
Reaction 7: Negative transformation control (Competent cells on Lb_{amp} Agar)
Reaction 8: Positive transformation control (Competent cells on Lb Agar)

The ligation reactions were incubated at room temperature for 2 hours followed by transformation into XL-1 blue *E.coli* competent cells. These reactions were repeated for each of the promoter constructs.

The results from reactions 1, 2 and 3 showed that the ligation reactions were successful (table 4.3). The plates were expected to have some white colonies containing the insert and some blue colonies that do not contain the insert. As expected the three plates showed both blue and white colonies The 3:1 insert to vector ratio gave the highest number of white colonies. Five colonies from each of the plates were picked and grown overnight (refer to section 2.2.2.1) at 37 °C and shaken at 200 rpm for a diagnostic digest the following day.

The control reactions were used to ensure the accuracy of the ligation reactions. Reaction 4 was used to ensure that the linear pGEM®-T vectors were unable to ligate in the presence of T4 DNA ligase whilst reaction 5 was used to ensure the pGEM®-T vectors were unable to ligate in the absence of T4 DNA ligase. Reaction 6 was done to ensure the competency of the *E.* coli cells. Reaction 7 and reaction 8 were used as

negative and the positive controls respectively. The results from each of the control plates (refer to table 4.3) showed that the ligation and the transformation experiments were successful.

Plate	Blue	White
1	34	21
2	44	33
3	30	5
4	16	-
5	-	-
6	-	lawn
7	-	-
8	-	lawn

Table 4.3: Typical transformation results from PCR product to pGEM®-T vector ligation.

Each plate contains ampicillin antibiotic, IPTG (16.7 µL of 24 mg/ml) and X gal (50 µL of 20 mg/ml) and were incubated overnight at 37°C.

Plate 8 does not contain any antibiotic, as it was intended as a positive control to check the viability of the competent cells.

Five individual colonies were picked from plates 1, 2 and 3. These colonies were transferred into culture tubes containing 5 mL LB_{amp} broth (refer to section 2.2.2.1) and incubated overnight at 37 °C. The bacteria were extracted by centrifugation and the DNA was extracted using the rapid boil method (described in 2.2.1). The plasmid DNA was digested with restriction enzymes (Table 4.4) to check for the presence of insert DNA.

Digestion reaction	
DNA	5 µL
Kpn I	0.5 µL
Hind III	0.5 µL
Buffer 4	2.5 µL
H_2O	16.5 µL
Total	25 µL

Table 4.4: Diagnostic digestion reaction for the bovine ATP citrate lyase gene deletion constructs ligated to pGEM®-T vector.

Typical digestion reaction using *Kpn* I and *Hind* III restriction enzyme. For the negative control each plasmid (1513- pGEM®- T, 513- pGEM®- T, and 213- pGEM®- T) was left uncut.

The reactions were incubated for 2 hours at 37°C to ensure complete digestion

4.2.3.1 Sequencing of the three construct

Each of the constructs cloned into the pGEM®-T vectors were sequenced by the Allan Wilson Centre DNA sequencing facility at Massey University using SP6 and T7 primers. Clones containing insert DNA with the correct sequence (Appendix 3) were selected and used to obtain the DNA sequence for cloning into the pGL3Basic vector.

4.2.4 pGL3Basic vector DNA ligation

The method to clone the ACLY promoter deletion constructs into the pGL3Basic vectors was as follows: the generated PCR deletion constructs of the bovine ACLY promoter were ligated through the T over hang of the pGEM®- T vector. The 3 vectors containing an insert were sequenced using the T7 and SP6 primers, the binding sites for these primers are located at each end of the multiple cloning site in pGEM®- T (Apendix 4).

Once the sequences were obtained, the 1513-pGEM®- T, 513- pGEM®- T and 213-pGEM®- T were digested using *Kpn* I and *Hin*d III enzymes to sub-clone the insert into a pGL3Basic vector. The fused bovine ACLY promoter in pGL3Basic was then used in transient transfection experiments.

4.2.4.1 Preparation of pGL3Basic vector

The pGL3Basic vector (Promega) was digested using *Kpn* I and *Hin*d III (Table 4.4), and gel electrophoresis was carried out to visualise the DNA under UV light (Figure 4.5 A), to ensure the vectors had completely linearised.

The band in lanes 3 and 4 (Figure 4.5 A), visualised under the UV light, was excised and purified using MinElute™ Gel purification kit (QIAGEN) following the manufacturers' protocol. The phosphate groups from the 5′ end of plasmid DNA were removed as indicated in section 2.2.1.10.

4.2.4.2 Insert preparation for ligation

The PCR products ligated in the pGEM®- T vectors were digested as shown in table 4.4. Agarose gel electrophoresis was carried out in 1x TAE buffer for approximately 1 hour at 80 V. DNA was visualised by incorporation of ethidium bromide and exposure under UV light. The appropriate bands were cut out and the DNA from

these bands was purified using MinElute™ Gel purification kit (QIAGEN) according to the manufacturers' protocols.

4.2.4.3 Ligation reaction of purified insert into pGL3Basic vector.

The ligations of the purified insert were carried out in a similar manner to the PCR product ligation into pGEM®- T vectors. The pGL3Basic vector does not contain a β-galactosidase gene; therefore blue/white selection could not be carried out. The insert to vector ratios were calculated as shown in 2.2.1.11 of the method section. The ligation reactions (Table 4.5) were incubated for 2 hours at room temperature. After the incubation period, 5 µL of the ligation reactions were used to transform 50 µL of XL-1 blue *E. coli* competent cells (refer to section 2.2.2.3).

Five individual colonies were picked from each plate. These colonies were transferred into culture tubes containing 5 mL LB_{amp} broth. These culture tubes were incubated overnight in a shaker (200 rpm) at 37 °C. DNA from each culture tube was extracted using the rapid boil method, as described in 2.2.1.1 of the method section.

The extracted plasmids were checked for insert by digestion with *Kpn* I and *Hin*d III (refer to table 4.4). 5 µL of the digestion reaction was loaded into a 1 % agarose together with 1 Kb DNA marker. Agarose gel electrophoresis was carried out in 1x TAE buffer for approximately 1 hour at 80 V (figure 4.5 B).

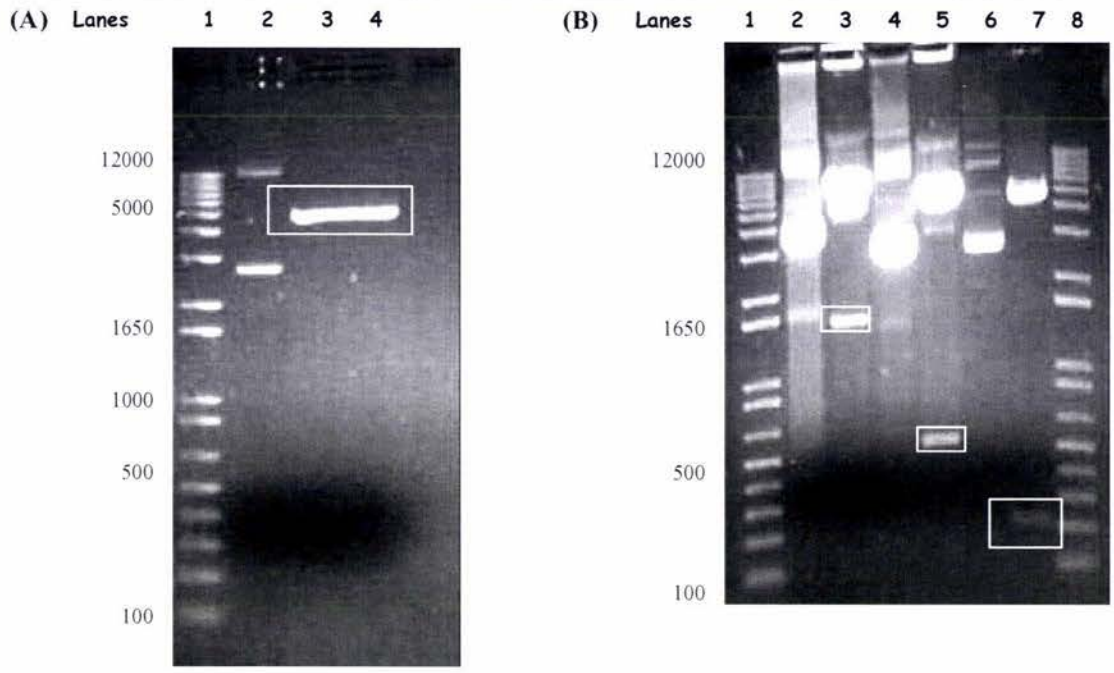


Figure 4.5 A: Digestion of pGL3Basic Plasmid
pGL3Basic plasmid was digested for 1 hour at 37°C using *Kpn* I and *Hin*d III in a 25 µL of reaction mix.

The digested plasmid (25 µL of reaction mix) was loaded into a 1% agarose gel and 1x TAE for 1 hour at 80V (lane 3 and 4) along with 5 µL the uncut plasmid DNA (lane 2) and 5 µL of 1 Kb DNA ladder (lane 1).

The linearised plasmid indicated by the white rectangle was isolated (referrer to section 2.2.1.6) and purified using QIAGEN Gel purification kit, according to the manufacturers' protocols.

Figure 4.5 B: pGL3Basic Plasmid Digest
5 µL (0.4 - 0.5 µg/µL of DNA) of each digest was loaded togetherinto a 1% agarose gel with 2 µL of DNA loading dye. Gel electrophoresis was carried out in 1x TAE for 1 hour at 80 V.

Lane 1 and 8 contain 5 µL of 1 Kb DNA ladder. Lane 2, 4 and 6 are negative controls where the 1513-pGL3, 513-pGL3 and 213-pGL3 plasmids respectively were not digested with the restriction enzymes.

Lane 3, 5 and 7 contain the pGL3Basic plasmid (1513-pGL3Basic, 513-pGL3Basic and 213-pGL3Basic respectively) cut with *Kpn* I and *Hin*d III. The insert can be seen in the lower part of the gel at 1650, 500 and 200 Kb area in lane 3, 5 and 7 respectively (indicated by the white box). The linear plasmid can be seen near the top of the gel.

Reaction	**1**	**2**	**3**	**4**	**5**	**6**	7	**8**
5X Ligase buffer	4	4	4	4	4	-	-	-
pGL3Basic vector (1.29 µg/µl)	1	1	1	1	1	-	-	-
T4 Ligase	1	1	1	1	-	-	-	-
Insert DNA (32.23 ng/µl	0.8	3.9	1	-	-	-	-	-
TE Buffer	-	-	-	-	-	4	-	-
Uncut vector (pGL3 Basic)	-	-	-	-	-	1	-	-
Water	13.2	10.1	13	14	15	15	20	20
Total volume (µL)	20	20	20	20	20	20	20	20

Table 4.5: Ligation reaction for cloning into pGL3Basic vector.
A typical ligation reaction generating 1513-pGL3b, 513-pGL3b and 213-pGL3b constructs, which will be use for the transient transfection experiments.

The reactions are:
Reaction 1: Insert : Vector (3:1)
Reaction 2: Insert : Vector (1:1)
Reaction 3: Insert : Vector (1:3)
Reaction 4: Background control (cut vector + ligase)
Reaction 5: Cut vector without ligase Negative control
Reaction 6: Uncut vector (pGL3Basic plasmid)
Reaction 7: Negative transformation control (Competent cells on Lb_{amp} Agar)
Reaction 8: Positive transformationcontrol (Competent cells on Lb Agar)

The ligation reactions were incubated at room temperature for 2 hours followed by transformation into XL-1 blue *E.coli* competent cells. These reactions were repeated for each of the promoter constructs.

Each of the bacterial culture containing one of the three plasmids were used to inoculate a 500 mL LB_{amp} broth. A large scale plasmid preparation (Midi plasmid preparation kit (QIAGEN)) was carried out the following day.

The purified plasmid was quantified using Ultrospec 3000 Spectrophotometer (Pharmacia biotech) (refer to section 2.2.1.7) (table 4.6).

Plasmids	Concentration (μg/μL)	Purity (A_{260}/A_{280})
1513-pGL3Basic	1.352	1.75
513-pGL3Basic	1.172	1.71
213-pGL3Basic	1.016	1.60

Table 4.6: The concentration of the plasmids used for Transient transfection.
The plasmids were quantified using Ultrospec 3000 Spectrophotometer (Pharmacia Biotech) after purification using midiprep plasmid purification kit (QIAGEN).

The 1513-pGL3Basic, 513-pGL3Basic and 213-pGL3Basic plasmids were diluted to a concentration of 0.5 μg/μL, whilst the pCMV-SPORT-βGal and pGL3Basic plasmid were diluted to a concentration of 0.25 μg/μL. Dilution of the plasmids to the same relative concentration is important to ensure the same amount of plasmid was distributed to each well, thus allowing more accurate transient transfection assays.

4.3 Transient transfection

4.3.1 Introduction

The promoter constructs fused into the pGL3Basic plasmid (described in section 4.2.2) generated three different constructs containing different elements that may be important in the regulation of the bovine ACLY gene (Figure 4.6). The longest construct made was the -1513 construct (the wild type construct), containing the full sequence of the bovine ACLY promoter region. The intermediate length construct is the -513 construct and it contains all of the putative *cis* elements in the wild type construct except for CCAAT box 2 and GC6. The shortest construct is -213 base pairs in length and contains the GC1 box and the TRE (Figure 4.6). These deletion constructs were made to investigate the minimal promoter activity of the bovine ACLY promoter. The 1 Kb region which contain the two putative *cis* elements (-513 construct) was deleted to investigate the importance of these elements in the regulation of the gene. If these elements play an important role in the up-regulation of bovine ACLY gene, a decrease in the relative luciferase activity will be observed.

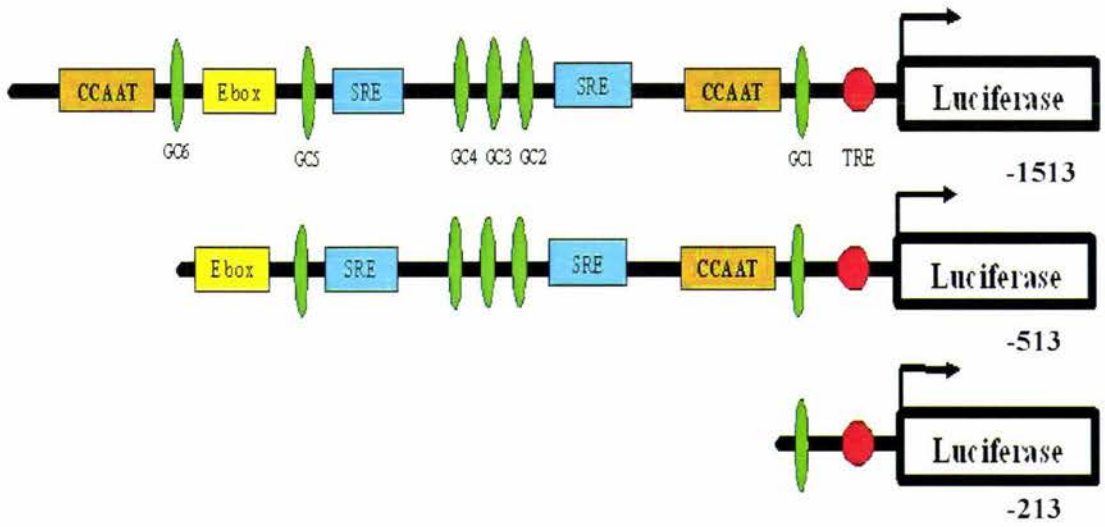


Figure 4.6: Deleted promoter region of bovine ACLY gene fused to pGL3Basic plasmid.
The -1513 constructs contains all of the putative *cis* elements in the 5' regulatory region of the bovine ACLY gene. The -513 construct is lacking in the CCAAT box and GC6 box, whilst the shortest construct (-213) contains only the GC1 box and a TRE.

The smallest construct was made to investigate if the two *cis* elements (GC1 box and TRE) were sufficient to drive the expression of the luciferase reporter gene.

4.3.2 Results and discussion

Alexander cells (human Hepatoma) cultured in 12 well plate (refer to section 2.2.3.5) were transfected together with a lucifrease reporter construct, pCMV-SPORT-βgal and pBlue-Script plasmid. The pCMV-SPORT-βgal act as an internal control for plasmid uptake. The amount of FUGENE : DNA ratio (refer to section 2.2.3.5) is critical for optimal transfection efficiency. To keep the amount of FUGENE added to each transfection reaction mix constant, the amount of DNA in the transfection reaction was equalised by the addition of the plasmid pBlue-Script plasmid.

0.5 µg of each construct (-1513, -513, -213) and 0.25 µg of pCMV-SPORT-βgal was used in each transient transfection experiment. A range of different concentrations were assayed and showed that 0.5 µg of the construct and 0.25 µg of pCMV-SPORT-βgal plasmid was enough to give the reproducible luciferase activities.
The cells were seeded into 12 well plates and were left to incubate over night at 37°C under 5% CO_2 and humid conditions. Each experiment was done in triplicate, and

each triplicate was repeated 3 times to ensure the accuracy of the data. The transient transfection reactions are as listed below.

pCMV-SPORT-βgal

This reaction mix contains 0.25 μg of pCMV-SPORT-βgal and the amounts of DNA were balanced with the addition of 0.5 μg of pBlue-script. This control is important to monitor the level of β-galactosidase protein produced in the absence of the reporter gene vector. The amount of β-galactosidase should be approximately equal in all the reactions, because the same amount of pCMV-SPORT-βgal (0.25 μg) plasmid was added to each reaction. This corrects for transfection efficiency in each reations.

pGL3Basic

This reaction contains 0.5 μg of pGL3Basic empty vector and 0.5 μg of pBlue-Script empty vectors. This reaction was used monitor the basal expression of luciferase in the absence of the promoter region of the ACLY gene.

-1513-pGL3Basic

Alexander cells were transfected with 0.5 μg of the full length promoter construct fused to the pGL3B vector together with 0.25 μg of the pCMV-SPORT-βgal plasmid. In this reaction, the level of luciferase expression was expected to be the highest, due to the intact promoter region of bovine ACLY. The amount of β-galactosidase expressed in this reaction was expected to be the same as the pCMV-SPORT-βgal reaction.

-513-pGL3Basic

Alexander cells were transfected with 0.5 μg of the middle length construct (513 base pairs) and fused into the pGL3B vector and 0.25 μg of pCMV-SPORT-βgal. The level of luciferase expression was expected to either be less than that the 1513 construct if there were enhancer elements in the deleted region, or the luciferase expression might be higher than the 1513 construct if suppressor elements were deleted and the enhancer elements were still intact.

Reactions X 3	**pCMV (µL)**	**pGL3B (µL)**	**-1513 (µL)**	**-513 (µL)**	**-213 (µL)**
pCMV (0.25 µg/µL)	3	-	3	3	3
pGL3basic (0.25 µg/µL)	-	3	-	-	-
pBS (0.5 µg/µL)	3	3	-	-	-
1513-pGL3Basic (0.5 µg/µL)	-	-	3	-	-
513-pGL3Basic (0.5 µg/µL)	-	-	-	3	-
213-pGL3Basic (0.5 µg/µL)	-	-	-	-	3
FUGENE	3.375	3.375	3.375	3.357	3.375
Serum Free Media (SFM)	296.625	296.625	296.625	296.625	296.625

Table 4.7: The transient transfection reactions.
A representative table of the components used in each transient transfection experiment. Each experiment was done in triplicate. 100 µL of DNA and FUGENE/ SFM mix was added to the 80% confluent cells in a 12 well plate containing 800 µL of media. The cells were incubated in humid condition for 24 hours at 37°C with 5% CO_2.

-213-pGL3Basic

This construct was the shortest construct, containing the 213 base pairs immediately upstream of the transcription start site. Alexander cells were transfected with 0.5 µg/µl of the -213-pGL3Basic plasmid. The level of luciferase expression in the 213 construct was expected to either be diminished if these elements were not enough to drive the expression of the gene, or higher if suppressor binding elements were deleted.

4.3.2.1 β-Galactosidase assays and Luciferase assays

Both of the β-Galactosidase (β-gal) assays and the luciferase assays were done as in section 2.2.6 and 2.2.7 respectively. 5 μL of the cell extract was used in both the β-galactosidase assays and the luciferase assays.

The results were collected and calculated as follows; the luciferase activity in the blank control (contains cell lysis buffer only) was substracted from the lucifrease activities to remove any background. β-galactosidase values were shown to be similar in each sample (Table 4.8), which shows that the rate of plasmid uptake by the cells between the samples is relatively constant.

The luciferase activities were normalised against the β-galactosidase activities by dividing the luciferase values by the β-galactosidase values. The normalising of the lucifrease value is important to compensate for any internal luciferase expression variation within the Alexander cells. The normalized luciferase value for the wild type constructs (-1513) was set to 100% and therefore the normalized luciferase values for the deleted constructs (-513 and -213) will be relative to the wild type construct (-1513).

The sample containing only the pCMV-SPORT-βgal plasmid and the empty pGL3Basic plasmid do not produce luciferase and so no luciferase activity was expected. This is because the pCMV-SPORT-βgal plasmid does not contain the gene to produce the luciferase enzymes and the empty pGL3Basic plasmid does not contain a promoter sequenceto drive the expression of the gene.

Transient transfection results show that with the deletion of the 1 Kb region of the wild type construct (compare the luciferase activity of the -1513 to -513), the luciferase activity was reduced by approximately 50% (Figure 4.7). A further 300 base pairs deletion (-213 construct) reduced the luciferase activity to almost zero (0.5 %) (Figure 4.7).

Constructs	Maximum Luciferase	Corrected Maximum Luciferase	ß-Gal	Corrected ß-gal	Normalised Luciferase activities	Average Normalised Luciferase	Relative activities to WT %
Blank	10	-	0.071	-	-	-	-
	58	-	0.071	-	-	-	-
	61	-	0.071	-	-	-	-
Avg Blank	43	-	0.071	-	-	-	-
	35733	35690	0.278	0.207	172415.5		
WT-1513	17882	17839	0.215	0.144	123881.9	141857	100
	19046	19003	0.218	0.147	129272.1		
	10569	10526	0.222	0.151	69708.61		
-513	11950	11907	0.227	0.156	76326.92	84559	59.61
	18988	18945	0.247	0.176	107642		
	160	117	0.179	0.108	1083.333		
-213	98	55	0.18	0.109	504.5872	827	0.58
	136	93	0.175	0.104	894.2308		

Table 4.8: A representative luciferase calculation for transient transfection using the bovine ACLY gene promoter region.
This table is a representative result of a transient transfection experiment using bovine ACLY promoter deletion constructs. Each transfection experiment was done in triplicate and the results were analysed as described above. Each set of transient transfection experiments were repeated an additional two times.

Both of the luciferase and the ß-galactosidase maximum values were subtracted from the average blank (cell lysis buffer) values. This step is important to accommodate any background signals that may occur in the assays. Each of the corrected luciferase values were normalised by dividing the corrected luciferase value by the corrected ß-galactosidase values.

The normalised luciferase values for the -513 and the -213 constructs were displayed relative to the -1513 (wild type construct), setting the normalised luciferase value of -1513 to 100%.

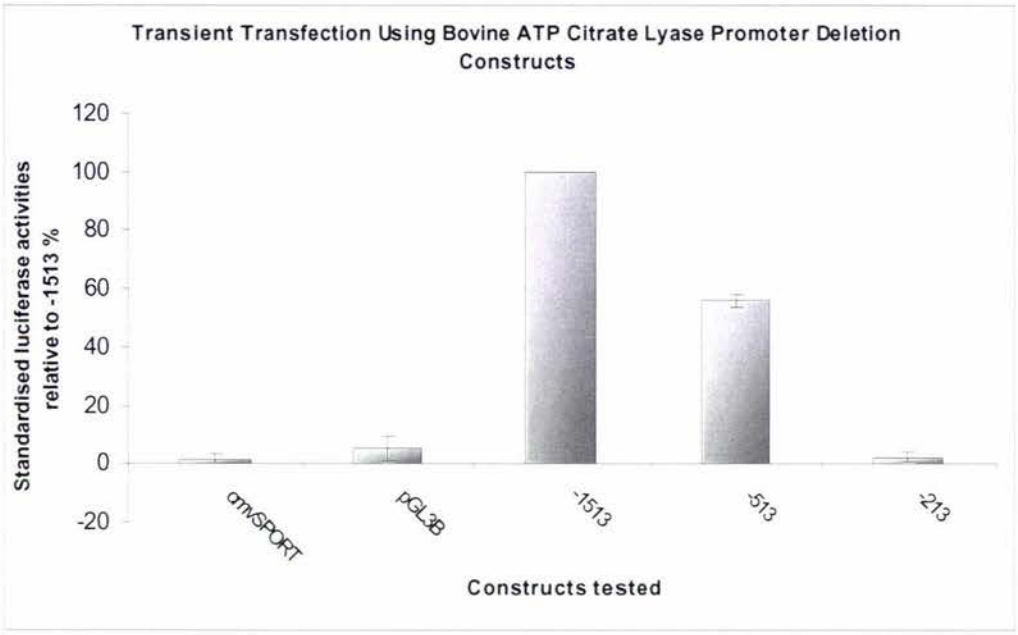


Figure 4.7: The transient transfection experiment using bovine ACLY gene promoter deletion constructs.
The graph shows the normalised luciferase value expressed by each constructs. The luciferase value of the -513 and the -213 constructs are relative to the -1513 construct (wild type) as it was set to be 100%.

The 1 Kb deletion (-513 construct) shows a 50 % in the luciferase activity, whilst the deletion of 1300 bp (-213 construct), abolished the expression of the gene.

4.3.2.2 Student t-test

The t-test was used to verify whether the differences between the two sets of data were statistically significant. The t-test deals with small and equal sample sizes, where it compares the two sets of data, with the null hypothesis being that the mean of one set of data is equal to the mean of the second set of data ($\mu_1 = \mu_2$). The P values resulting from the t-test calculation will provide the evidence to support or reject the null hypothesis (Norman and Streiner, 1994).

The results show that the P-values for the normalised luciferase activities were less than 0.05 for both of the deletion constructs (table 4.9). This shows that there is a significant difference between the normalised luciferase activities for the wild type construct (-1513) and the two deletion constructs (513 and 231).

Construct tested	Normalised luciferase activities relative to the WT %	P Value	Significant Differences
pCMV-SPORT	0.53 ± 2.30 %	-	-
pGL3Basic	-3.94 ± 8.62 %	-	-
-1513	100 ± 0.00 %	-	-
-513	55.92 ± 2.46 %	0.004	++
-213	2.23 ± 1.86 %	4.41×10^{-5}	++

Table 4.9: Table of analysed luciferase values using T student test.
Each of the luciferase activities was shown relative to the -1513 (wild type) and analysed using the t-test to determine if there is a significant difference between the deleted constructs (-513 and -213) and the wild type construct (-1513). The t-test was performed using Excel (XP, Microsoft) program.

The t-test shows that there is evidence for a significant decrease in the luciferase activity after the deletion of 1 Kb region in the bovine ACLY promoter, which contains the GC6 element and the CAAT box 2 (Table 4.9). This result suggests that these two elements bind transcription factors that act as enhancers in the expression of the ACLY gene.

The smallest construct (-213) contains only the thyroid hormone response element and GC1 box. The luciferase activity of the -213 construct when compared to wild type construct (100%), was only measured to 0.5 %. The t-test shows that there is a significant difference between these two samples with the P-value of 4.41×10^{-5}. These finding show that the thyroid hormone response element and the GC1 element are not sufficient to sustain the expression of bovine ACLY gene.

The GC6 and GC1 elements were shown to bind the same proteins in EMSAs which are Sp1 and Sp3 (refer to section 5.2.6). This, together with the results from the transient transfection experiments, suggest that the binding of these transcription factors Sp1 and Sp3 may cause the up-regulation of the gene. This up-regulation may be facilitated by a change in DNA topology caused by the binding of Sp1 and Sp3.

Other experimental evidence has shown that the binding of Sp1 may induce looping of the promoter to up-regulate gene expression (Mastrangelo *et al.*, 1991).

The looping of DNA is often observed in other promoters, where an activator may change the topology of the DNA upon binding to the promoter region. The looping of the promoter region is able to stimulate transcription of the gene by facilitating the binding of other transcription factors and RNA polymerase to the promoter. The looping of the bovine ACLY promoter may aid in the recruitment of the DNA polymerase enzyme, as the promoter region of bovine ACLY is TATA-less. In a TATA-less promoter, the TATA binding protein (TBP) is not able to bind to the TATA box directly and recruit the RNA polymerase and general transcription factor to initiate the transcription of the gene.

4.4 Chapter summary

The bovine ACLY gene promoter was used to makeinto a series of three deletion constructs by PCR. The first 1513 bp construct was referred to as the wild type construct and it contains all of the putative *cis* elements that may be important binding sites for transcription factors. The second construct is 513 base pairs long, and lacks 1000 base pairs of the 5' upstream region of bovine ACLY gene. This deleted region contains the GC6 box and the second CCAAT box. The last construct is the shortest construct; with only 213 base pairs, containing the thyroid hormone receptor response element and the GC1 box.

Each of the PCR products was ligated into the pGEM®-T vector. The ligated PCR products were sequenced uisng the SP6 and T7 primers. After sequencing of the constructs, the insert was cut out of the pGEM®-T vector and ligated into the pGL3 basic vector for transient transfection experiments.

The transient transfection results show that the deletion of the 1000 base pair from the wild type construct (-1513) reduced the luciferase activity by 50%. A further 300 base pair deletion was shown to abolish the luciferase activity reducing it to 0.5% of the wild type luciferase level which is equivalent to the promoter less pGL3Basic vector. These results show that the GC6 box and the CCAAT box 2 were crucial in regulating the transcription of the gene, and the presence of GC1 and the thyroid hormone receptor response element alone is not sufficient to initiate transcription of the gene.

5.0 Electrophoretic Mobility Shift Assays

5.1 Introduction

As discussed earlier, the promoter region of the bovine ACLY gene contains several putative *cis* elements that may play an important role in the regulation of the bovine ACLY gene expression. Some of these elements are: GC boxes, SREs, CAAT boxes and E boxes. Transcription factors Sp1 and Sp3 bind to GC boxes and have been shown to regulate the expression of the ACLY gene in rat and mouse (Fukuda *et al.*, 1999). Studies of the promoter region of the human ACLY gene have shown that the E boxes (also known as Carbohydrate responsive elements (ChoRE)) regulate the expression of the ACLY gene (Kim *et al.*, 1996).

The results from the alignments (discussed further in 3.1) showed that the human's and the rat's ACLY 5' regulatory region have a high sequence identity to that of the bovine 5' regulatory region. These results suggest that although these promoters have different sequences, all of these promoters possess similar *cis* elements that may be regulated in a similar manner. Elements, such as SRE, multiple GC boxes, E box and CCAAT boxes, have been shown to be important for the regulation of the bovine ACLY gene through reporter gene assays (discussed further in section 4.3.2.2).

EMSAs are one of the techniques used to demonstrate the sequence specific binding of DNA to protein. EMSAs use native polyacrylamide gel electrophoresis (PAGE) and labelled double stranded oligonucleotide. If the labelled double stranded oligonucleotide binds to a protein component in the nuclear extract, then the labelled oligonucleotide protein complex will have a lower mobility (Figure 5.1A) and this change in mobility can be visualised by exposure to X-ray film. Native PAGE providing stability to the DNA/protein complexes by the use of a low ionic strength buffer that can increase the binding stability and the gel matrix provides a stabilised cage for the DNA/protein complex.

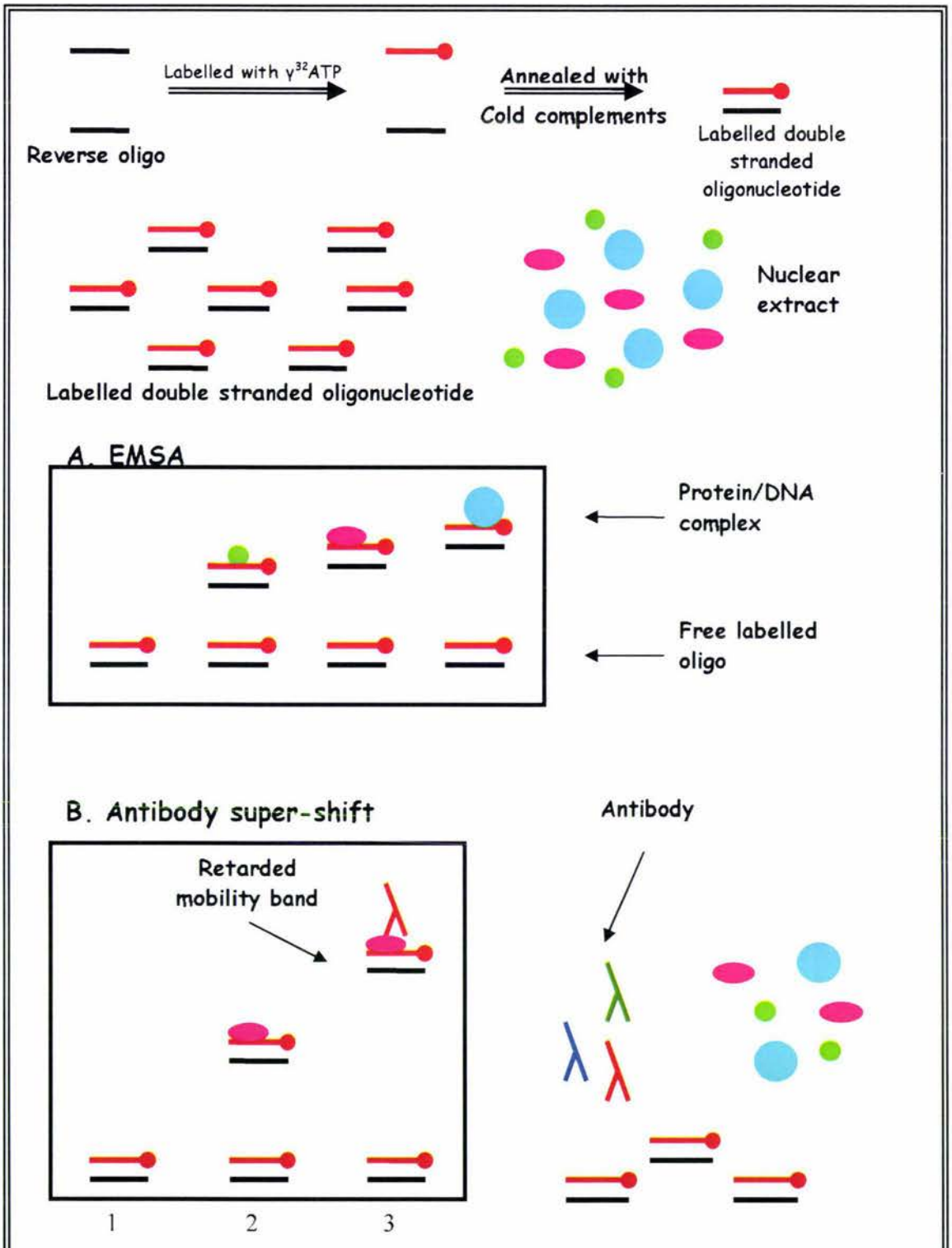


Figure 5.1: Schematic representation of Electrophoretic Mobility Shift Assay.
The radioactively labelled DNA probe will travel slower when bound to a specific protein in comparison to the free oligonucleotide (A). By the addition of specific antibody to the reaction, the antibody-protein-DNA complex will be super-shifted (lane 3 of figure 5.1 B) and therefore the specific protein that binds to that particular oligonucleotide can be identified.

To investigate the DNA binding proteins that may associate with the putative bovine ACLY *cis* elements, Electrophoretic Mobility Shift Assays (EMSA) were carried out. Crude nuclear extract was extracted from Alexander cells (PCL/PRF/5) and used in standard EMSA reactions (refer to section 2.2.19. Alexander cells are human hepatoma cell line (www.atcc.org) that are known to be able to provide the required DNA binding proteins to drive the bovine ACLY gene promoter (refer to section 4.3.2.2).

Synthetic polymers poly (dI-dC), were used to minimise non-specific binding of protein to labelled double stranded oligonucleotide. Therefore an appropriate amount of poly (dI-dC) is added to each reaction. This should be enough to prevent non-specific protein binding to the oligonucleotide but not so great as to prevent the specific binding of proteins that recognise the oligonucleotide sequence.

5.1.1 Variation on EMSAs

5.1.1.1 Competitor Assays

Competitor assays are used to assess the binding specificity of the protein/DNA interaction. This is necessary because the extract preparation will contain a large number of proteins that can associate with the labelled oligonucleotide. Both the wild type (which contains the intact sites for transcription factor recognition) and the mutant (having the transcription factor binding site mutated) unlabelled competitors are added in excess to determine the specificity of the protein interaction with the DNA. If an unlabelled oligonucleotide competes for the binding of any protein then a reduction in band intensity is expected.

5.1.1.2 Antibody super-shift Assays

Antibody super-shift assays were carried out by adding specific antibody to the EMSA reactions. This was done to identify the proteins present in some of the protein/DNA complexes. Addition of antibodies may have several effects on the protein/DNA interactions. If the antibody recognises a protein that is not involved in DNA binding, the addition of antibody should have no affect on observed binding patterns.

However, if the antibody recognises a protein that is involved in an observed protein/DNA complex, then the antibody can either block complex formation or can form an antibody-protein-DNA complex that will migrate even less than just a protein/DNA complex. This is seen a shift in bands (super-shift) due to the higher molecular mass of this complex (Figure 5.1B).

5.2 Protein/DNA interaction in bovine ACLY promoter

The bovine ACLY promoter contains several elements that are speculated to be involved in the regulation of ACLY gene transcription. For some of these elements a 20 to 25 base pair oligonucleotide was designed. Both the human and the bovine E box, bovine GC1 and GC6 were used (Figure 5.2).

These elements have been characterised and previously studied in different species. For example, the carbohydrate response element binding proteins (ChREBP) are known to bind to E boxes (ChoRE) in the promoter of many genes that are involved in fatty acid biosynthesis(Kim *et al.*, 1996; Towle *et al.*, 1997), whilst Sp1 and Sp3 have been shown to regulate the ACLY gene and several other genes by binding to the GC boxes located in the promoter (Fukuda *et al.*, 1999; Mastrangelo *et al.*, 1991).

Both labelled and unlabelled oligonucleotide sequences are shown in figure 5.2. The GC boxes are highlighted in green, while the E boxes are highlighted in yellow. All of the substituted base pairs are written in red font.

The human ACLY carbohydrate binding element contains two E box sequences separated by 12 base pairs. The corresponding element in the bovine ACLY consists of a single E box sequence. The mutant human E box differs from the wild type human E box (5'-CACGTGCTAAAGGAAAGCGGCGA-3) by having three base pairs in both elements substituted with three adenines (5'-CA**AAA**GCTAAAGGAAA**AAA**CT-3'). This base pair substitution with the three adenines is necessary because there are many variations in the sequences of E boxes (Yamada *et al.*, 1999). In order for the elements to loose its binding ability, three base pairs in both sites of wild type human E box were substituted with adenines (Figure 5.2). The single bovine E box (incomplete E box) was also mutated from 5'-

CACGTG-3' into 5'-CA**AAA**G-3', to see if this change causes the loss of protein binding.

Both the wild type and the mutated topoisomerase-2β GC1 box oligonucleotides were taken from (Willingham, 2004). The wild type sequence of 5′-CCCGCCCC-3′ binds Sp1 and Sp3 whilst the mutated sequence 5′-C**GA**GC**T**CC-3′ is no longer able to bind these two proteins.

Human E box
Forward 5′ TCCACGTGCTAAAGGAAAGCGGCCT 3′
Reverse 3′ AGGTGCACGATTTCCTTTCGCCGGA 5′

Human E box Mutant
Forward 5′ TCCAAAAGCTAAAGGAAAGCAAACT 3′
Reverse 3′ AGGTTTTCGATTTCCTTTCGTTTGA 5′

Bovine E box
Forward 5′ GAGAGTCCACGTGCTAAAGG 3′
Reverse 3′ CTCTCAGGTGCACGATTTCC 5′

Bovine E box Mutant
Forward 5′ GAGAGTCCAAAAGCTAAAGG 3′
Reverse 3′ CTCTCAGGTTTTCGATTTCC 5′

Bovine *GC1* box
Forward 5′ CCGATGGGGGCGGGGAAAAG 3′
Reverse 3′ GGCTACCCCCGCCCCTTTTC 5′

Bovine *GC6* box
Forward 5′ TAGTGGGGGCAGGGCGGAGA 3′
Reverse 3′ ATCACCCCCGTCCCGCCTCT 5′

Topo2β *GC1* box
Forward 5′ CGGGTCCCGCCCCTCCA 3′
Reverse 3′ GCCCAGGGCGGGGAGGT 5′

Topo2β *GC1* box mutant
Forward 5′ CTCGGGTCGAGCTCCTCCA 3′
Reverse 3′ GAGCCCAGCTCGAGGAGGT 5′

Figure 5.2: Oligonucleotides designed for EMSAs.
The sequences of the human E box, bovine E box, Bovine GC1box, bovine GC6 box and topo 2β GC1 box are shown. Also shown are the mutant of Human E box, the Bovine E box and the mutant of topo 2β GC1 box. The elements are highlighted in yellow and green whilst the mutant nucleotides were shown in red.

5.2.1 Oligonucleotide labelling

The oligonucleotides were labelled with γ^{32}P [ATP] (refer to 2.2.7.1). The labelled double stranded oligonucleotides were gel purified by 10% non-denaturing polyacrylamide gel electrophoresis to remove any single stranded oligonucleotides and other contaminants. An autoradiograph of a gel showing the labelled oligonucleotides is shown in figure 5.3. After elution, the amount of γ^{32}P [ATP] incorporation was determined by scintillation counting (Cherenkov emission).

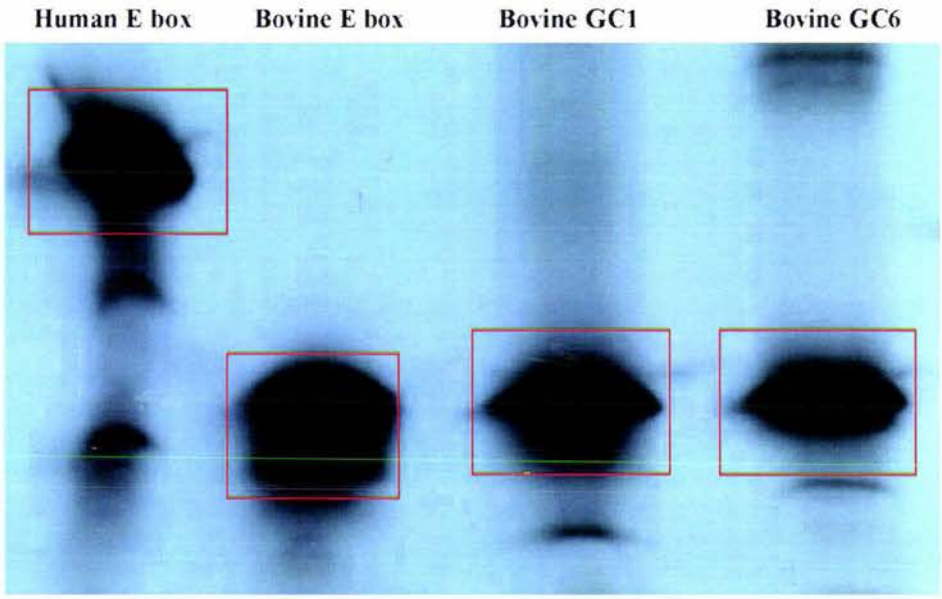


Figure 5.3: Gel purification of ^{32}P labelled oligonucleotides for EMSA.
The forward strand of each oligonucleotide was labelled with γ-^{32}P [ATP]. The labelled oligonucleotide was then annealed to its cold complement. The double stranded oligonucleotides were gel purified using 10% of non-denaturing polyacrylamide in 1 x TBE for 1 hour at 30 Watts (1500V).

The gel was exposed to X-ray film for 1 minute to locate the position of the labelled oligonucleotide and the major bands were excised. The DNA was eluted in 50 mM KCL overnight at 37°C.

Oligonucleotide	Counts per minutes (1 µL)
Blank	93
Human E box	73913
Bovine E box	72021
Bovine GC1	34514
Bovine GC6	44814

Table 5.1: Radioactivity of labelled oligonucleotides probe.
1 µL of each labelled oligonucleotide was transferred to a 0.5 ml microcentrifuge tube and the radioactivity of each oligonucleotide was determined using Beckman LS3801 Scintillation counter.

5.2.2 Preparation of Alexander cell extract and HeLa cell extract

The preparation of bovine liver nuclear extract was described in section 2.2.4 and the Alexander and HeLa cell extracts were prepared as described in section 2.2.3.7. The protein content of the nuclear extract was quantified using the Bradford assay (described in section 2.2.5). The protein concentration of HeLa extract was estimated to be 4.04 µg/µL and the Alexander cell extract was estimated to have a protein concentration of 4.48 µg/µL. In order to examine the overall protein content of these extracts, each sample was subjected to denaturing polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 5.4). The intensity of the staining directly correlates with the relative protein concentration of each extract and the amount of protein in the gel correlates with the estimated concentration of the proteins from the Bradford assay.

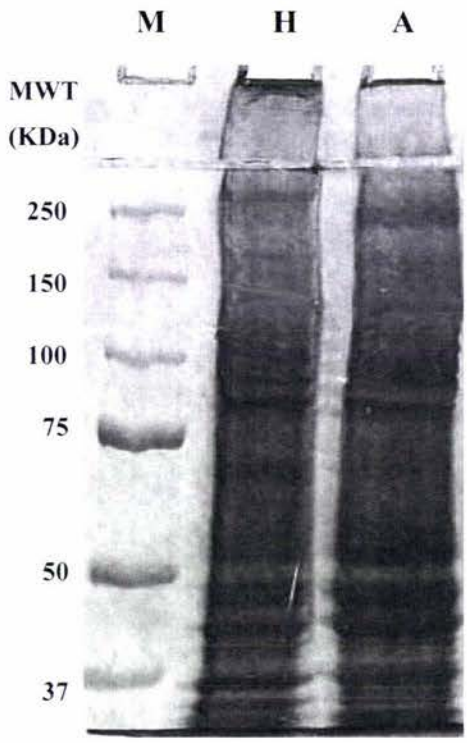


Figure 5.4: SDS PAGE of cell extract
The HeLa cell extract (H) and the Alexander cell extract (A) were loaded together with a protein molecular weight marker (M).

15 µL (4.04 µg/µl for HeLa and 4.48 µg/µl for Alexander) of each extract was boiled for 5 minutes with 7.5 µL of sample buffer containing (6 % w/v) SDS before loading on a 6 % SDS-Polyacrylamide gel , and electrophoresis was carried out for 1 hour at 120 V (~80 mA).

The gel was stained using comassie blue and was destained using 10 % acetic acid.

5.2.3 Titration of Alexander cell nuclear extracts

The amount of oligonucleotide after purification can only be estimated and consequently it is necessary to optimise the amount of DNA relative to the amount of cell extract in order to examine the protein/DNA interactions.

Increasing amounts of Alexander cell extracts between 0 - 16 µg of protein (0, 1, 2, and 4 µL) were added to standard EMSA reactions (refer to 2.2.19.3). In lanes 1, 5, 9 and 13 (Figure 5.5) only free oligo was observed on the gel. This was expected as no cell extract was added to the reaction mix i.e. no protein complex was present. With increasing amounts of extract, there are distinct banding patterns shown in the gel as the protein from the Alexander cell extract forms DNA/protein complexes. These bands indicate the different DNA binding proteins that interact with the labelled oligonucleotide, the increasing intensity of the bands correlates with the increasing amount of extract added to each reaction.

The human E box sequence element shows three distinct bands in each lane (lanes 2-4), whilst the bovine E box sequence element only shows two major bands (lanes 6-8). This result may suggest that the band with the higher mobility in lane 2-4 is represented by a DNA binding proteins which is specific to the human E box. The bands for the human E box complex are fainter than those for the bovine E box (compare lanes 1-4 with lanes 5-8 of figure 5.5) suggesting that the bovine E box interacts more strongly with the DNA binding proteins from the cell extract than the human E box. The similarity in the two bands present in both the bovine and the human E boxes may represent identical protein/DNA interactions.

Both the bovine GC1 and GC6 boxes appear to have the same binding pattern, there are 3 distinct bands in each lane (refer to lanes 9-16 of figure 5.5). They also seem to have greater band intensity when compared to both the human and the bovine E box. This result may suggest that either that the nuclear proteins that bind to the GC boxes are present in higher amounts than the proteins that bind to the E boxes or that the binding efficiency of the DNA binding protein to these elements is greater.

EMSAs using HeLa cell extracts were carried out to compare the banding pattern of the DNA/protein complexes formed with those of the Alexander cell extract. The DNA binding proteins Sp1 and Sp3 isolated from the HeLa cells are known to binds to the GC boxes (Magan, 2003). The results from the HeLa cell extract, except that the intensity of the bands was slightly higher, showed the same binding pattern as the Alexander cell extract (Figure not shown). This similarity suggests that the interactions in the Alexander extracts are consistent with the binding patterns found in other cell lines and that are the same proteins that bind in the HeLa extract are also present in the Alexander cell extract.

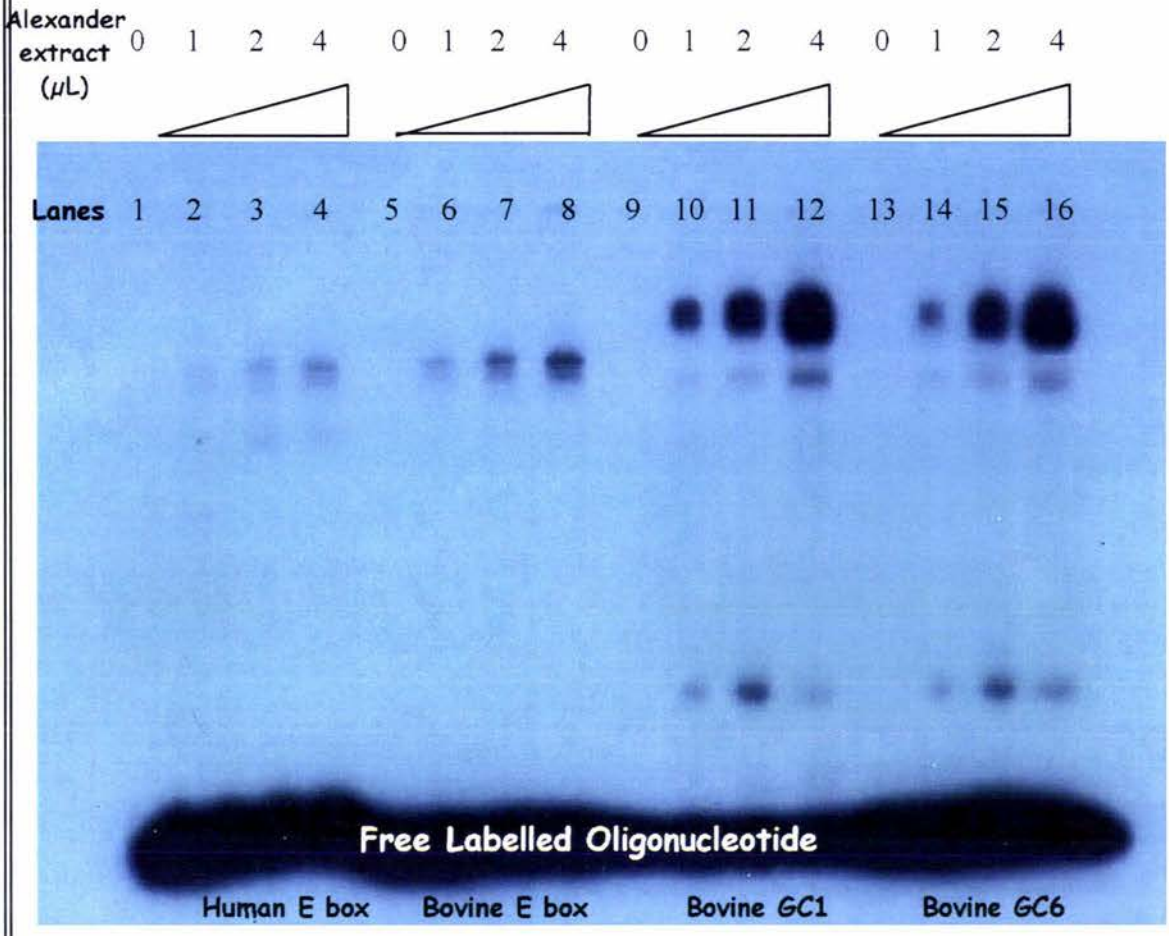


Figure 5.5: EMSA showing the effect of increasing amount of Alexander cell extract.
1µL of labelled oligonucleotide was added to increasing amounts of Alexander extract, poly (dI-dC) and gel shift buffer (20 µL of reaction mix). Half of each reaction was loaded onto a 4 % non-denaturing polyacrylamide gel and electrophoresis was carried out in 0.25 X TBE for 1 hour and 5 minutes at 200V.

The gel was dried on DE-81 paper, exposed to X-ray film for approximately 18 hours at -70°C in a cassette with intensifying screens.

Lane 1, 5, 9 and 13 are the negative control lanes, where no Alexander cell extract was added to the reaction. The dark band at the bottom of the gel is the free (unbound) labelled oligonucleotide that migrates faster than the protein bound oligonucleotides.

Increasing amounts of Alexander cell extract are represented by the triangles at the top of the gel.

5.2.4 EMSA using Alexander cell extract and bovine GC box elements

5.2.4.1 Unlabelled competitor EMSA using GC1 and GC6 elements

The competitor assays are used to determine the specificity of DNA binding proteins interacting with the oligonucleotides. The unlabelled competitors used in this study are: bovine GC1, bovine GC6, topo2β_GC1_WT and topo2β_GC1_MT. These competitors will provide an indication not only to the specificity of the binding, but also to the strength of the interactions.

Increasing amounts of unlabelled double stranded DNA were added to each binding reaction containing the labelled bovine GC1 oligonucleotide (Figure 5.6). The bovine GC1 competitor as expected, is a strong competitor against its homologue labelled oligonucleotide (lane 2-5 of figure 5.6) and the addition of 5 ng of the bovine GC1 unlabelled competitor decreases the bands intensity (lane 3). When 50 ng of the unlabelled competitor was added to the reaction (lane 4), all the 3 bands shown in lane 2 disappear completely.

The bovine GC6 element also competes strongly with the bovine GC1 elements (lane 6-9 of figure 5.6). However, unlike the bovine GC1 competitor, the bovine GC6 competitor only competes for the 2 bands that have the lowest mobility. The band with the highest mobility appears to interact only with the GC1 unlabelled competitor (compare lanes 4 and 5 with lanes 6-12), which may suggest that this third protein binds with greater affinity to GC1.

The GC1 element from the Topoisomerase 2β promoter (Topo2β_GC1_WT) is a known bonafide Sp protein binding element (Wilingham, 2004). The addition of increasing amount of the unlabelled Topo2β_GC1_WT competitor to the reactions caused a decrease in the 2 bands of lower mobility, suggesting that the proteins that bind to bovine GC1 elements may be the Sp proteins (lane 10-13). When the sequence of the GC1 element of the topoisomerase 2β competitor is mutated, it no longer competes with the bovine GC1 element (lane 14-17) meaning that the protein binding to this element is specific.

GC boxes have been shown to be recognised by Sp1 and Sp3 transcription factors (Suske, 1999), it could therefore be predicted that these proteins that bind to both GC1 and GC6 boxes may be Sp1 and Sp3 transcription factors. To determine the identity of these proteins to the bovine GC boxes, antibodies super-shift experiments were carried out (Figure 5.10).

The bands with the lowest mobility were shown to be inconsistent (Figure 5.6). This inconsistency was also observed when increasing amount of Alexander extract was added into the standard EMSA reaction (Figure 5.5). Further experiments are needed to investigate the specificity of this interaction.

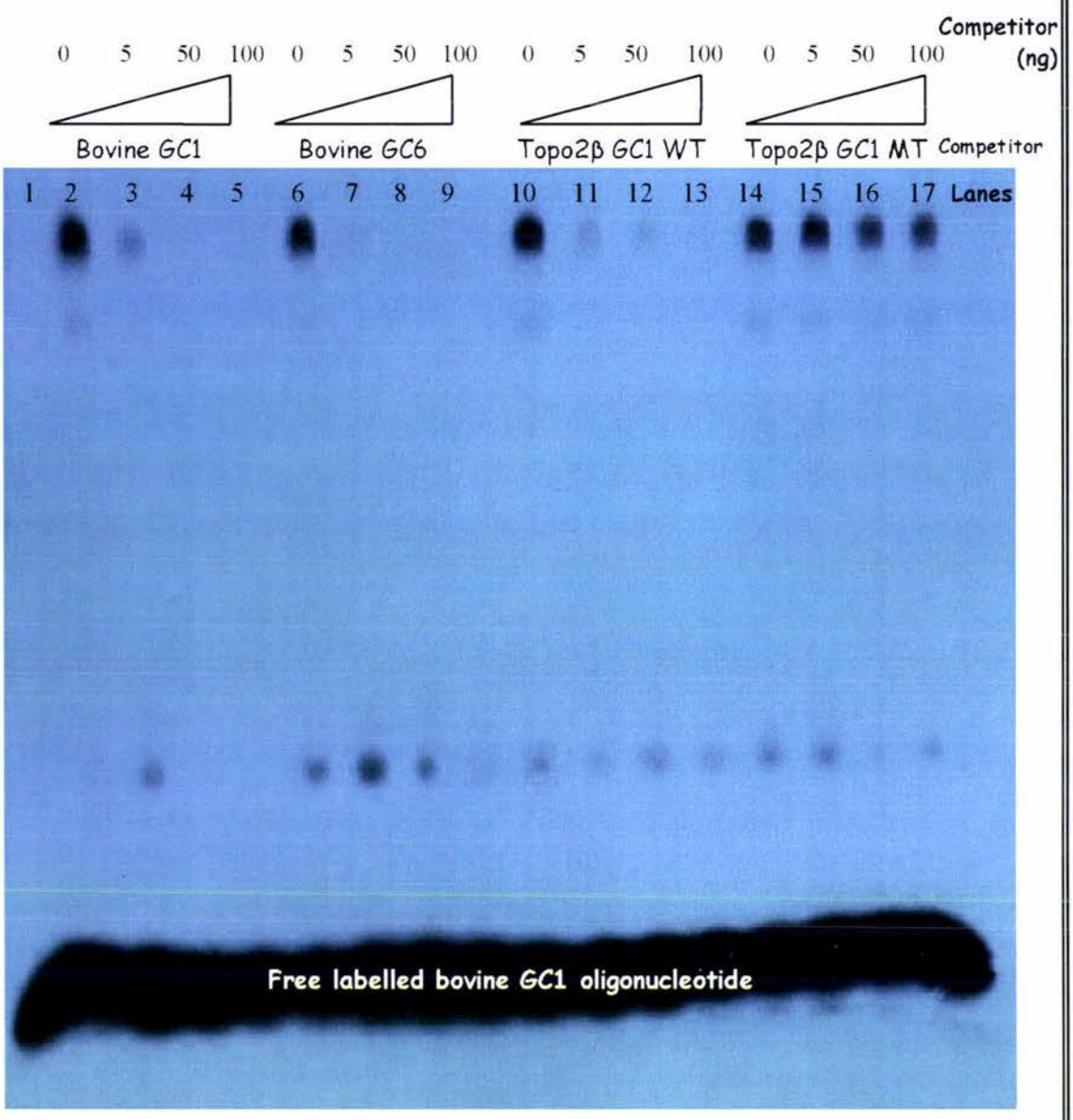


Figure 5.6: Competitor assay using Alexander cell extract.
1µL of labelled bovine GC1 oligonucleotide was added to each reaction containing increasing amounts (0 ng, 5 ng, 50 ng and 100 ng) of unlabelled double stranded competitor. Each reactions contain 1 µL of 50 fold diluted (2.24 µg) Alexander extract. 12.5 µL (half) of each reaction mix was loaded into a 4 % non-denaturing polyacrylamide gel and electrophoresis was carried out in 0.25 X TBE for 1 hour and 5 minutes at 200V.

The gel was dried on DE-81 paper and exposed to X-ray film for about 25 hours at -70°C in a cassette with intensifying screen.

This gel is a representative of both the bovine GC1 oligonucleotide and the bovine GC6 oligonucleotide competitor assay.

5.2.4.2 Antibody super-shifts assays using Sp1 and Sp3 antibody

As mentioned earlier, Sp1 and Sp3 are known to bind to GC boxes and appear to be involved in regulating the ACLY gene in human and rats (Fukuda *et al.*, 1999). Therefore, Sp1 and Sp3 antibodies were used in attempt to identify the type of proteins present in protein/DNA complexes shown in figure 5.5.

Lanes 1, 5, 9 and 13 contain only the labelled oligonucleotide. The natures of the labelled oligonucleotides are indicated at the bottom of the gel (Figure 5.10).
Lane 2, 6, 10 and 14 (Figure 5.10) contain only Alexander cells extract and the labelled oligonucleotide. The banding pattern in these lanes shows the bands similar to the banding pattern in figure 5.5.

The addition of antibody to Sp3 (lane 3 and lane 7) in the human and bovine E boxes did not appear to have any effect. No bands appear to be reduced in intensity or to be super-shifted (Figure 5.10). The addition of antibody to Sp1 (lane 4 and lane 8) also had no apparent effect on the binding of proteins to both the human and bovine E boxes.

The Sp3 antibody was added to the standard EMSA reaction containing labelled oligonucleotide for bovine GC1 (lane 11), and a decrease in intensity was observed for band B, which may indicate that the Sp3 antibody recognised and formed an interaction with the protein in B. Similar results were shown by a decrease in the observed band intensity when Sp3 antibody was added to the reaction containing the GC6 sequence element (lane 15).

The addition of Sp1 antibody decreases the intensity of bands A, C and D (lane 12), which may suggest that these bands are caused by Sp1 proteins forming a complex with the GC1 elements in A, C and D. However when Sp1 antibody was added to the reaction containing the labelled bovine GC6 oligonucleotide (lane 16), only the intensity of band A was reduced. These results suggest that the binding of the Sp1 proteins in C and D may be specific to the GC1 element.

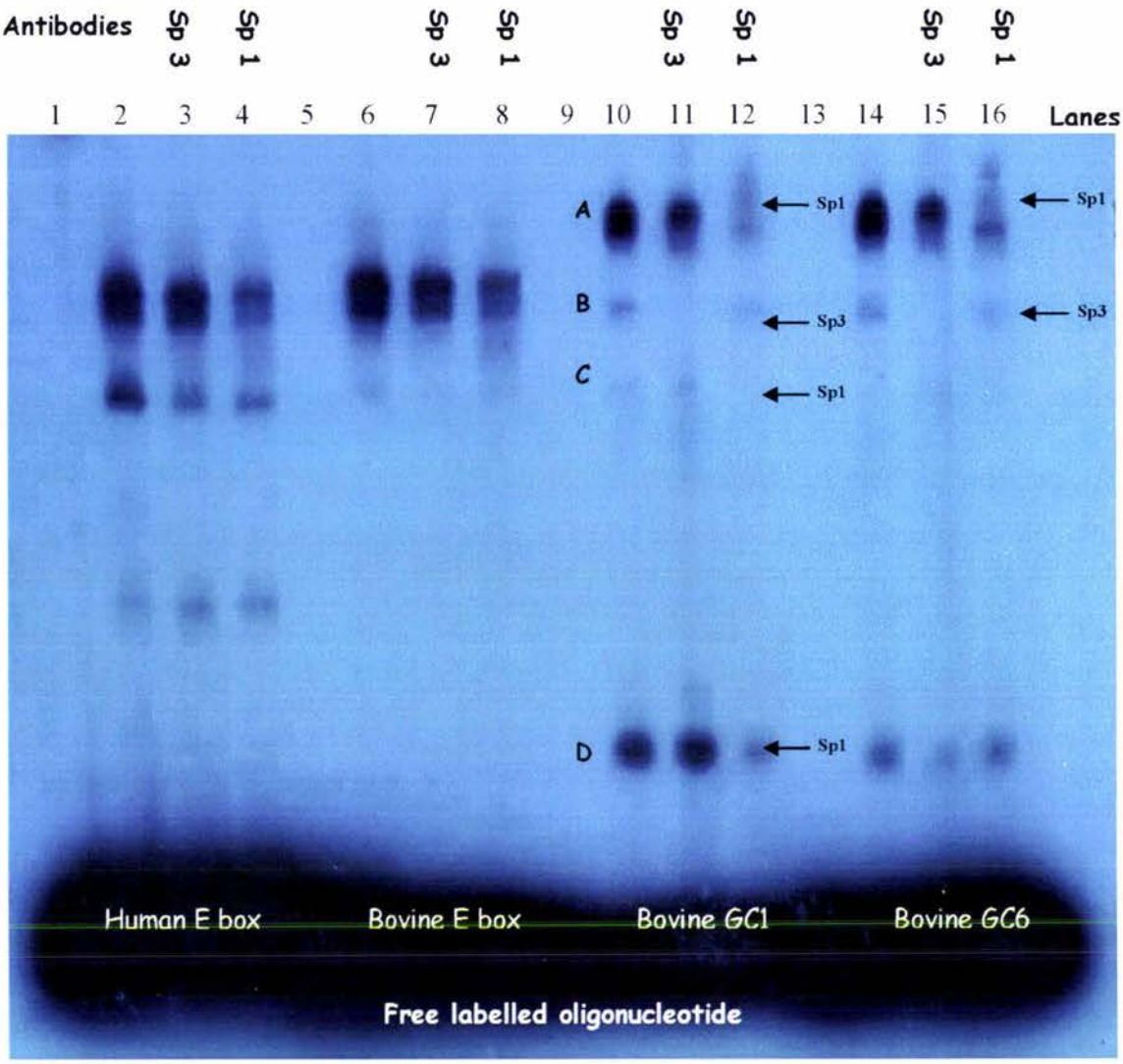


Figure 5.7: Antibody super-shift assays using Sp1 and Sp3 antibodies, the four labelled oligonucleotide and Alexander cellextract

Reactions contained 1µL of the appropriate labelled probe as indicated and 4µL of extract from Alexander cells. 5µL of antibody to either Sp1 or Sp3 was added to the reaction as indicated The 25 µL binding reactions were left to incubate at room temperature before half of each reaction mix was loaded onto a 4 % non-denaturing PAGE and electrophoresis was carried out in 0.25 X TBE for 1 hour and 5 minutes at 200V.

After the gel was dried on DE-81 paper, it was exposed to X-ray film for approximately 18 hours at -70°C in a cassette with intensifying screen.

Lane 1, 5, 9 and 13 are negative control lanes, where no Alexander extract was added to the reaction mix.

5.2.5 EMSA using Alexander cell extract and E boxes elements

5.2.5.1 Unlabelled competitor EMSA using bovine E boxes sequence elements

The competitors used in this assay were; the wild type bovine E box, the wild type human E box, the bovine E box mutant and the human E box mutant (Figure 5.8). These competitors were used to demonstrate the differences or similarities that the bovine E box elements (incomplete) may have in comparison to the human E box element (complete). The unlabelled competitors were added in increasing amount (0 ng - 100 ng) to each reaction mix together with an equal amount of labelled bovine E box, the result are shown in figure 5.8.

The unlabelled bovine E box is an effective competitor to the labelled probe (lanes 1 – 5) as it competes for the same DNA binding proteins as expected. When the unlabelled competitor oligonucleotide is mutated (lanes 6 – 9) it becomes ineffective as a competitor, as there is no change in the bands intensity observed. These results suggest that the observed banding pattern of the bovine E box element is specific as the nucleotides changes in the bovine E box element abolished any competition for the DNA binding proteins.

The unlabelled human E box oligonucleotide is somewhat less effective as a competitor to the bovine homolog (compare lane 10 – 13 with lanes 2-5). This may suggest that the proteins that bind to the bovine E box are recognised by the human E box element; however the binding of these proteins to the human E box is less effective. The mutant human E box competitor is also ineffective as a competitor (lane 14 – 17 of figure 5.8), suggesting that the mutant element does not have the ability to bind any of the specific proteins that bind to the bovine E box this confirms the specificity of the interaction observed.

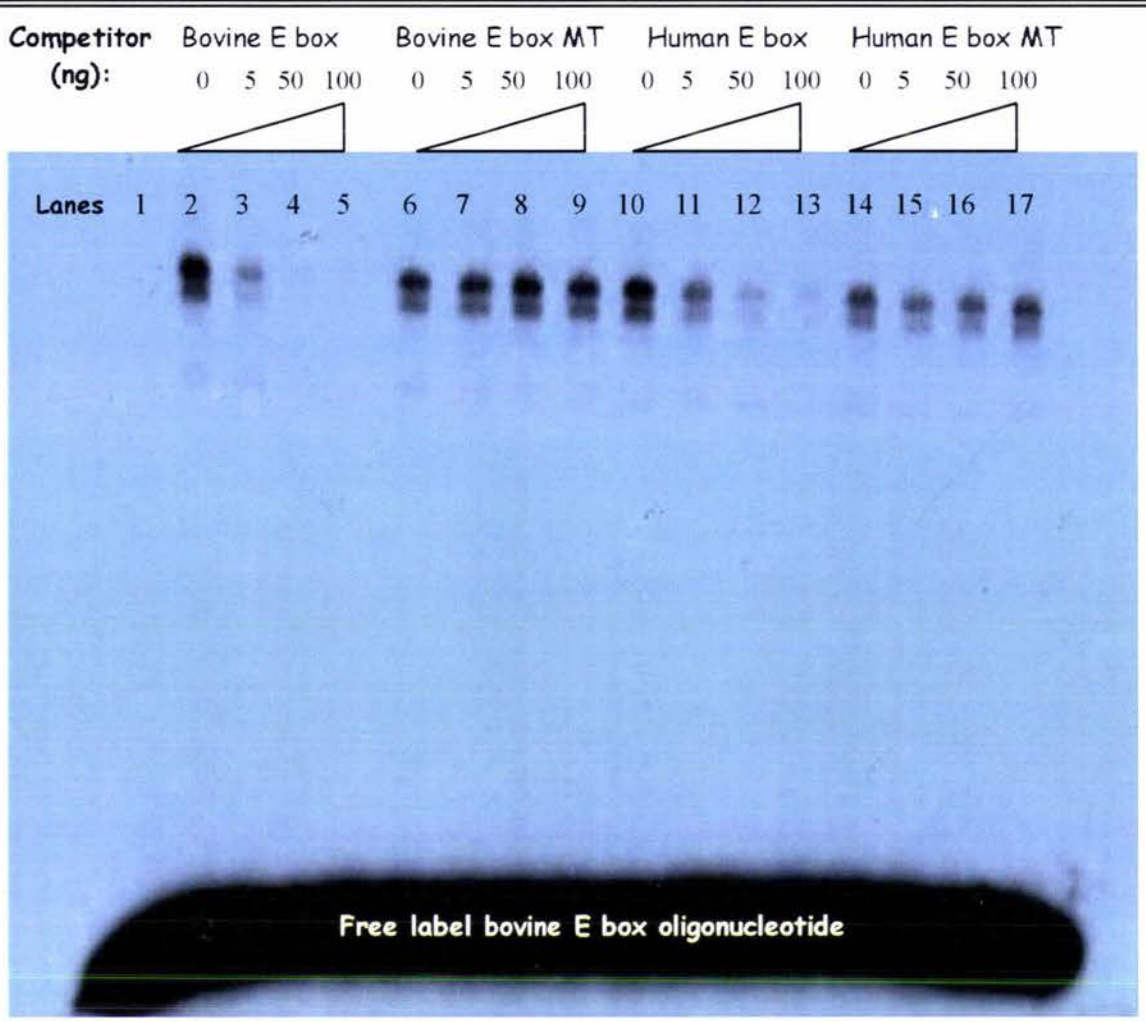


Figure 5.8: Competitor assay using wild type and mutated E box oligonucleotides.
Increasing amounts of unlabelled competitor (0 ng, 5 ng, 50 ng and 100 ng) were added to the binding reactions before the addition of 1µL of Bovine E box labelled oligonucleotide. In each reaction, 1 µL (4.48 µg) of Alexander extract was added.

12.5 µL (half) of each reaction mix was loaded into a 4 % non-denaturing polyacrylamide gel and electrophoresis was carried out in 0.25 X TBE for 1 hour and 5 minutes at 200V. The gel was dried on DE-81 paper and exposed to X-ray film for about 20 hours at -70°C in a cassette with intensifying screen.

The free labelled oligonucleotides are shown as the dark band at the bottom of the gel. The triangle at the top of the gel indicates the increasing amounts of unlabelled competitor.

5.2.5.2 Unlabelled competitor EMSA using Human E box elements

Increasing amount of the unlabelled competitor (0 ng - 100 ng) was added to each reaction mix together with 1μL labelled human E box oligonucleotide (73913 cpm) (Figure 5.9). The competitors contained the sequences of the wild type bovine E box, thw wild type human E box, the bovine E box mutant and the human E box mutant. This experiment is important to investigate the specificity of the proteins binding to the human E box element.

Lanes 2, 6, 10 and 14 (Figure 5.9) are positive control lanes, where 0 ng of competitor was added to the standard EMSA reaction. These lanes showed the distinct banding patterns that are formed by the DNA binding proteins from Alexander cell extract and the labelled oligonucleotide. As expected the addition of increasing amounts of unlabelled wild type human E box oligonucleotide decreases the intensity of all bands (lanes 11–13). This result suggests that the binding of these proteins to the human E box sequence element is specific. A similar result was shown after the addition of the unlabelled mutant human E box to the binding reaction (lanes 14-17). The binding of the proteins to the mutant elements is somewhat less effective when compared to the wild type elements, as there were faint bands observed (compare lanes 10-13 with lanes 14-17). The decrease in the band intensity in these lanes suggests that the mutant human E box sequence still has the ability to bind the same DNA binding proteins. A possible explanation for this is that the human E box sequence element consists of two sequences separated by several nucleotides (Figure 5.2), the presence of this middle sequence may be important for the binding of the proteins.

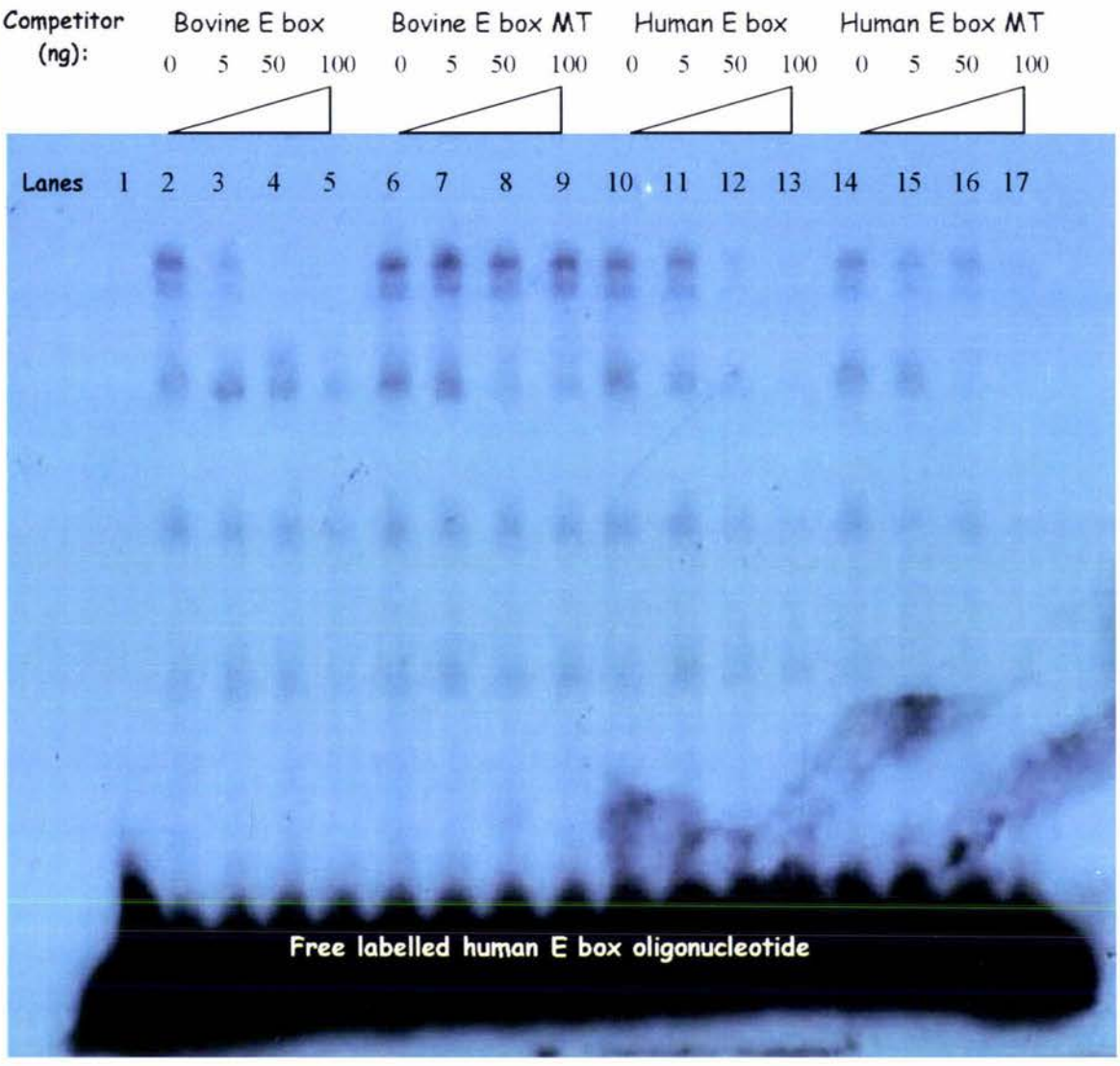


Figure 5.9: Competitor assay using unlabelled wild type and mutated E box oligonucleotide sequences
Increasing amounts of unlabelled competitor (0ng, 5 ng, 50 ng and 100 ng) were added to the binding reaction. 1µL of labelled human E box was added to each binding reaction, along with 1 µL (4.48 µg) of Alexander extract.

Half of each binding reaction (12.5 µL) was loaded into 4 % non-denaturing PAGE and gel electrophoresis was carried out in 0.25 X TBE for about 1 hour at 200V. The gel was dried on DE-81 paper and exposed to X-ray film for about 24 hours at -70°C in a cassette with intensifying screen.

The free labelled oligonucleotide is shown as the dark band at the bottom of the gel. The triangle at the top of the gel indicates the increasing amounts of unlabelled competitor.

When increasing amount of the unlabelled bovine E box competitor was added to the reactions, a decrease in the band intensity was observed (Lane 2-5). The bovine E box sequence element was shown to compete for the binding of proteins with the human E box by removing the top two bands with the lowest mobility. The three bands with the higher mobility were still present after the addition of 100 ng of the bovine E box competitor, which may suggest that these DNA binding proteins are specific to the human E box element.

Increasing amount of the unlabelled bovine E box mutant competitor was added in lanes 6 – 9 (Figure 5.9). The mutated bovine E box sequence was shown to be an ineffective competitor, as there was no decrease in the bands intensity observed. The fact that the change in the nucleotide sequence of the bovine E box abolished any DNA/protein interaction (shown in figure 5.8 lanes 6-10 and figure 5.9 lanes 6-10) suggests that the presence of the middle sequence in the complete E box is important.

The results from these competitor assays demonstrate that the mutated complete (human) E box element was able to compete for the DNA binding proteins (lane 14-17 of figure 5.9) which supports the idea that there are several variations of the complete E box sequence that are functional (Yamada *et al.*, 1999). However the incomplete (bovine) E box element was unable to bind the proteins after the nucleotide changes which may show the importance of the centre region of the element.

5.2.5.3 Antibody super-shift assays using ChREBP antibodies

The ACLY gene promoter was found to have an E box element in the promoter region of the gene that is thought to have a role in the regulation of the gene through the binding of carbohydrate response element binding proteins (Kim *et al.*, 1996). Carbohydrate Response element binding proteins (ChREBP) is a member of the b/HLH/LZ family of transcription factors that are known to bind to E box elements in many genes (Uyeda *et al.*, 2002). To determine the interaction of ChREBPs to the bovine and the human E box elements, the ChREBP antibody was added to each standard EMSA reactions (Figure 5.11).

The addition of ChREBP antibody (2 µL) to the reaction containing the labelled human E box oligonucleotide and undiluted Alexander cell extract (1.5 µL) caused a significant decrease in the intensity of the top two bands (a and b) in lane 3 (Figure 5.11). A decrease in the intensity of bands a and b was also observed when the ChREBP antibody was added to the reaction containing the labelled bovine E box oligonucleotide (lane 6). The decrease in band intensity, observed in lanes 3 and 6 suggests that ChREBP may be present in each of the DNA/protein complexes and that this protein may be forming interaction with other proteins.

2 µL of ChREBP antibody was added to lane 9 and 12 together with 1.5 µL of diluted (2 fold) Alexander extract. The dilution of the extract for these lanes is necessary for optimal interaction between the protein and the bovine GC1 and GC6 sequence elements (shown in figure 5.5). The intensity of the bands in lane 9 and 12 decreases slightly, in particular in band b upon the addition of antibody. A previous EMSA showed that the intensity of band c was reduced in the presence of Sp1 antibody; these results suggest that there may be an interaction between Sp1 and ChREBP. The intensity of bands a, b and c were also reduced upon the addition of ChREBP antibody (lanes 9), this suggest that ChREBP may also be interacting with other DNA binding proteins in these complexes. Sp1 proteins are known to interact with a number of proteins from the bHLH protein family to form multiprotein transcriptional complexes (Biesiada *et al.*, 1999). ChREBPs are a member of bHLH proteins family and therefore it is possible that there may be an interaction between ChREBP and Sp1 and this interaction may regulate the transcription of ACLY gene.

Both of the human and the bovine E boxes appear to have the similar protein binding pattern in EMSA, but the addition of the ChREBP antibody appears to diminish the intensity of both bands (a and b) in the presence of the human E box element (compare lane 3 and 6). A possible explanation for this could be that there may be other proteins that are able to form a complex with ChREBP. ChREBP was shown to form a heteromeric complex with the bHLH/LZ transcription factor Mlx (Max-like protein X) which is ubiquitously expressed in order to activate transcription (Ma *et al.*, 2005; Stoeckman *et al.*, 2004).

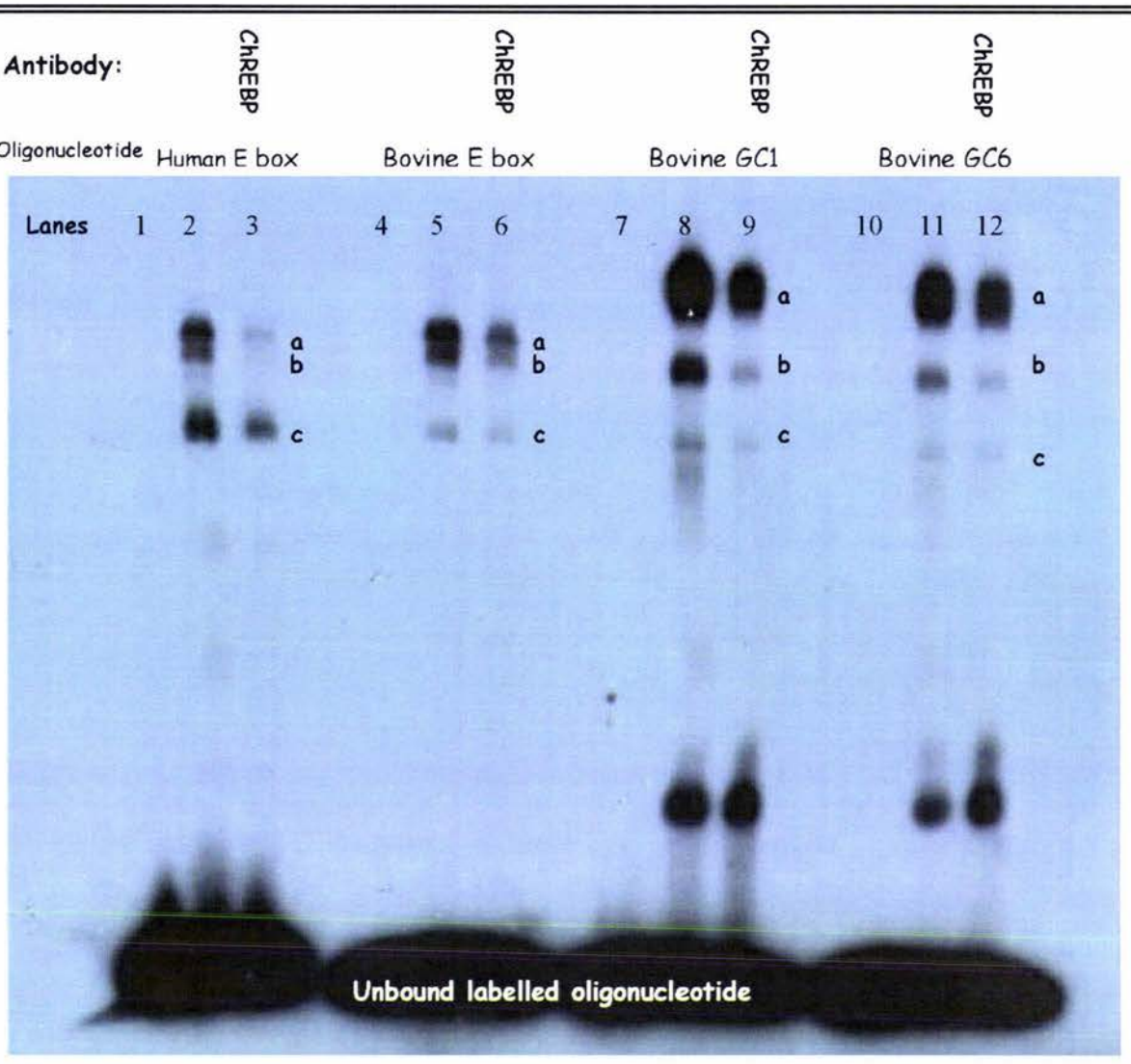


Figure 5.10: Antibody super-shift using ChREBP antibody.
2 µL of ChREBP antibody was added to the reaction mix in lanes 3, 6, 9 and 12 containing 1.5 µL of Alexander cell extract (undiluted for lane 2, 3, 5 and 6 and 2 fold dilution for lane 8, 9, 11 and 12). Lane 1, 4, 7 and 10 are negative control lanes, with no cell extract.

1 µL of labelled oligonucleotide was added to the reaction mix before incubation at room temperature for 10 minutes. The entire sample was loaded onto a 4% non-denaturing PAGE and gel electrophoresis was carried out in 0.25 X TBE for approximately 1 hour at 200V.

The gel was dried onto DE-81 paper and was exposed to X-ray film for approximately 24 hours at -70°C.

Stoeckman *et al* (2004) also showed that the ChREBP/Mlx complex is able to bind to oligonucleotides with single E box sites and act as a transcription repressor. This findings correlates with the fact that the bovine 5' regulatory region was found contain a single E box site (refer to section 3.2.1) and that the E box element was found to interact with proteins from the cell extract (lane 6 of figure 5.5) which may be the key to the down regulation of bovine ATP citrate lyase gene.

5.3 Summary

The specificity of these protein/DNA interactions were demonstrated by the competitor assays where the unlabelled wild type oligonucleotide competitors were able to bind to the proteins present, establishing the bonafide protein/DNA interactions. Further more, the addition of increasing amounts of unlabelled mutant competitor seems to have no effect on the band intensity meaning there is no protein interaction with the mutant oligonucleotides. These assays also indicate that the presence of the central sequence of the human E box is important in the binding of the DNA binding proteins.

The antibody super-shift assay demonstrate the nature of the proteins that bind to the putative *cis* elements in the bovine ACLY promoter. The bovine GC1 and GC6 elements appear to recruit both Sp1 and Sp3 (Figure 5.10). In addition, both the bovine and human E boxes appear to recruit the carbohydrate response element binding protein (Figure 5.11). The addition of the ChREBP antibody also showed that the putative protein-protein interactions may be recruiting proteins to the DNA and it may even be possible that there are other proteins binding to the bovine E box. These proteins may be the Mlx proteins that are known to form a heteromeric protein complexes with the ChREBP (lane 6, figure 5.10).

6.0 Summary and Future Directions

6.1 Introduction

Ruminants are large mammals that have a very different glucose metabolism in comparison to the monogastric animal, due to the low glucose uptake from the diet. Instead of using glucose as the main precursor for fatty acids biosynthesis, ruminants utilise the volatile fatty acid acetate as the main precursor for fatty acid biosynthesis (Houtert, 1993). As a result, the regulation of the enzymes involved in these pathways is expected to be different to that of enzymes in the monogastric animals. ATP citrate lyase is a cytosolic enzyme that catalyses the cleavage of cytosolic citrate into acetyl CoA and oxaloacetate and it catalyses the only unique step in the pathway from glucose to fatty acids. In ruminants, ATP citrate lyase activity is down-regulated which were thought to be due to the adaptation of the animal toward low glucose uptake from feed.

The isolation of the 5' region of the bovine ACLY gene was carried out at Massey University (Tong, 2000). This allowed further investigation of the molecular mechanisms of bovine ACLY gene regulation. The techniques used in this study were the alignment of bovine ACLY gene promoter against other ACLY gene promoter regions, the assays of reporter gene constructs to investigate the minimal promoter activity and electrophoretic mobility shift assays to investigate the DNA binding proteins that may be involved in the transcriptional regulation of the ACLY gene.

These preliminary results help in the understanding of the transcriptional regulation of bovine ATP citrate lyase gene. Further studies are required in this area, and some of these methods outlined in this chapter may be of use to fully understand the molecular mechanisms that down regulate ATP citrate lyase in ruminants.

6.2 Characterisation of ATP citrate lyase gene promoter

6.2.1 Summary and conclusion

The alignment of the bovine ATP citrate lyase, the human ATP citrate lyase and the rat ATP citrate lyase 5' region using clustal W, showed that the three promoters are very similar in term of the putative *cis* elements located in their promoter (discussed further in section 3.1), which suggest that ACLY gene in these species may be regulated in a similar manner.

One of the key differences between the three promoters is the E box sequence elements. Both the bovine and the rat ACLY gene promoter was found to only have a single E box elements, whist the human ACLY gene promoter contains the complete E box motif of 5' – CACGTGNNNNNGCC - 3'. However, both rat and human E box sequence element was found to be glucose responsive. These differences in the E box sequence element may result in the difference in the expression of ATP citrate lyase between monogastric and ruminant as ACLY gene in ruminant was thought to be down regulated as a mean of physiological adaptation for glucose conservation.

6.2.2 Future directions

As a future project, it would be interesting to analysed ACLY gene expression in different tissues such as in bovine liver (ruminant), human Alexander cells (monogastric), calf liver (monogastric) and calf liver during the transition from monogastric of the young calf to the ruminant type of the adult cow (calf transition), as the ACLY gene expression levels may be different in these tissue due to the different glucose metabolism in each animal. The Level of ACLY gene expression in ruminant were thought to be lower than in monogastrict, therefore by using Northern analysis and reverse transcription polymerase chain reaction different levels of expression of ACLY gene in these tissues may be confirmed. Both of these methods measure mRNA levels.

6.2.2.1 Northern analysis

Northern analysis gives a comparative quantification of mRNA levels. For future studies the purification of mRNA from each of the three different tissues (Alexander cells, bovine liver and calf liver) by using oligo (dT) to bind and isolate the poly (A) tail of the mRNA, would allow a comparison of mRNA levels between these tissues. Hybridisation of a radioactively labelled probe (RNA, DNA or oligodeoxynucleotide) with the purified mRNA bound to a solid membrane enables the detection and the quantification of the mRNA level (www.ambion.com). The results from the Northern analysis may confirm that the different level of ACLY gene activity is due to a reduction in the level of mRNA.

6.2.2.2 Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR is the most sensitive technique for the detection and quantification of mRNA. RT-PCR is sensitive enough to quantify mRNA from very small samples when compared to other methods such as Northern blot analysis or RNase protection assays (www.ambion.com).

The RT-PCR method requires a specific enzyme (reverse transcriptase), where it catalyses the conversion of mRNA to cDNA using primers specific to the desired gene. The results from the RT-PCR reactions may be used to determine the level of ATP citrate lyase mRNA from the three different tissues (bovine liver, calf liver, calf liver transition and Alexander cells).

6.3 Transient transfection and luciferase assays

6.3.1 Summary and conclusion

To investigate the minimal promoter activity of bovine ACLY, a series of 5' deletion constructs were made through a PCR deletion method by designing specific primers to produce desired length products. Three deletion constructs were made and ligated into the pGL3B reporter vector which is lacking a promoter but contains the cDNA encoding luciferase (*luc*$^{+}$) cloned from the North American firefly *Photius pyralis* (www.promega.com).

Alexander cells (PCL/PRF/5) were transfected with the three expression reporter constructs. Two of the fused 5′ upstream regions of the bovine ACLY gene were able to drive the expression of the luciferase gene, demonstrating that bovine ACLY gene promoter was functionally active as promoter.

The luciferase assays showed a 50% decrease in luciferase activity when 1000 base pairs from the wild type construct (-1513) were absent from the promoter region. By deleting a further 300 base pairs from the -513 construct, the luciferase activity was totally eliminated (discussed further in section 4.3.2 of chapter 4). The reduction of the relative luciferase activity might be caused by the loss of DNA elements capable of binding activators in the 5′ upstream region of the bovine ACLY gene.

Further experiments are required to give more accurate to define specific regions that influence ACLY gene transcription. These experiments may also identify the minimal promoter region of bovine ACLY gene. Therefore more constructs and further transients transfection experiments chould be carried out (outlined in figure 6.1).

6.3.2 Future directions

6.3.2.1 Additional deletion constructs of ACLY gene promoter and transient transfection assays

Each of the new constructs may be made using the PCR deletion method as discussed in section 4.2. PCR deletion enables the promoter region to be deleted one *cis* element at a time, which would produce a wider range of deletion constructs (Figure 6.1). Each of the deletion constructs may be ligated into pGL3Basic reporter vector (Promega) to be used in transient transfection experiments as done in this study.

The six deletion constructs (figure 6.1) could be used to transfect Alexander cells (as done in 2.2.3.5) and subsequent measurement of relative luciferase activity may be used to accurately pinpoint the key regulatory elements in the 5′ upstream region of the bovine ACLY promoter.

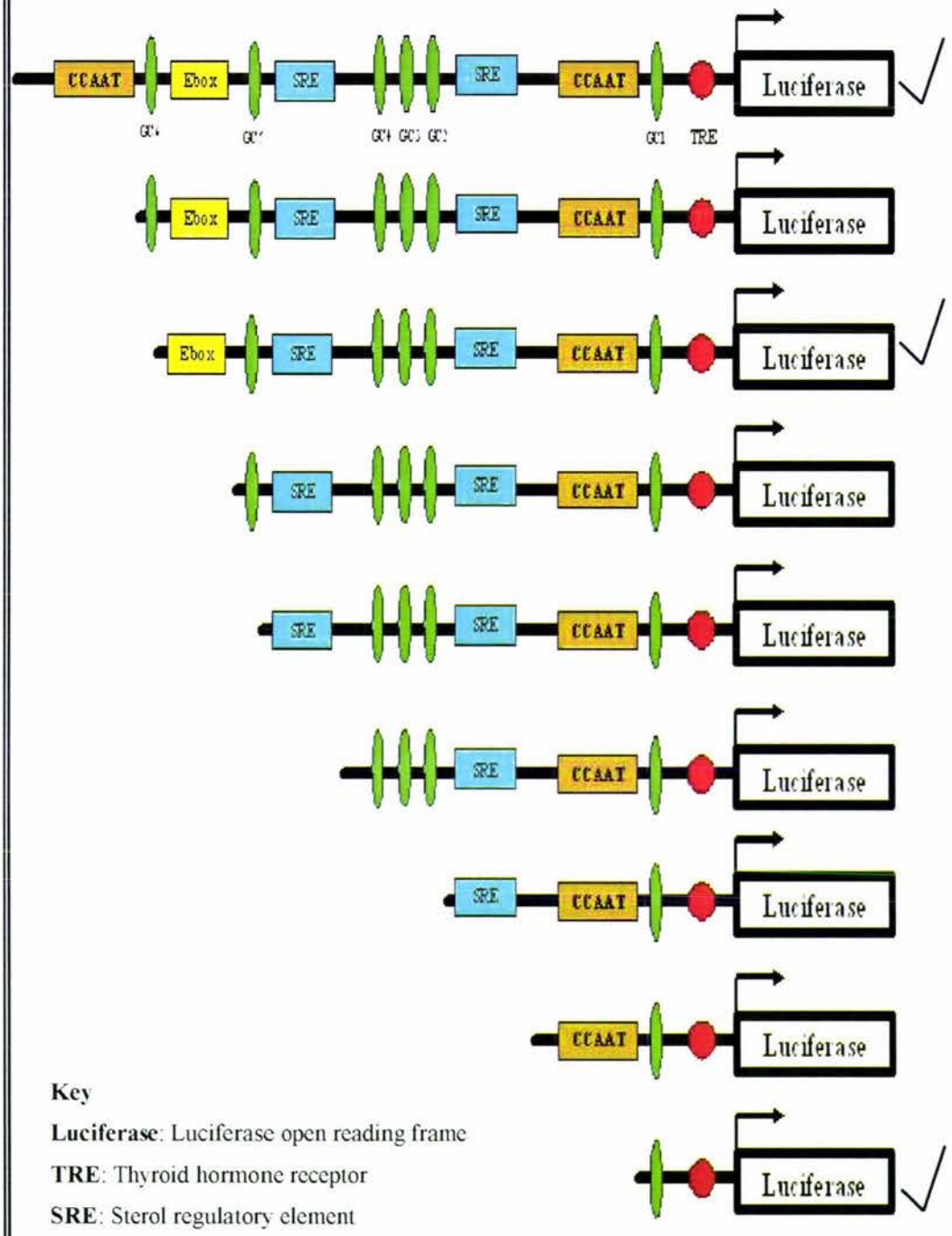


Figure 6.1 ACLY gene promoter deletion constructs showing the predicted elements.
Suggested deletion constructs (made by the PCR deletion method). Lucifrease reporter gene assays may be used to identify regions in the bovine ACLY gene promoter region that are important in the regulation of the gene.
The deletion constructs that were constructed and assayed in this study are indicated by the black tick on the right hand side of the picture

Additional experiments may be done by transfecting expression plasmids which contain cDNA for the transcription factors; Sp1, Sp3, ChREPB and SREBP into the cells used for the transient transfection reactions. The addition of these proteins would enable the investigation of the change in the luciferase gene expression level with the wild type constructs in the presence of excess transcription factors. For example addition of excess Sp1 proteins may decrease the relative luciferase activity as shown by Fukuda *et al.*, (1999), whilst the addition of excess ChREBP may increase the relative luciferase activity (Kim *et al.*, 1996)

The results from assaying each of the nine constructs will give a better understanding of the functions of each of the *cis* elements located in the 5′ upstream region of the bovine ACLY gene.

6.4 Electrophoretic mobility shift assay (EMSA)

6.4.1 Summary and conclusion

Eukaryote gene transcription is usually regulated by many DNA binding proteins that are able to form complexes with each other, such as the TATA binding protein that may form an activation complex with general transcription factors to help recruit RNA polymerase during activation of transcription (Roeder, 1991).

To investigate the association of DNA binding proteins with the ACLY promoter, electrophoretic mobililty shift assays (EMSA) were carried out. Extract from Alexander cells and HeLa cells were used as the protein source for this study . The labelled oligonucleotides used contained sequences for bovine E box, human E box, and bovine GC1 and bovine GC6 elements. These elements were chosen, because previous studies have shown that they regulate ACLY in humans (Kim *et al.*, 1996).

The bovine ACLY promoter has been shown to bind to several DNA binding proteins (refer to chapter 5), the nature of the interactions between these proteins is unknown and it might be the key to different transcriptional regulation between the ruminant and the monogastric animal. The alignment of the bovine ACLY promoter region with the human ACLY promoter shows that the bovine E box sequence is incomplete and that the human ACLY promoter is lacking in the GC6 element (discussed

previously in chapter 3). However, the results from the EMSA and antibody supper shift assays showed that both bovine and human E box interact with the carbohydrate response element binding protein and that the bovine GC1 and GC6 elements interact with both Sp1 and Sp3 (discussed in section 5.2.6.1). The results from the competitor assays demonstrated that the protein/DNA interactions were specific in all four sequence elements that were tested.

6.4.2 Future directions

The preliminary results from EMSA experiments may be extended with further EMSA experiments to give a more thorough understanding of protein/DNA interactions occurring during the regulation of bovine ACLY gene.

Western blot analysis may also be carried out, to investigate and quantify the amount of DNA binding protein such as Sp1, Sp3 and ChREBP that were found to bind to bovine ACLY promoter. Immunoprecipitation experiments may be carried out to investigate any protein – protein interactions at the promoter to influence the transcriptional regulation of bovine ACLY gene.

6.4.2.1 EMSA

Further EMSA experiments could be carried out using oligonucleotides specific to each element (shown in figure 6.2.). As sources for DNA binding proteins Alexander cell extracts, bovine liver nuclear extract, calf liver nuclear extract and calf liver transition nuclear extract may be used. The results from using the four different extracts may be compared and any differences identified. The results from this could also be compared to the western blot analysis. The bovine liver nuclear extracts may be used to compare with the monogastrict cell nuclear extract. The difference in the type of DNA binding protein and the binding pattern of protein/DNA complex between the monogastric animal (human and bovine calf) and ruminant can be analysed which may be able to demonstrate the difference in the regulation between the two animals.

6.4.2.2 Western blots

To determine the molecular weight of a protein and to measure the relative amount of protein in different samples, western blot analysis could be preformed. As a future study, quantitative immunoblotting may be used to investigate the amount of transcription factor (Sp1, Sp3 and ChREBP) presence in the four different nuclear extract (Alexander cell extract, bovine liver nuclear extract, calf liver nuclear extract and calf liver transition nuclear extract). This may confirm the hypothesis that the bovine ACLY gene is down regulated in mature bovine liver by the down-regulation of the essential transcription factors. The transcription factors that may be regulating bovine ACLY promoter are: Sp1 and Sp3, which were found to bind to human and bovine GC1 and GC6 elements; ChREBP that binds to both bovine and human E boxes (Refer to section 5.2.6); NFY that is known to bind to inverted CCAAT boxes (Mantovani, 1998) and SREBP that is known to bind to the human and rat SRE (Sato *et al.*, 2000)

In addition this work shows that the bovine ACLY promoter was able to drive the expression of luciferase gene in Alexander cell lines. The western blot results should be able to confirm whether the down regulation of ATP citrate lyase in ruminant is due to the down-regulation of essential transcription factors involved in the regulation of gene expression.

6.4.2.3 Immunoprecipitation

Immunoprecipitation of ChREBP could be used to determine protein-protein interactions between ChREBP and other proteins such as USF1, Sp1, Sp3 or NFY. This method involves the reversible cross-linking step of the proteins in the cell using formaldehyde to isolate the DNA/protein complex. This complex may then be analysed using immunodetection to identify the DNA binding protein-protein interaction by using a number of antibodies to various transcription factors. The immunoprecipitation experiments may be able to give insights to the transcription factors that regulate the bovine ACLY gene.

6.5 Conclusion

The molecular regulation of bovine ATP citrate lyase is expected to be different when compared to the regulation of ATP citrate lyase from other monogastric animals due to the different fatty acid biosynthesis pathway in ruminant. ATP citrate lyase was thought to be down-regulated as a physiological adaptation for glucose conservation. However, this study demonstrates that the bovine ATP citrate lyase gene promoter was able to drive the expression of luciferase reporter gene in Alexander cell line by transient transfection and luciferase assays. The EMSA experiments showed that transcription factor Sp1, Sp3 and ChREBP interact with bovine ACLY promoter suggesting that these transcription factors may play roles in the regulation of the gene. This study may be the first step in the understanding of the molecular regulation of bovine ATP citrate lyase gene and which may lead toward further understanding for the differences in the molecular regulation of fatty acid biosynthesis between ruminant and monogastric animals.

7.0 References

Allen, M. S., Bradford, B. J., and Harvatine, K. J. (2005). The cow as a model to study food intake regulation. Annual Review of Nutrition *25*, 523-547.

Ammanamanchi, S., Freeman, J. W., and Brattain, M. G. (2003). Acetylated Sp3 Is a Transcriptional Activator. Journal of Biological Chemistry *278*, 35775-35780.

Armstrong, S. A., Barry, D. A., Leggett, R. W., and Mueller, C. R. (1997). Casein kinase II-mediated phosphorylation of the C terminus of Sp1 decreases its DNA binding activity. The Journal of Biological Chemistry *272*, 13489-13495.

Berwick, D. C., Hers, I., Heesom, K. J., Moule, K., and Tavare, J. M. (2002). The identification of ATP-citrate lyase as a protin kinase B (Akt) substrate in primary adipocytes. Journal of Biological Chemistry *277*, 33895-33900.

Biesiada, E., Hamamori, Y., Kedes, L., and Sartorelli, V. (1999). Myogenic basic helix-loop-helix proteins and Sp1 interact as components of a multiprotein transcriptional complex required for activity of human cardiact α-actin promoter. Molecular and Cellular Biology *19*, 2577-2584.

Brown, M. S., and Goldstein, J. L. (1997). The SREBP pathway: Regulation of cholesterol metabolism by proteolysis of membrane-bound transcription factor. Cell *89*, 331-340.

Brun, T., Roche, E., Kim, K. H., and Prentki, M. (1993). Glucose regulates acetyl-CoA carboxylase gene expression in pancreatic beta-cell lines (INS-1). Journal of Biological Chemistry *268*, 18905-18911.

Crabtree, B., Taylor, D. J., Coombs, J. E., Smith, R. A., Templer, S. P., and Smith, G. H. (1981). The activities and intracellular distribution of enzymes of carbohydrate, lipid and ketone-body metabolism in lactating mammary glands from ruminants and non-ruminants. Biochemical Journal *196*, 747-756.

Dooley, K. A., Millinder, S., and Osborne, F. T. (1998). Sterol regulation of 3-Hydroxy-3-Methylglutaryl-coenzyme A Synthase gene through a direct interaction between Sterol Regulatory Element Binding Protein and the trimeric CCAAT-binding factor/Nuclear factor Y. Journal of Biological Chemistry *273*, 1349-1356.

Eaton, S., Bartlett, K., and Pourfarzam, M. (1996). Mammalian mitochondrial beta-oxidation. Biochemical Journal *320*, 345-357.

Elshourbagy, N. A., Near, J. C., Kmetz, P. J., Sathe, G. M., Southan, C., Strickler, J. E., Gross, M., Young, J. F., Wells, T. N. C., and Groot, P. H. E. (1990). Rat ATP Citrate-Lyase. The Journal of Biological Chemistry *265*, 1430-1435.

Ferre, P., Foretz, M., Azzout-Marniche, D., Becard, D., and Foufelle, F. (2001). Sterol-regulatory-element-binding protein 1c mediates insulin action on hepatic gene expression. Biochemical Society transactions *29*, 547-552.

Fliers, E., Kereier, F., Voshol, P. J., Havekes, L. M., Sauerwein, H. P., Kalsbeek, A., Buijst, R. M., and Romijn, J. A. (2003). White Adipose Tissue: Getting Nervous. Journal of Neuroendocrinology *15*, 1005-1010.

Forest, C., Tordjman, J., Glorian, M., Duplus, E., Chauvet, G., Quette, J., Beale, E. G., and Antoine, B. (2003). Fatty Acid Recycling in Adipocytes: a Role for Glyceroneodenesis and Phosphoenolpyruvate Carboxykinase. Biochemical Society transactions *31*, 1125-1160.

Foufelle, F., Gouhot, B., Pegorier, J., Perdereau, D., Girard, J., and Ferre, P. (1992). Glucose stimulation of lipogenic enzyme gene expression in cultured white adipose tissue. A role for glucose 6-phosphate. Journal of Biological Chemistry *267*, 20543-20546.

Foufelle, F., Gouhot, B., Perdereau, D., Girard, J., and Ferre, P. (1994). Regulation of lipogenic enzyme and phosphoenolpyruvate carboxykinase gene expression in cultured white adipose tissue: Glucose and insulin effects are antagonized by cAMP. European Journal of Biochemistry *223*, 893-900.

Fukuda, H., and Iritani, N. (1999). Regulation of ATP citrate lyase gene expression in hepatocytes and adipocytes in normal and genetically obese rats. Journal of Biochemistry *126*, 437-444.

Fukuda, H., Iritani, N., Katsudara, A., and Noguchi, T. (1996). Insulin and polyansaturated fatty acid responsive region(s) of rat ATP citrate lyase gene promoter. FEBS Letter *308*, 204-207.

Fukuda, H., Iritani, N., and Noguchi, T. (1999). Transcriptional regulation of fatty acid synthase gene and ATP citrate lyase gene by SP1 and SP3 in rat hepatocytes. FEBS Letter *464*, 112-117.

Girard, J., Ferre, P., and Foufelle, F. (1997). Mechanism by which carbohydrates regulates expression of gene for glycolytic and lipogenic enzymes. Annual Review of Nutrition *17*, 325-352.

Goodwin, G. W., Ahmad, F., Doenst, T., and Taegtmeyer, H. (1998). Energy provision from glycogen, glucose, and fatty acids on adrenergic stimulation of isolated working rat hearts. Am J Physiol Heart Circ Physiol *274*, H1239-1247.

Hagen, G., Muller, S., Beato, M., and Suske, G. (1994). Sp1-mediated transcriptional activation is repressed by Sp3. EMBO J *13*, 3843-3851.

Han, I., and Kudlow, J. E. (1997). Reduced O glycosylation of Sp1 is associated with incread proteosome susceptibility. Molecular and Cellular Biology *17*, 2550-2558.

Hanahan, D., Jessee, J., and Bloom, F. R. (1991). Plasmid transformation of *Escherichia coli* and other bacteria. Methods in enzymology *204*, 63-84.

Hanson, R. W., and Ballard, F. J. (1967). The relative significance of acetate and glucose as precursors for lipid synthesis in liver and adipose tissue from ruminants. Biochemical Journal *105*, 529-356.

Holmes, D. S., and Quigley, M. (1981). A rapid boiling method for the preparation of bacterial plasmid. Analytical Biochemistry *114*, 193-197.

Hornton, J. D., Goldstein, J. L., and Brown, M. S. (2002). SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. Journal of clinical investigation *109*, 1125-1131.

Horowitz, K. B., Jackson, T. A., Bain, D. L., K., R. J., S., T. G., and L., T. (1996). Nuclear receptors coactivators and corepressors. Molecular Endocrinology *10*, 1167-1177.

Horton, J. D. (2002). Sterol regulatory element-binding proteins: transcriptional activators of lipid synthesis. Biochemical Society *30*, 1091-1095.

Horton, J. D., Goldstein, J. L., and Brown, M. S. (2002). SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. Journal of clinical investigation *109*, 1125-1131.

Houtert, M. F. J. V. (1993). The production and metabolism of volatile fatty acids by ruminants feed roughages: A review. Animal Feed Science & Technology *34*, 189-225.

Kawaguchi, T., Takenoshita, M., Kabashima, T., and Uyeda, K. (2001). Glucose and cAMP regulate the L-type pyruvate kinase gene by phosphorylation/ dephosphorylation of the carbohydrate response element binding protein. PNAS *98*, 13710-13715.

Kennett, S. B., Udvadia, A. J., and Horowitz, M. J. (1997). Sp3 encodes multiple proteins that differ in their capacity to stimulate or repress transcription. Nucleic acid research *25*, 3110-3117.

Khan, S. A., and Heuvel, J. P. V. (2003). Role of Nuclear Receptors in The Regulation of Gene Expression ob Dietary Fatty Acids. Journal of Nutritional Biochemistry *14*, 554-556.

Kim, K.-S., Kang, J.-G., Moon, Y.-A., Park, S.-W., and Kim, Y.-S. (1996). Regulation of the ATP-Citrate Lyase Gene Transcription. Yonsei Medical Journal *37*, 214-224.

Kim, K.-S., Park, S.-W., Moon, Y.-A., and Kim, Y.-S. (1994). Organisation of the 5' region of the rat ATP citrate lyase gene. Biochemical Journal *302*, 759-764.

Kim, S.-Y., Kim, H.-i., Kim, T.-H., Im, S.-S., Park, S.-K., Lee, I.-K., Kim, K.-S., and Ahn, Y.-H. (2004). SREBP1c Mediates the Insulin-dependent Hepatic Glucokinase expression. The Journal of Biological Chemistry *279*, 30823-30829.

Lania, L., Majello, B., and Luca, P. D. (1997). Transcriptional regulation by the Sp family proteins. International journal of biochemistry and cell biology *29*, 1313-1323.

Ma, L., Tsatsos, N. G., and Towle, H. C. (2005). Direct role of ChREBP/Mlx in regulating hepatic glucose-responsive genes. Journal of Biological Chemistry *280*, 12019 - 12027.

Magan, N. (2003) Basal transcription of human toposiomerase II, Massey university, Palmerston north.

Mantovani, R. (1998). A survey of 187 NF-Y binding CCAAT boxes. Nucleic acid research *26*, 1135-1143.

Mastrangelo, I. A., Courey, A. J., Wall, J. S., Jackson, S. P., and Hough, P. V. C. (1991). DNA looping and Sp1 multimeric links: a mechanism for transcriptional synergism and enhancement. PNAS *88*, 5670-5674.

Moncur, J. T., Park, J. P., Memoli, V. A., Mohandas, T. K., and Kinlaw, W. B. (1998). The "Spot 14" gene reside on the telomeric end of the 11q13 amplicon and is expressed in lipogenic breast cancers: Implication for control of tumor metabolism. PNAS *95*, 6989-6994.

Moon, Y.-A., Kim, K.-S., Park, S.-W., and Kim, Y.-S. (1996). Cloning and identification of exon-intron organistion of the rat ATP Citrate Lyase gene. Biochimica et Biophysica acta *1307*, 280-284.

Moon, Y.-A., Lee, J., Park, S.-W., Ahn, Y., and Kim, K.-S. (2000). The role of sterol regulatory element binding proteins in the transactivation of the rat ATP Citrate Lyase promoter. Journal of Biological Chemistry *275*, 30280-30286.

Moon, Y.-A., Park, S.-W., and Kim, K. (2002). Characterisation of cis-acting elements in rat ATP citrate lyase gene promoter. Experimental & Molecular Medicine *34*, 60-68.

Nadeau, K. J., Leitner, J. W., Gurerich, I., and Draznin, B. (2004). Insulin Regulation of Stereol Regulatory Element-binding Protein-1 expression in L-6 Muscle Cells and 3T3 Adipocytes. The Journal of Biological Chemistry *279*, 34380-34387.

Norman, G. R., and Streiner, D. L. (1994). Biostatistic; The Bare Essential (St. Louis, missourie, Mosby).

Park, S.-W., Moon, Y.-A., Kim, K., Ahn, Y., and Kim, Y.-S. (1997). Cloning and characterisation of the 5' flanking region of human ATP citrate lyase gene. Biochimica et Biophysica acta *1353*, 236-240.

Ramakrishna, S., and Benjamin, W. B. (1979). Fat cell portein phosphorylation: identification of phosphoprotein-2 as ATP-citrate lyase. Journal of Biological Chemistry *254*, 9232-9236.

Ramakrishna, S., Murthy, K. S., and Benjamin, W. B. (1989). Effect of insulin on ATP-citrate lyase phosphorylation: Regulation of peptide A and peptide B phosphorylation. Biochemistry *28*, 856-860.

Remmers, E. F., Goldmuntz, E. A., Cash, J. M., Crofford, L. J., Misiewicz-Poltorak, B., Zha, H., and Wilder, R. L. (1992). Genetic map of the nine polymorphic loci comprising a single linkage group on rat chromosome 10: Evidence for lingkage conservation with human chromosome 17 and mouse chromosome 11. Genomic *14*, 618-623.

Roeder, R. G. (1991). The complexities of eukaryotic transcription initiation: regulationof preinitiation complex assembly. TIBS *16*, 402-408.

Saffer, J. D., Jackson, S. P., and Annarella, M. B. (1991). Developmental expression of Sp1 in the mouse. Molecular and Cellular Biology *11*.

Sato, R., Okamoto, A., Inoue, J., Miyamoto, W., Sakai, Y., Emoto, N., Shimano, H., and Maeda, M. (2000). Transcriptional regulation of the ATP citrate lyase gene by sterol regulatory element binding proteins. The Journal of Biological Chemistry *275*, 12497-12502.

Shimomura, I., Shimano, H., Hornton, J. D., Goldstein, J. L., and Brown, M. S. (1997). Elevated levels of SREBP-2 and cholesterol synthesis in livers of mice homozygous for a target distruption of SREBP-1 gene. Journal of clinical investigation *99*, 838-845.

Stoeckman, A. K., Ma, L., and Towle, H. C. (2004). Mlx in the functional heteromeric partner of the carbohydrate response element-binding protein in glucose regulation of lipogenic enzyme genes. Journal of Biological Chemistry *279*, 15662-15669.

Sul, H. S., and Wang, D. (1998). Nutritional and hormonal regulation of enzymes in fat synthesis: Studies of fatty acid synthase and mitochondrial glycerol-3-phosphate acyltransferase gene trancription. Annual Review of Nutrition *18*, 331-351.

Suske, G. (1999). The Sp-family of transcription factors. Gene *238*, 291–300.

Tong, X. (2000) Isolation and characterisation of the 5' region sequence of the bovine ATP citrate lyase gene, MSc, Massey University, Palmerston North.

Towle, H. C., Kaytor, E. N., and Shih, H.-M. (1997). Regulation of the Expression of Lipogenic Enzyme Gene by Carbohydrate. Annual Review of Nutrition *17*, 405-433.

Uyeda, K., Yamashita, H., and Kawaguchi, T. (2002). Carbohydrate Responsive Element-Binding Protein (ChREBP). Biochemical Pharmachology *63*, 2075-2080.

Walt, J. G. V., and Linington, M. J. (1989). A review of energy metabolism in producing ruminant. Journal of the South African Veterrinary Association *60*, 223-227.

Wang, H., and Wollheim, C. B. (2002). ChREBP Rather than USF2 Regulates Glucose Stimulation of Endogenous L-pyruvate Kinase Expression in Insulin-secreting Cells. The Journal of Biological Chemistry *277*, 32746-32752.

Willingham, M. (2004). Regulation of the human topoisomerase II beta promoter, Massey University, Palmesrton North.

Yamada, K., Tanaka, T., and Noguchi, T. (1999). Characterization and purification of carbohydrate response element binding protein of the rat L-type pyruvate kinase gene promoter. Biochemical and Biophysical Research Communication *257*, 44-49.

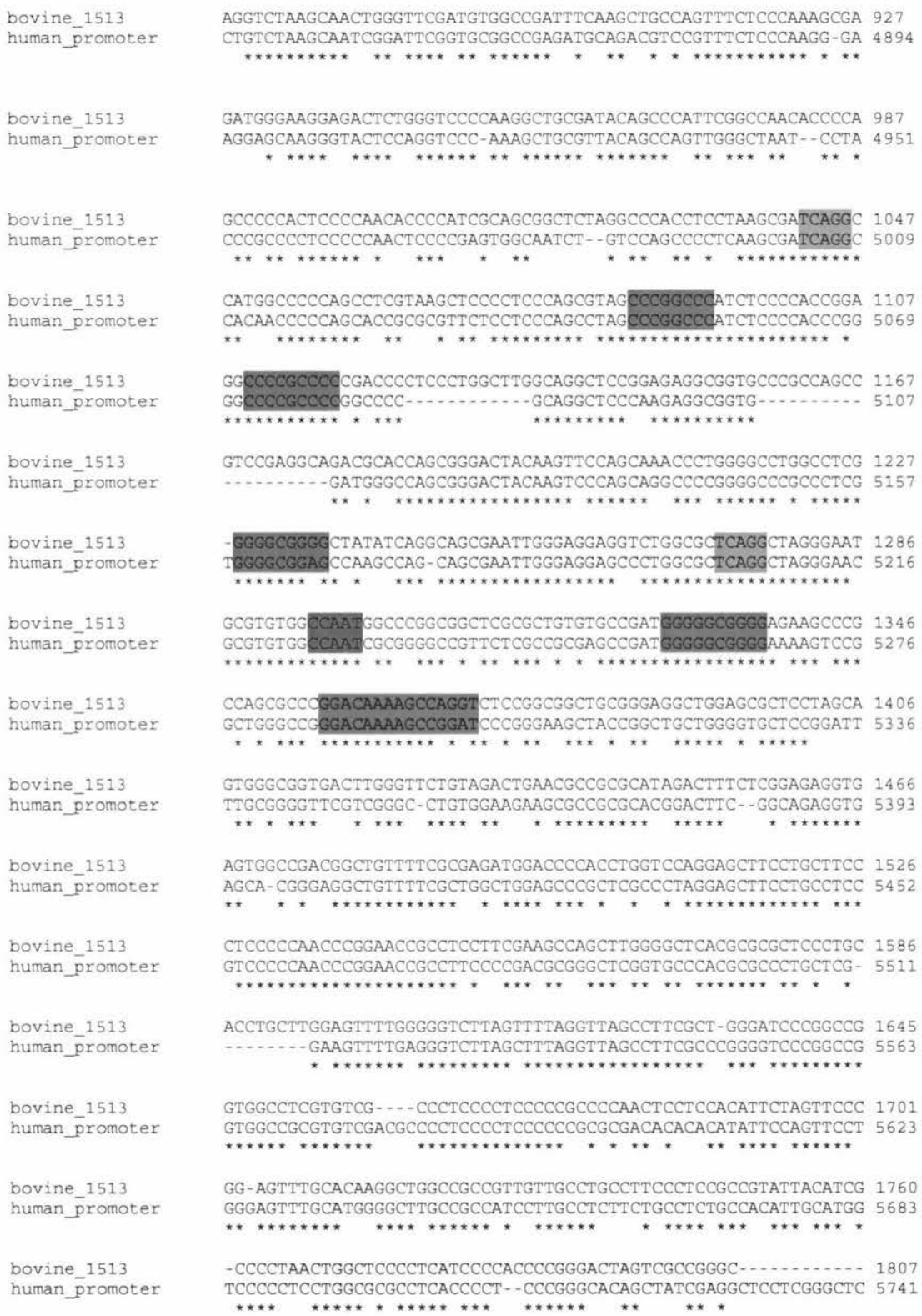
Yamashita, H., Takenoshita, M., sakurai, M., Bruick, R. K., Henzel, W. J., Shilinglaw, W., Arnot, D., and Uyeda, K. (2001). A glucose-responsive transcripion factor that regulates carbohydrate metabolism in the liver. PNAS *98*, 9116-9121.

Young, J. W. (1977). Gluconeogensis in cattle: Significance and methodology. Journal of Dairy Science *60*, 1-15.

Appendix 1 – CLUSTAL W

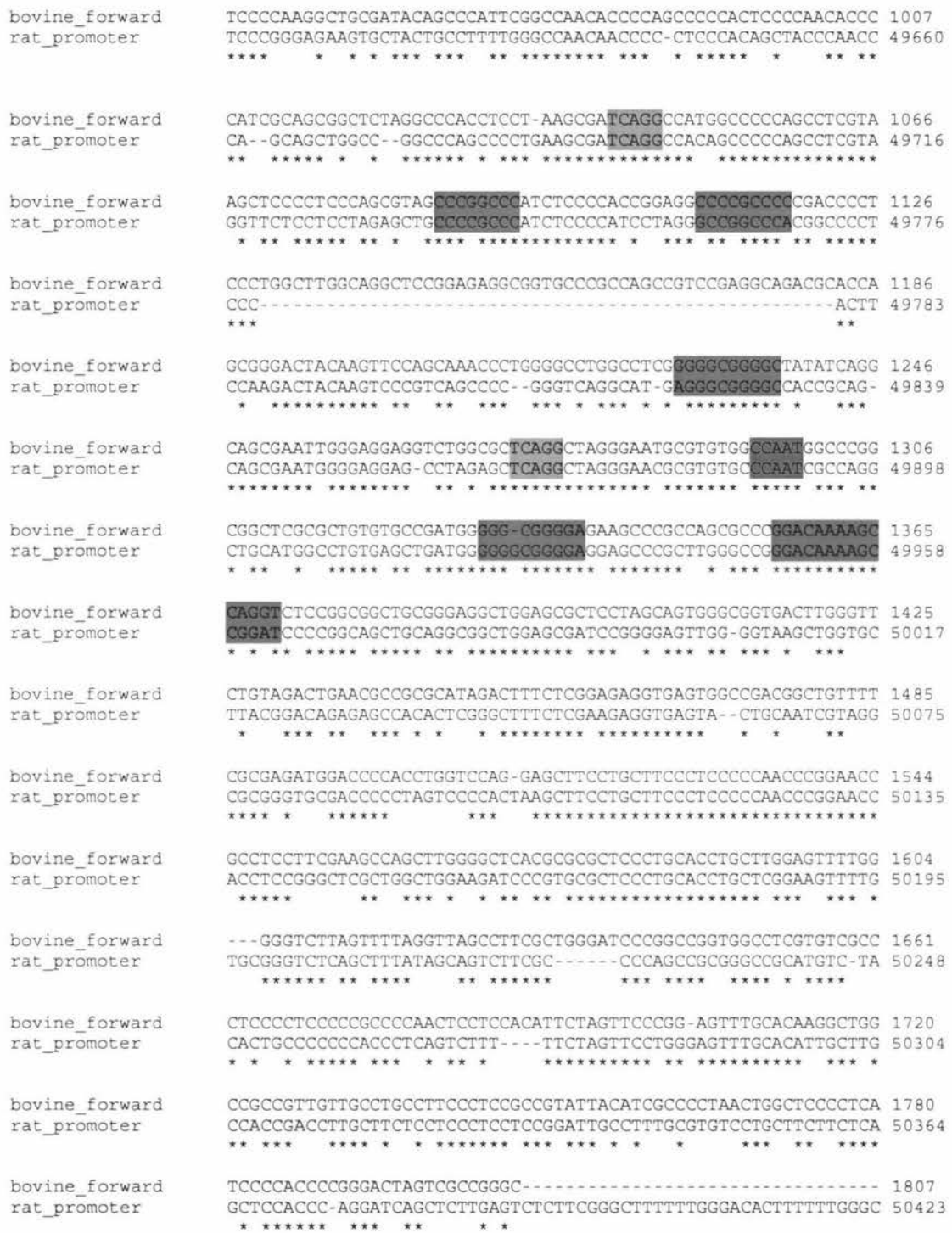
A. Sequence alignment for bovine and human promoter region

```
bovine_1513        ------------------------------------------------------------A 1
human_promoter     TTTGTAGAGATGGGGTTTTGCCTTGTTGGTCTTGAACTCCTGACCTCAAGTGATCCCCCA 3900
                                                                               *

bovine_1513        TCTGAGTA----GGGGTGGAGCTATTCCATGTAGGAGTGG--AAGGCT------CTCTAT 49
human_promoter     CCTTGGCCTTCCAAAGTGCTGGGATTACAGGTGTGAGCCACCAAGACTGGCCACCCCCAT 3960
                    **  *          ***  *  *** ** **  ***      *** **      * * **

bovine_1513        ----GACCAACAGGAGAGAGGAGCTGAGGAAATGCTGTTTCAAAAAGATGCCTGTGAGGG 105
human_promoter     TTTAGGACAAGAGAAGAGAGGTGATAAGGAACTGCTATTTCAAAAAGATGAATGGGAGGG 4020
                       *  *** ** ******* * * ***** **** ************  ** *****

bovine_1513        AAGGGACCAGAAACTGACATCATCAGGGATCACTTTGTTCAGGCATGGCCAACAAGTGTA 165
human_promoter     AAGGGACCAGAAAGTGACATCATCAGGGAACACTTGATTCAGGCATGGGTAGCAAGTTCA 4080
                   ************ *************** *****  ***********  * *****  *

bovine_1513        TTG-------------------------GGGGGAGAAGGATTTGGCCAGGTTTTTGTGT 199
human_promoter     GTTACCTTACTCCTGGCAGCAACAGGATTGGGGGAGGGGGTCTTGGAGAAGATTTTGTCT 4140
                    *                          *******  **  ****  * * ****** *

bovine_1513        ACAGCAATGTGGTAGCACTGTTACAGTGTGA-------CCCCAGCGCCTGGCAGGAACAC 252
human_promoter     GCA--AATGTCGCAACACTGTTGCAGTGTGAGTATGACCCAAAGTGCTTGGCAGGGGGAC 4198
                    **  ***** * * ******* ********       **  ** ** *******   **

bovine_1513        AGACATGTGGTCTGTTTGCACACTGCGCTGACCCTTTCACCTGAGCTGCTCAGCGCATGA 312
human_promoter     AGGCCTGCTGCTTCCCTGCATTCTGGGCTGACCTCTTCACCTGAGTCGGTCAGTGGACGC 4258
                   ** * **  *  *   ****  *** *******  **********  * **** * * *

bovine_1513        T-----CCTCCTTCCTGGAGCTAGACAGCTGTTAGGGTCAGGGAGCAGCTCGAGAGACCT 367
human_promoter     TTTTGGCCTCCTTCCTGGAGCTAGACAGCTGTTAGGCTTAGGGAGCAGCTCCGGA--CCT 4316
                   *     ******************************** * ************  **  ***

bovine_1513        AGAGGACTGGGATGGACATAGGAAAG----CTCCAGGATGTCTCAGCAGTAAATCTATGT 423
human_promoter     ACAGGGTTGGGGTGCAGGTAGGGAGGGAGGCTGCAGCATGTCAGAGTGGTAAATACATGT 4376
                   * ***  **** ** *  **** * *    ** *** *****  **  ******  ****

bovine_1513        CTTGGTTTCTAGGACTGTCTGATTTTAAAAATTCTGTCCCACAAGTTCACCAATACCTGT 483
human_promoter     CCTGATTTCCAGGACT----GATTTCACATATTCTGCCCCACAAGTTCACCAGCACCTGT 4432
                   * ** **** ******    ***** * * ****** ***************  ******

bovine_1513        CAGACCAGGTGTCCCAGTTTTTGATTTGGAAAATGCACTCTGTTTCCCCGGTAATGGAGA 543
human_promoter     CAGATTGGATGTCCCAGTTTTTGGTTTGGAAAATTCGATCTGTCTC--AGGTAATGGAGA 4490
                   ****   * ************** ********** *  ***** **   ***********

bovine_1513        AGGGGAAGGCAGCAGTTCATTCATTCCTCTAGTCTAAGCGTGAGAT-AGTGGGGGCAGGG 602
human_promoter     AGGGGGAGACAGCAGTTCATTCATTCCAGT-GTCTAAGCCTGTGGTTGGTGGGAACAAGA 4549
                   ***** ** ******************  * ******** ** * *  *****  ** *

bovine_1513        CGGAGAGCCCGAGCAGCAGAGATGGCCCCAGCCTTGCGCTAGAATCTCTCATTGAATTCT 662
human_promoter     ----------GACTGTCTGGGATGGCTCCAGCCCTTCGCTGGAATCTCGCATTGAATTCC 4599
                             **    * * ****** ****** * **** ******* **********

bovine_1513        CTGCTCTGTGCGCCCAGGTGAGTGGATGAGTGGTGGTGACTGACGCGAGGTG--GTGATT 720
human_promoter     TAGCTCCGCCCTGATCCGTGCTGAGGCGAGCAGGTGAGTGGGACGGACAGCGCTGTGCCT 4659
                     **** *  *      ***    *  ***  *  * *   ****    * *  ***  *

bovine_1513        GACCCCTCCTAGCCCCACCTGCTCACGCGGTCCTCCGCCCTATCCCATTTACAGTGGGAG 780
human_promoter     GCACCCTCCTAGCCCCACTTGCTTACCGCGGCCCCTGCCTTATCCCA----CACTGGGAA 4715
                   *  *************** **** **   * ** * *** *******    ** *****

bovine_1513        GTCTCATCTATC--TCTGCTCTCGGCCTCGCTCCACCAGCTTAAGGAAGAGAGTCCA--- 835
human_promoter     GTATGCTCTGTTAGCCTCGGCTCAGCCTTGCCTCATCGGCCCAGTGGGGAGAGTCCAGGG 4775
                   ** *  *** *    **   *** **** **  ** * **  *  *  *********

bovine_1513        ---------------CGTGCAAATGGG-----------CAAGCAGTCTTGC--AAAAAGT 867
human_promoter     AGGAGAGAGGCTCCACGTGCTAAAGGAAAGCGGCCTGCCAGACACTGCTGCTTAAGGAGA 4835
                                 ***** ** **           **  ** *  ***  **  **
```


bovine_1513 AGGTCTAAGCAACTGGGTTCGATGTGGCCGATTTCAAGCTGCCAGTTTCTCCCAAAGCGA 927
human_promoter CTGTCTAAGCAATCGGATTCGGTGCGGCCGAGATGCAGACGTCCGTTTCTCCCAAGG-GA 4894
bovine_1513 GATGGGAAGGAGACTCTGGGTCCCCAAGGCTGCGATACAGCCCATTCGGCCAACACCCCA 987
human_promoter AGGAGCAAGGGTACTCCAGGTCCC-AAAGCTGCGTTACAGCCAGTTGGGCTAAT--CCTA 4951
bovine_1513 GCCCCCACTCCCCAACACCCCATCGCAGCGGCTCTAGGCCCACCTCCTAAGCGATCAGGC 1047
human_promoter CCCGCCCCTCCCCCAACTCCCCGAGTGGCAATCT--GTCCAGCCCCTCAAGCGATCAGGC 5009
bovine_1513 CATGGCCCCCAGCCTCGTAAGCTCCCCTCCCAGCGTAGCCCGGCCCATCTCCCCACCGGA 1107
human_promoter CACAACCCCCAGCACCGCGCGTTCTCCTCCCAGCCTAGCCCGGCCCATCTCCCCACCCGG 5069
bovine_1513 GGCCCCGCCCCCGACCCCTCCCTGGCTTGGCAGGCTCCGGAGAGGCGGTGCCCGCCAGCC 1167
human_promoter GGCCCCGCCCCGGCCCC----------GCAGGCTCCCAAGAGGCGGTG---------- 5107
bovine_1513 GTCCGAGGCAGACGCACCAGCGGGACTACAAGTTCCAGCAAACCCTGGGGCCTGGCCTCG 1227
human_promoter ----------GATGGGCCAGCGGGACTACAAGTCCCAGCAGGCCCCGGGGCCCGCCCTCG 5157
bovine_1513 -GGGGCGGGGCTATATCAGGCAGCGAATTGGGAGGAGGTCTGGCGCTCAGGCTAGGGAAT 1286
human_promoter TGGGGCGGAGCCAAGCCAG-CAGCGAATTGGGAGGAGCCCTGGCGCTCAGGCTAGGGAAC 5216
bovine_1513 GCGTGTGGCCAATGGCCCGGCGGCTCGCGCTGTGTGCCGATGGGGGCGGGGAGAAGCCCG 1346
human_promoter GCGTGTGGCCAATCGCGGGGCCGTTCTCGCCGCGAGCCGATGGGGGCGGGGAAAAGTCCG 5276
bovine_1513 CCAGCGCCCGGACAAAAGCCAGGTCTCCGGCGGCTGCGGGAGGCTGGAGCGCTCCTAGCA 1406
human_promoter GCTGGGCCGGGACAAAAGCCGGATCCCGGGAAGCTACCGGCTGCTGGGGTGCTCCGGATT 5336
bovine_1513 GTGGGCGGTGACTTGGGTTCTGTAGACTGAACGCCGCGCATAGACTTTCTCGGAGAGGTG 1466
human_promoter TTGCGGGGTTCGTCGGGC-CTGTGGAAGAAGCGCCGCGCACGGACTTC--GGCAGAGGTG 5393
bovine_1513 AGTGGCCGACGGCTGTTTTCGCGAGATGGACCCCACCTGGTCCAGGAGCTTCCTGCTTCC 1526
human_promoter AGCA-CGGGAGGCTGTTTTCGCTGGCTGGAGCCCGCTCGCCCTAGGAGCTTCCTGCCTCC 5452
bovine_1513 CTCCCCCAACCCGGAACCGCCTCCTTCGAAGCCAGCTTGGGGCTCACGCGCGCTCCCTGC 1586
human_promoter GTCCCCCAACCCGGAACCGCCTTCCCCGACGCGGGCTCGGTGCCCACGCGCCCTGCTCG- 5511
bovine_1513 ACCTGCTTGGAGTTTTGGGGGTCTTAGTTTTAGGTTAGCCTTCGCT-GGGATCCCGGCCG 1645
human_promoter --------GAAGTTTTGAGGGTCTTAGCTTTAGGTTAGCCTTCGCCCGGGGTCCCGGCCG 5563
bovine_1513 GTGGCCTCGTGTCG----CCCTCCCCTCCCCCGCCCCAACTCCTCCACATTCTAGTTCCC 1701
human_promoter GTGGCCGCGTGTCGACGCCCCTCCCCTCCCCCCGCGCGACACACACATATTCCAGTTCCT 5623
bovine_1513 GG-AGTTTGCACAAGGCTGGCCGCCGTTGTTGCCTGCCTTCCCTCCGCCGTATTACATCG 1760
human_promoter GGGAGTTTGCATGGGGCTTGCCGCCATCCTTGCCTCTTCTGCCTCTGCCACATTGCATGG 5683
bovine_1513 -CCCCTAACTGGCTCCCCTCATCCCCACCCCGGGACTAGTCGCCGGGC------------ 1807
human_promoter TCCCCCTCCTGGCGCGCCTCACCCCT--CCCGGGCACAGCTATCGAGGCTCCTCGGGCTC 5741

B. Sequence alignment for bovine and rat promoter region

```
bovine_forward      ----------------------------ATCTGAGTAGGGGTGGAGCTATTCCATGTAGG 32
rat_promoter        CCTGAATTTGAGCAACTTCATAGAAGACATTTACTTAGGGA-GAAGTGATCCCTCACAGA 48599
                                             ** *   *****  * **  ** **    **

bovine_forward      AGT---GGAAGGCTCT--------CTATGACCAACAGGAGAGAGGAGC-TGAGGAAATGC 80
rat_promoter        GATTTGGGTAGGTTCTAGGCCTTTCTCTCAACTTCAGGAGCAAGGGGCACTATAAACTGT 48659
                      *   ** *** ***        ** * * *  ******  *** **   *  ** **

bovine_forward      TGTTTCAAAAAGATGCCTGTGAGGGAAGGGACCAGAAAC-TGACATCATCAGGGATCACT 139
rat_promoter        TATTTCTAAAGAATAAGTATGAGGGAAGGGACCAAAAAAATGACATCATCAAGGAGCACA 48719
                    * **** ***  **   * ***************** ***  *********** *** ***

bovine_forward      T--TGTTCAGGCATGGCC--AACAAGT-------------------------GTATTGG 169
rat_promoter        CGCTGGTCAAGCATGGGTGTAACAAGTTCAGCCAGCCTTTGGAGGAGGGCTTGGGAGTGG 48779
                       ** *** ******    *******                          * * ***

bovine_forward      GGGGAGAAGGATTTGGCCAGGTTTTTGTGTACAGCAATGTGGTAGCACTGTTACAGTG-- 227
rat_promoter        GGGAGGGGGGCTTGGGAAAAGTGTTTGCCCAGGAGAGTGTTGCAACATTGTTGCAGGGAG 48839
                    *** *  ** ** **  * ** ****   *    * *** * * ** **** *** *

bovine_forward      ----TGACCCCAG-CGCCTGGCAGGAACACAGACATGTGGT-----CTGTTTGCACACTG 277
rat_promoter        CTTTTGACCCAGGGTTTTTGCCAGGGGCATAGCCATGCCACGCTGATTCTCTGCACTCTG 48899
                        ******  *     ** ****  ** ** ****          * * ***** ***

bovine_forward      CGCTGACCCTTTCACCTGAGCTGCTCAGCGCATGAT-----CCTCCTTCCTGGAGCTAGA 332
rat_promoter        GGCTGATGCAT-CACCTGAGCCGATTAGAGGATGATTCTGGCCTCCTTCCTAGAGCTGGA 48958
                     *****  * * ********* * * ** * *****     ********** ***** **

bovine_forward      CAGCTGTTAGGGTCAGGGAGCAGCTCGAGAGACCTAG-AGGACTGGGATGGACATAGGAA 391
rat_promoter        AAATTGTTTGTTTCTGGGACCGTCTGGGAAGACATACTGGGACTAAGGTACAAGCAAAAG 49018
                     *  **** *  ** **** *  ** *  **** **   *****  * *  *   *  *

bovine_forward      AG--CTCCAGGATGTCTCAGCAGTAAATCTATGTCTTGGTTTCTAGGACTGTCTGATTTT 449
rat_promoter        AGGCTTTCACTCCAGGGAAGCAGTAAATCGGAGTTCTGATTTCCAGGACGTTCTGATTTT 49078
                    **   * **         ***********   **  ** **** *****  *********

bovine_forward      AAAAATTCTGTCCCACAAGTTCACCAATACCTGTCAGACCAGGTGTCCCAGTTTTTGATT 509
rat_promoter        ATATATTCTGCTCCACAAGT---CGGGCACCTGTTAGACTGGGTGCTCCACTTTTGGATT 49135
                    * * ******  ********   *    ****** ****  ****  *** **** ****

bovine_forward      TGGAAAATGCACTCTGTTTCCCCG--GTAATGGAGAAGGGGAAGGCAGCAGTTCATTCAT 567
rat_promoter        TGGAAAATATGTTCTACCTCTCATATGTAACAGAAAAGAAGAGGA-AGCTGTTCATTGCT 49194
                    ********    ***   ** *    ****  ** ***  ** *  *** *******  *

bovine_forward      TCCTCTAGTCTAAGCGTGAGATAGTGGGGGCAGGGCGGAGAGCCCGAGCAGCAGAGATGG 627
rat_promoter        TTCAGT-GTCTTAGTTTGAG-TTGTGGGAG-GGAGCAAGAGGTTGGA----CAGGGATGG 49247
                    * *  * **** **  **** * ***** *  * **     *   **    *** *****


bovine_forward      CCCCAGCCTTGCGCTAGAATCTCTCATTGAATTCTCTGCTCTGTGCGCCCAGGTGAGTGG 687
rat_promoter        CCCCAGTGCTAGGTTTTAATCTCAGAT-AAATGATCAGCTCCGCCCAGATCCGCGCTCAG 49306
                    ******   *  * *  ******  **  ***  ** **** *  *      * *    *

bovine_forward      ATGAGTGGTGGTGACTGACGCGAGGTGGTGATTGACCCCTCCTAGCCCCACCTGCTCACG 747
rat_promoter        GTGAGCC-CGTAAGAGGACTCGCGTTGGAACTGGCGTCCTTCTGGCCTCACTAGTTTACC 49365
                     ****    *      *** ** * ***   * *   *** ** *** ***  * * **

bovine_forward      CGGTCCTCCGCCCTATCCCATTTACAGTGGGAGGTCTCATC---TATCTCTGCTCT-CGG 803
rat_promoter        TCTTCCTCCTATCTATGT--TCTACGTCGGGCAGTCTGATTGTGTAGCGTGGTCATGTAG 49423
                       ******   ****    * ***   ***  **** **    ** *   *   *   *

bovine_forward      CCTCGCTCCACCA---------------GCTTAAGGA----AGAGAGTCCACGTGCAAAT 844
rat_promoter        TCTGATTCTAGTAAATTCTATGGGGTGGGTTTCAGGAGCGGAGAGGCGCCACGTGTGAAA 49483
                     **   ** *  *              * ** ****    ****   *******  **

bovine_forward      GG---GCAAGCAGTCTTGCAAAAAGTAGGTCTAAGCAACTGGGTTCGATGTGGCCGATTT 901
rat_promoter        GGAAAGCAGACGTAGCTTTCAAAAGCAGGTCTACCGCACTGG-TTTGGTGTGGCGGCTTA 49542
                    **   ***  *     *   ***** *******    ***** ** * ****** * **

bovine_forward      CAAGC--------------TGCCAGTTTCTCCCAAAGCGAGATGGGAAGGAGACTCTGGG 947
rat_promoter        CAAACGCCAGAGATAAGAACGTCAGTTGCTCCCAGGG-GAGGGAGGGAGGGGACGCCAGG 49601
                    *** *               * ***** ******  * ***   ** *** *** *  **
```


bovine_forward TCCCCAAGGCTGCGATACAGCCCATTCGGCCAACACCCCAGCCCCCACTCCCCAACACCC 1007
rat_promoter TCCCGGGAGAAGTGCTACTGCCTTTTGGGCCAACAACCCC-CTCCCACAGCTACCCAACC 49660
bovine_forward CATCGCAGCGGCTCTAGGCCCACCTCCT-AAGCGATCAGGCCATGGCCCCCAGCCTCGTA 1066
rat_promoter CA--GCAGCTGGCC--GGCCCAGCCCCTGAAGCGATCAGGCCACAGCCCCCAGCCTCGTA 49716
bovine_forward AGCTCCCCTCCCAGCGTAGCCCGGCCCATCTCCCCACCGGAGGCCCCGCCCCCGACCCCT 1126
rat_promoter GGTTCTCCTCCTAGAGCTGCCCCGCCCATCTCCCCATCCTAGGGCCGGCCCACGGCCCCT 49776
bovine_forward CCCTGGCTTGGCAGGCTCCGGAGAGGCGGTGCCCGCCAGCCGTCCGAGGCAGACGCACCA 1186
rat_promoter CCC----------ACTT 49783
bovine_forward GCGGGACTACAAGTTCCAGCAAACCCTGGGGCCTGGCCTCGGGGGCGGGGCTATATCAGG 1246
rat_promoter CCAAGACTACAAGTCCCGTCAGCCCC--GGGTCAGGCAT-GAGGGCGGGGCCACCGCAG- 49839
bovine_forward CAGCGAATTGGGAGGAGGTCTGGCGCTCAGGCTAGGGAATGCGTGTGGCCAATGGCCCGG 1306
rat_promoter CAGCGAATGGGGAGGAG-CCTAGAGCTCAGGCTAGGGAACGCGTGTGCCCAATCGCCAGG 49898
bovine_forward CGGCTCGCGCTGTGTGCCGATGGGGG-CGGGGAGAAGCCCGCCAGCGCCCGGACAAAAGC 1365
rat_promoter CTGCATGGCCTGTGAGCTGATGGGGGGCGGGGAGGAGCCCGCTTGGGCCGGGACAAAAGC 49958
bovine_forward CAGGTCTCCGGCGGCTGCGGGAGGCTGGAGCGCTCCTAGCAGTGGGCGGTGACTTGGGTT 1425
rat_promoter CGGATCCCCGGCAGCTGCAGGCGGCTGGAGCGATCCGGGGAGTTGG-GGTAAGCTGGTGC 50017
bovine_forward CTGTAGACTGAACGCCGCGCATAGACTTTCTCGGAGAGGTGAGTGGCCGACGGCTGTTTT 1485
rat_promoter TTACGGACAGAGAGCCACACTCGGGCTTTCTCGAAGAGGTGAGTA--CTGCAATCGTAGG 50075
bovine_forward CGCGAGATGGACCCCACCTGGTCCAG-GAGCTTCCTGCTTCCCTCCCCCAACCCGGAACC 1544
rat_promoter CGCGGGTGCGACCCCCTAGTCCCCACTAAGCTTCCTGCTTCCCTCCCCCAACCCGGAACC 50135
bovine_forward GCCTCCTTCGAAGCCAGCTTGGGGCTCACGCGCGCTCCCTGCACCTGCTTGGAGTTTTGG 1604
rat_promoter ACCTCCGGGCTCGCTGGCTGGAAGATCCCGTGCGCTCCCTGCACCTGCTCGGAAGTTTTG 50195
bovine_forward ---GGGTCTTAGTTTTAGGTTAGCCTTCGCTGGGATCCCGGCCGGTGGCCTCGTGTCGCC 1661
rat_promoter TGCGGGTCTCAGCTTTATAGCAGTCTTCGC------CCCAGCCGCGGGCCGCATGTC-TA 50248
bovine_forward CTCCCCTCCCCCGCCCCAACTCCTCCACATTCTAGTTCCCGG-AGTTTGCACAAGGCTGG 1720
rat_promoter CACTGCCCCCCCCACCCTCAGTCTTT----TTCTAGTTCCTGGGAGTTTGCACATTGCTTG 50304
bovine_forward CCGCCGTTGTTGCCTGCCTTCCCTCCGCCGTATTACATCGCCCCTAACTGGCTCCCCTCA 1780
rat_promoter CCACCGACCTTGCTTCTCCTCCCTCCTCCGGATTGCCTTTGCGTGTCCTGCTTCTTCTCA 50364
bovine_forward TCCCCACCCCGGGACTAGTCGCCGGGC---------- 1807
rat_promoter GCTCCACCC-AGGATCAGCTCTTGAGTCTCTTCGGGCTTTTTTGGGACACTTTTTTGGGC 50423

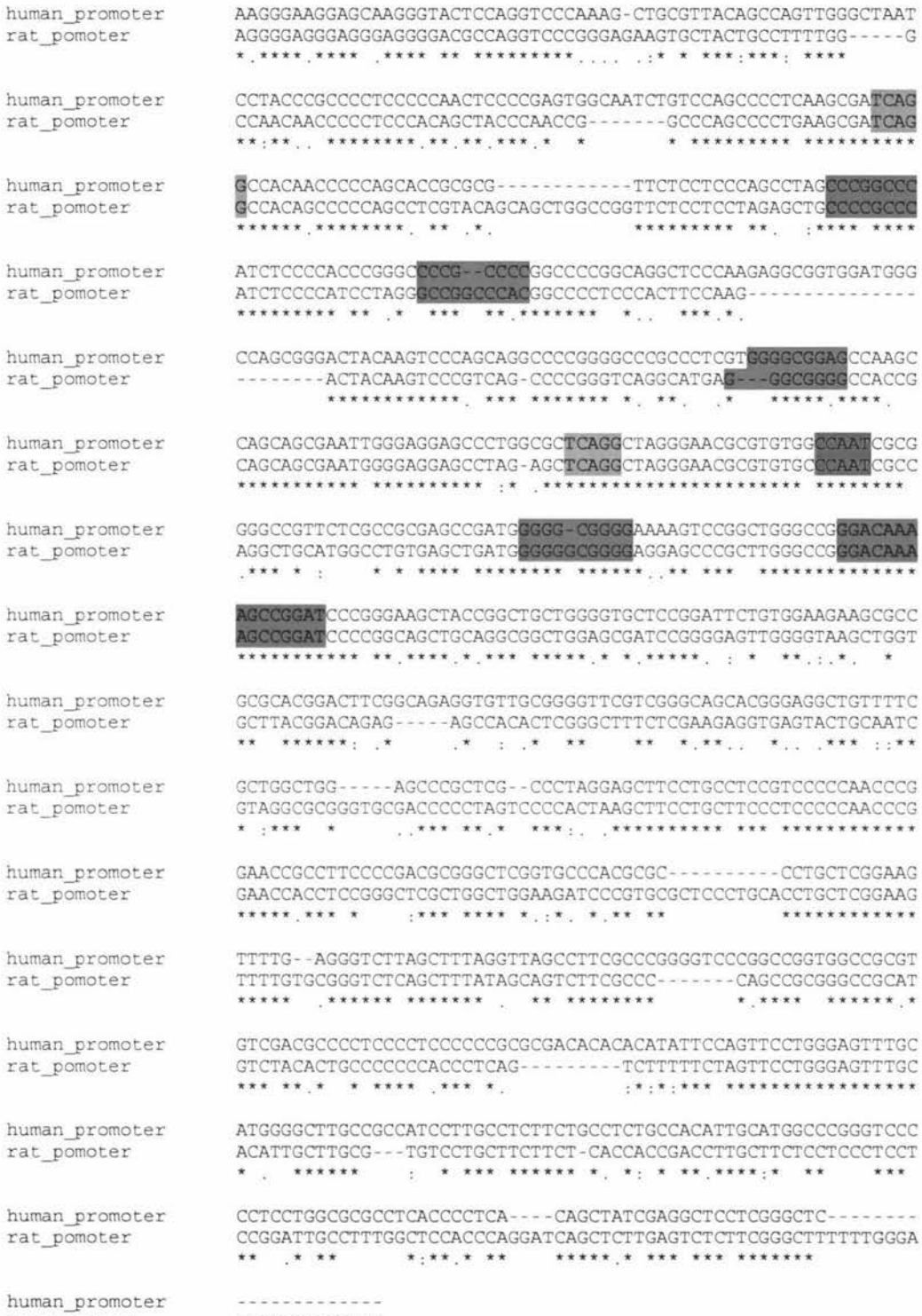
C. Sequence alignment for mouse and bovine promoter region

```
mouse_promoter      GAGATTAAAAACAGCAGCTACTCTGTCTTCAAGGCCTCCTTTTACTGCCCCCCCCCCCCG 60
bovine_forward      ------------------------------------------------------------

mouse_promoter      CAGAGGGGAGCGTTTAAGCAGCTTTCTAAGAGGTGGAAATGATCTTTCCAGGA-CAGTTA 119
bovine_forward      --------------------ATCTGAGTAGGGGTGGAGCTATTCCATGTAGGAGTGGAAG 40
                                      *    ** ******  *  **  *  ****   *

mouse_promoter      TCTCTCGATAGTAAAGAAACCCGACATGCTGAGCAAAGTTAAAGCGGGGACAGACAGGGT 179
bovine_forward      GCTCTCTATGACCAACAGGAGAGAGGAGCTGAGGAAAT-----GCTGTTTCAAAAAGA-- 93
                     ***** **    ** *     **   ****** ***      ** *   ** * **

mouse_promoter      TGGCTGGCAGACTAAAGTGCCTTATGCTGTCCTTTCCAAA-ATTATGTCAGG-AGGGATC 237
bovine_forward      TGCCTGTGAGGG-AAGGGACCAGAAACTGACATCATCAGGGATCACTTTGTTCAGGCATG 152
                    ** ***  **   ** *  **  *  *** * *   **   ** *  *     *** **

mouse_promoter      CCTCATAGCAGAATTTCAAGACTGAAGAAAGCAGCAAGCTTTGGGGACCAGACCTCTGCC 297
bovine_forward      GCCAACAAGTGTATTGGGGGGAGAAGGATTTGGCCAGGTTTTTGTGTACAGCAATGTGGT 212
                     *  * *   * ***    *    * **      ** * *** * *  ***   * **

mouse_promoter      AGGCCTGACAGGAGGTAGAGGTAGGTAGCATCCTTTGCACACACGCGCGCCCGCGCACAT 357
bovine_forward      AGCACTGTTACAGTGTGACCCCAGCGCCTGGCAGGAACACAGACATGTGGTC-----TGT 267
                    **  *** *    **       **        *     **** **  * *  *       *

mouse_promoter      ACACACACCCTAGGTGCTTGTCTCATCCAGACTCCTGAGCACTCCGACCATCTGTTGTTT 417
bovine_forward      TTGCACACTGC-GCTGACCCTTTCACCTGAGCTGCTCAGCGC----ATGATCCTCCTTCC 322
                       *****    * **    * *** *    ** ** *** *    *  ***     *

mouse_promoter      GGGGGCTACACCAGTAGGGAGCTCCTTAGCAGGGAGAGCCCATCCAGCGCCGTCGCTCCG 477
bovine_forward      TGGAGCTAGAC--------AGCTGTTAGGGTCAGGGAGCAGCTCGAGAGACCTAG---AG 371
                     ** **** **        ****  *  *    * ****   ** ** * * * *    *

mouse_promoter      GAGCCAAGTCCTCGCAGCAAGGCCTCAGGCACTCTGAAGCAGCACGACCCCGCGTGGCCA 537
bovine_forward      GACTGGGATGGACATAGGAAAGCTCCAGGATGTCTCA-GCAGTAAATCTATGTCTTGGTT 430
                    **      *  *  ** ** **  ****   *** * **** *   *   *  * *

mouse_promoter      GGGCCCGCTCCCTACACTTCGACATAGAGCTTAAGGGATTCACCACAGAACTAACAGGAC 597
bovine_forward      TCTAGGACTGTCTGATTTTAAAAATTCTGTCCCACAAGTTCACCA-ATACCTGTCAGACC 489
                          **  **    **  * **   *    *    ******* * * **  ***  *

mouse_promoter      AGGCCTGCTTGAGTCCGGCTTGCGAGCGGC---------CGGCGTGGAGAGAGTAGCCAC 648
bovine_forward      AGGTGTCCCAGTTTTTGATTTGGAAAATGCACTCTGTTTCCCCGGTAATGGAGAAGGGGA 549
                    ***  * *  *  *  *  ***  *   **         *  **   *  *** **

mouse_promoter      CGCCATCTCTACTACCCGCCCCGGCATCCGGTGGGGAACCCGAATGGGGCCCAAAAAAGT 708
bovine_forward      AGGCAGCAGT-TCATTCATTCCTCTAGTCTAAGCGTGAGATAGTGGGGGCAGGGCGGAGA 608
                     * ** *  *   *  *   **   *  *   * *  *       *****       **

mouse_promoter      GTCCCAAAAAAAGCCCGACTAGACTCGAGAGCTGGCCCTGGGTGGAGCTGAGAAGAAGCA 768
bovine_forward      GCCCGAGCAG----CAGAGATGGCCCCAGCCTTGCGCTAGAATCTCTCATTGAATTCTCT 664
                    * ** *  *     * **   * * * **   **  *  *  *    *   ***    *

mouse_promoter      GGACGCGCAAAGGCAATCCGGAGGAGGGAGAAGTAAGGTCGGTGGCAAGCAATGTGCAAA 828
bovine_forward      GCTC-TGTGCGCCCAGGTGAGTGGATGAGTGGTGGTGACTGACGCGAGGTGGTGATTGAC 723
                    *  *  *      **     * *** *         *   *  *  * *   **    *

mouse_promoter      CTCCCAGGAACTAGAATAGGATTGGGAGGGTGTAGACATGCGGCCCGCGGCTGGGGAGAA 888
bovine_forward      CCCTCCTAGCCC--CACCTGCTCACGCGGTCCTCCGC-CCTATCCCATTTACAGTGGGAG 780
                    * * *     *    *   * *   * **   *   *      ***       * * **

mouse_promoter      GACTACTATAAAGCTTAGACCCGCACAAAACTTCCGAGCAGGTGCAGGGAG--CGCGCGG 946
bovine_forward      GTCTCATCTATCTCTGCTCTCGGCCTCGCTCCACCAGCTTAAGGAAGAGAGTCCACGTGC 840
                    * **  * **   **     * **      *  **        * ** ***  * ** *

mouse_promoter      GAGCGCCCAGCCAGGGAGCCCGGAGGTGGTTCCGGGTTGGGGGAGGGAAGCAGGAAGCTT 1006
bovine_forward      AAATGGGCAAGCAGTCTTGCAAAAAGTAGGTCTAAGCAACTGGGTTCGATGTGGCCGATT 900
                     *  *  **  ***     *   * ** * **   *     **     *   **  * **

mouse_promoter      AGTGGGTACTGGGGGGTCGCACCCGCGCCTACGACTGCCGTACTCACCTCTTCGAGAAAG 1066
bovine_forward      TCAAGCTGCCAG----TTTCTCCCAAAGCGAGATGGGAAGGAGACTCTGGGTCCCCAAGG 956
                        * * *   *   * * ***    * *     *  * *  * *    **   ** *
```

```
mouse_promoter      CCTGAGTGTGGCTCTCTGTCCGTAAGCACCAGCTCACCCCAACTCTTCGGATCGCTCCAG 1126
bovine_forward      CTGCGATACAGCCCATT--CGGCCAACACC--CCAGCCCCCACTCCCCAA--CACCCCA- 1009
                    *     *   ** *  *  * *  * **** *   **** **** *    * * ***

mouse_promoter      CCGCCTGCAGCAGCCGGGGATCCGGCTTTTGTTCCGGCCCAAGTGGGCTCCTCCCCGCCC 1186
bovine_forward      ----TCGCAGCGGCTCTAGGCCCACCTCCTAAGC--GATCA---GGCCATGGCCCCCAGC 1060
                          ***** **    *  **  **  *   *  *  **   ** *    ****   *

mouse_promoter      CCCATCAGCTCACAGGCTAA-GCAGCCCGGCGATTGGGCACAC--GCGTTCCCTAGCCTG 1243
bovine_forward      CTCGTAAGCTCCCCTCCCAGCGTAGCCCGGCCCATCTCCCCACCGGAGGCCCCGCCCCCG 1120
                    * * * ***** *   * *  * ********   *   * ***  * *  ***   ** *

mouse_promoter      AGCTCTAGGCTCCTCCCCATTCGCTGCTGTGGTGGCCCCGCCCTCAGGCCTGACCTGGGG 1303
bovine_forward      ACCCCT---CCCTGGCTTGGCAGGCTCCGGAGAGGCGGTGCCCGCCAGCC--GTCCGAGG 1175
                    * * **   * *   *      *   * *  * ***   **** *  ***    * * **

mouse_promoter      CTGATGGGACT-TGTAGTCCCGGACGTGGGCGGGGCCGTGGGCGGGGCCTAGGATGGGGA 1362
bovine_forward      CAGACGCACCAGCGGGACTACAAGTTCCAGCAAACCCTGGGGCCTGGCCTCGGGGGCGGG 1235
                    * ** *   *   *      *        **    **  ****  ***** **  * **

mouse_promoter      GATGGGCCGGGCGGCT--CTAGGAGGAGAACCTACG---AGGCTGGGGGCTGTGGCCTGA 1417
bovine_forward      GCTATATCAGGCAGCGAATTGGGAGGAGGTCTGGCGCTCAGGCTAGGGAATGCGTGTGG- 1294
                    * *    * *** **    * *******  *   **   ***** ***  ** *    *

mouse_promoter      TCGTTTCAGGGGCTGGGCCGGCCAGCCGCCGGGTTGGGTAGCTGAGGGAGGGGGTTGTTG 1477
bovine_forward      -----CCAATGGCCCGGCGGCTCGCGCTGTGTGCCGATGGGGGCGGGGAGAAGCCCGCCA 1349
                         **  ***  *** * *  *   * * *   *    *****  *   *

mouse_promoter      GCCCAAAAGGCTATAGCACTTCTCCCTGGAC--CTGGCGTCCTCTTCCTCCCTCCCAGGA 1535
bovine_forward      GCGCCCGGACAAAAGCCAGGTCTCCGGCGGCTGCGGGAGGCTGGAGCGCTCCTAGCAGTG 1409
                    ** *        *   **  *****   * *  * ** * *     *   ***  ***

mouse_promoter      GA--GCAACTGACGTTCT-TATCCCAGGCTCT---TGTAAGCCGCCACACCAAACCCAGT 1589
bovine_forward      GGCGGTGACTTGGGTTCTGTAGACTGAACGCCGCGCATAGACTTTCTCGGAGAGGTGAGT 1469
                    *   *  ***   ***** **  *    * *      **  *   * *    *    ***

mouse_promoter      GCGTTAGACCTGCTTTTGAAAAGCTACGTCTGCTTTCCTTTCACACGTGTCGCCTCTCCG 1649
bovine_forward      GGCCGACGGCTGTTTTCGCGAGATGGACCCCACCTGGTCCAGGAGCTTCCTGCTTCCCTC 1529
                    *    *   *** *** *  *         *  * *           * *   ** ** *

mouse_promoter      CTCC----TGGAACCCTCCCCAGGGAATTTACCAGAGCCAGTCTACACGACCACGCTACA 1705
bovine_forward      CCCCAACCCGGAACCGCCTCCTTCGAAGCCAGCTTGGGGCTCACGCGCGCTCCCTGCACC 1589
                    * **     ******  * **   ***   * *   *         * **  * *   **

mouse_promoter      CAATCAGACTGTCCGA--CCTAGGACATAGATTGGAG---GAAGGGGTA--AACTAGTGA 1758
bovine_forward      TGCTTGGAGTTTTGGGGGTCTTAGTTTTAGGTTAGCCTTCGCTGGGATCCCGGCCGGTGG 1649
                       *  ** * *  *    **  *   *** ** *     *  *** *     *  ***

mouse_promoter      GGCCAGAAGGACGCCAGTCCCAACGCGAGTCCTCTTACAGGCTCACCTGAGCGCGGATCT 1818
bovine_forward      CCTCGTGTCGCCCTCCCCTCCCCCGCCCCAACTCCTCCACATTCTAGTTCCCG-GAGTTT 1708
                       *     * *  *    **  ***     *** * **   **   *   ** *  * *

mouse_promoter      GGGCGGAGCTGATCATTTATCTGAGATTAAAACTTAGGACTGGGGCCATCCCTGCCCAAC 1878
bovine_forward      GCACAAGGCTGGCCGCCGTTGTTGCCTGCCTTCCCTCCGCCGTATTACATCGCCCCTAAC 1768
                    *  *   ****  *     * *    *     *      * *        *   ** ***

mouse_promoter      CTCTTGCTCCCTCCCCCAACCTCAAACTAAGACACCGAAAGCAATGAACAACCTCCTCTT 1938
bovine_forward      TGGCTCCCCTCATCCCCACCCCGGGACTA-GTCGCCGGGC------------------- 1807
                        * * * *  ***** **    **** * * ***
```

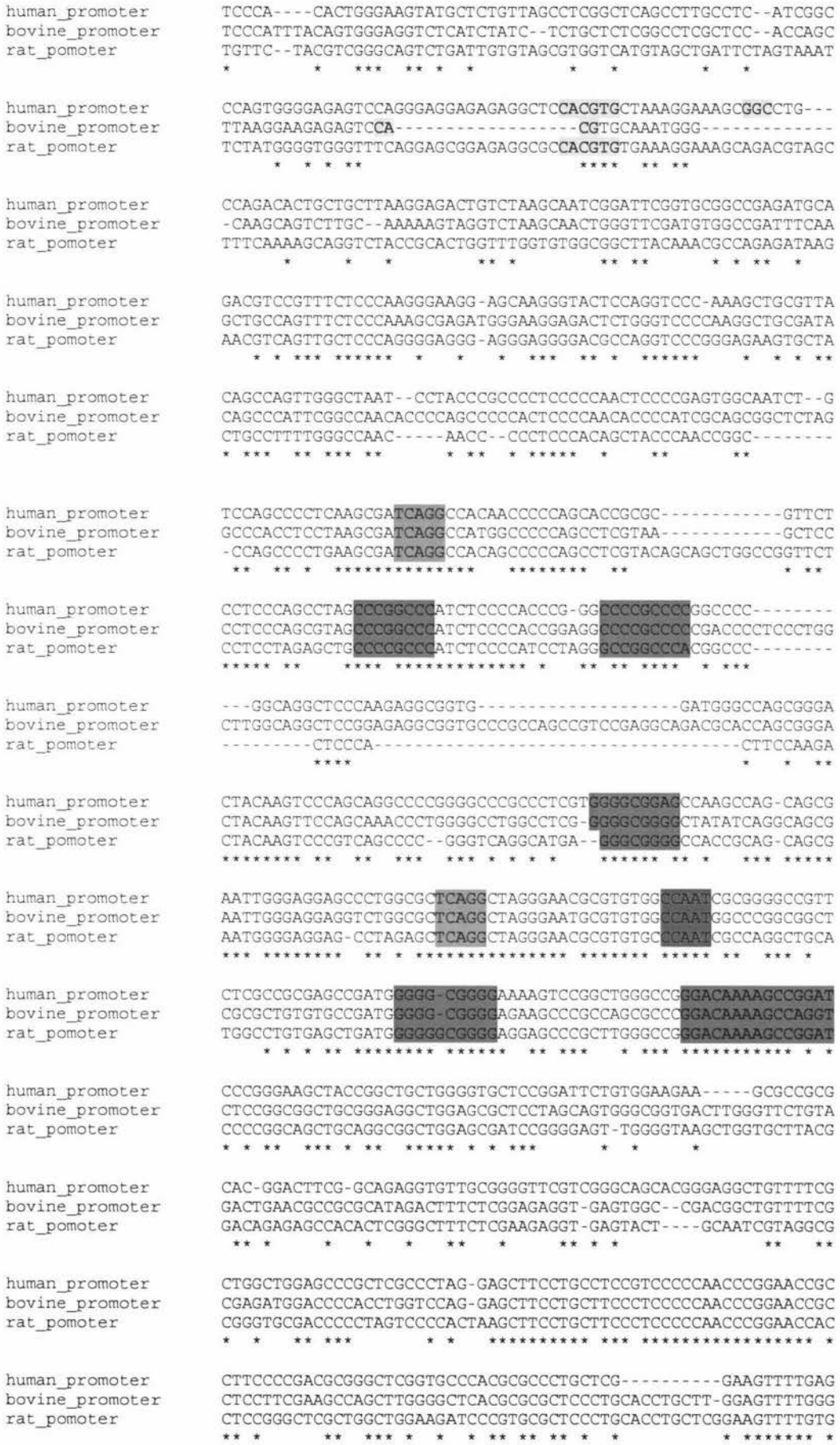
D. Sequence alignment for human and rat promoter region

```
human_promoter      TTTGTAGAGATGGGGTTTTGCCTTGTTGGTCTTGAACTCCTGACCTCAAGTGATCCCCCA
rat_pomoter         ---CCTGAATTTGAGCAACTTCATAGAAGACATTTACTTAGGGAG--AAGTGATCCCTCA
                         :**.:* *.* ::   *:*. :.*:*:* :*** . *..   ********** **

human_promoter      CCTTGGCCTTCCAAAGTGCTGGGATTACAGGTGTGAGCCACCAAGACTGGCCACCCCCAT
rat_pomoter         CAGAG----------------------ATTTGGGTAGGTTCTAGGCCTTTCTCTCAACT
                    *. :*                      *  ** *:.  : *:**.*    *:* *...*

human_promoter      TTTAGGACAAGAGAAGAGAGGTGATAAGGAACTGCTATTTCAAAAAGATGAATGGGAGGG
rat_pomoter         TCAGGAGCAAGG--------GGCACTATAAACTGTTATTTCTAAAGAATAAGTATGAGGG
                    * :.*..****.        *  * :* .***** ******:***..**.*.*. *****

human_promoter      AAGGGACCAGAAAG-TGACATCATCAGGGAACACTTGAT--TCAGGCATGG--GTAGCAA
rat_pomoter         AAGGGACCAAAAAAATGACATCATCAAGGAGCACACGCTGGTCAAGCATGGGTGTAACAA
                    *********.***. **********.***.***: *.*  ***.******  ***.***

human_promoter      GTTCAGTTACCTTACTCCTGGCAGCAACAGGATTGGGGGAGGGGGTCTTGGAGAAGATTT
rat_pomoter         GTTCAGCCAGCCTTTGGAGGAGGGCTTGGGAGTGGGGGAGGGGGGCTTGGGAAAAGTGTT
                    ******  * * *:   . *. .**:: .*..* ****..*****  * ***.***: **

human_promoter      TGTCTG--CAAATGTCGCAACACTGTTGCAGTGTGAGTATGACCCAAAGTGCTTGGCAGG
rat_pomoter         TGCCCAGGAGAGTGTTGCAACATTGTTGCAGGGAGCTTTTGACCCAGGGTTTTTGCCAGG
                    ** * .  ..*.*** ****** ******** *:*. *:*******..**  *** ****

human_promoter      GGGACAGGCCT-----GCTGCTTCCCTGCATTCTGGGCTGACCTCTTCACCTGAGTCGGT
rat_pomoter         GGCATAGCCATGCCACGCTGATTCTCTGCACTCTGGGCTGATG-CATCACCTGAGCCGAT
                    ** * ** *.*     ****.*** ***** **********   *:********* **.*

human_promoter      CAGTGGACGCTTTTGGCCTCCTTCCTGGAGCTAGACAGCTGTTAGGCTTAGGGAGCAGCT
rat_pomoter         TAGAGGATGATTCTGGCCTCCTTCCTAGAGCTGGAAAATTGTTTGTTTCTGGGACCGTCT
                     **:*** *.** **************.*****.**.*. ****:*  * :**** *. **

human_promoter      --CCGGACCTACAGG-GTTGGGGTGCAGGTAGGGAGGGAGGCTGCAGCATGTCAGAGTGG
rat_pomoter         GGGAAGACATACTGGGACTAAGGTACAAGCAAAAGAGGCTTTCACTCCAGGGAAGCAG-T
                       ..***.***:** . *..***.**.* *.....**.     .*: ** * .**..

human_promoter      TAAATACATGCCTGATTTCCAGGAC----TGATTTCACATATTCTGCCCCACAAGTTCAC
rat_pomoter         AAATCGGAGTTCTGATTTCCAGGACGTTCTGATTTTATATATTCTGCTCCACAAG---TC
                    :**: . *   ***************    ****** * ********* *******   :*

human_promoter      CAGCACCTGTCAGATTGGATGTCCCAGTTTTTGGTTTGGAAAATTCGATCTGTCTCAGGT
rat_pomoter         GGGCACCTGTTAGACTGGGTGCTCCACTTTTGGATTTGGAAAATATGTTCTACCTCTCAT
                     .******** *** ***.**  *** **** *.**********: *:***. ***: .*

human_promoter      AATGGAGA---AGGGGGAGACAGCAGTTCATTCATTCCAGTGTCTAAGCCTGTG--GTTG
rat_pomoter         ATGTAACAGAAAAGAAGAGGAAGCTGTTCATTGCTTTCAGTGTCTTAGTTTGAGTTGTGG
                    *:  .* *   *.*..***..***:******* .** ********:**  **:*  ** *

human_promoter      GTGGGAACAAGAGACTG-TCTGGGATGGCTCCAGCCCTTCGCTGGAATCTCGCATTGAAT
rat_pomoter         GAGGGAGCAAGAGGTTGGACAGGGATGGCCCCAGTGCTAGGTTTTAATCTCAGATAAATG
                    *:****.******. ** :*:******** ****  **: * *  ******. **:.*:

human_promoter      TCCTAGCTCCGCCCTGATCCGTGCTGAGGCGAGCAGGTGAGTGGGACGGACAGCGCTGTG
rat_pomoter         ATCAG-CTCCGCCCAGATCCGCGCTCAGGTGAGCCCGTAAGAGG----ACTCGCGTTGGA
                    : *:. ********:****** *** *** ****. **.**:**    .. .*** ** .

human_promoter      CCTG-CACCCTCCTAGCCCCACTTGCTTACCGCGGCCCCTG--CCTTATCCCACACTGGG
rat_pomoter         ACTGGCGTCCTTCTGGCCTCACTAGTTTACCTCTTCCTCCTATCTATGTTCTACGTCGGG
                    .*** *. *** **.*** ****:* ***** *  ** *    * :*.* * **.  ***

human_promoter      AAGTATGCTCTGTTAGCCTCGGCTC--AGCCTTGCCTCATCGGCCCAGTGGGGAGAGTCC
rat_pomoter         CAGTCTGATTGTGTAGCGTGGTCATGTAGCTGATTCTAGTAAATTCTATGGGGTGGGTTT
                    .***.**.*    **** * * *:   ***  :  **..*...  *:.*****:*.**

human_promoter      AGGGAGGAGAGAGGCTCCACGTGCTAAAGGAAAGCGGCC--TGCCAGACACTGCTGCTTA
rat_pomoter         CAGGAGCGGAGAGGCGCCACGTGTGAAAGGAAAGCAGACGTAGCTTTCAAAAGCAGGTCT
                    ..**** .******* *******  **********.*.*  :** : ..*.:**:* * :

human_promoter      AGGAGACTG-TCTAAGCAATCGGATTCGGTGCGGCCGAGATGCAGACGTCCGTTTCTCCC
rat_pomoter         ACCGCACTGGTTTGGTGTGGCGGCTTACAAACGCCAGAGATAAGAACGTCAGTTGCTCCC
                    *  . **** * *..  :. ***.**. .:.** *.*****....*****.*** *****
```


human_promoter AAGGGAAGGAGCAAGGGTACTCCAGGTCCCAAAG-CTGCGTTACAGCCAGTTGGGCTAAT
rat_pomoter AGGGGAGGGAGGGAGGGGACGCCAGGTCCCGGGAGAAGTGCTACTGCCTTTTGG-----G
human_promoter CCTACCCGCCCCTCCCCCAACTCCCCGAGTGGCAATCTGTCCAGCCCCTCAAGCGATCAG
rat_pomoter CCAACAACCCCCTCCCACAGCTACCCAACCG-------GCCCAGCCCCTGAAGCGATCAG
human_promoter GCCACAACCCCCAGCACCGCGCG------------TTCTCCTCCCAGCCTAGCCCGGCCC
rat_pomoter GCCACAGCCCCCAGCCTCGTACAGCAGCTGGCCGGTTCTCCTCCTAGAGCTGCCCCGCCC
human_promoter ATCTCCCCACCCGGGCCCCG--CCCCGGCCCCGGCAGGCTCCCAAGAGGCGGTGGATGGG
rat_pomoter ATCTCCCCATCCTAGGGCCGGCCCACGGCCCCTCCCACTTCCAAG---------------
human_promoter CCAGCGGGACTACAAGTCCCAGCAGGCCCCGGGGCCCGCCCTCGTGGGGCGGAGCCAAGC
rat_pomoter --------ACTACAAGTCCCGTCAG-CCCCGGGTCAGGCATGAG---GGCGGGGCCACCG
human_promoter CAGCAGCGAATTGGGAGGAGCCCTGGCGCTCAGGCTAGGGAACGCGTGTGGCCAATCGCG
rat_pomoter CAGCAGCGAATGGGGAGGAGCCTAG-AGCTCAGGCTAGGGAACGCGTGTGCCCAATCGCC
human_promoter GGGCCGTTCTCGCCGCGAGCCGATGGGGG-CGGGGAAAAGTCCGGCTGGGCCGGGACAAA
rat_pomoter AGGCTGCATGGCCTGTGAGCTGATGGGGGGCGGGGAGGAGCCCGCTTGGGCCGGGACAAA
human_promoter AGCCGGATCCCGGGAAGCTACCGGCTGCTGGGGTGCTCCGGATTCTGTGGAAGAAGCGCC
rat_pomoter AGCCGGATCCCCGGCAGCTGCAGGCGGCTGGAGCGATCCGGGGAGTTGGGGTAAGCTGGT
human_promoter GCGCACGGACTTCGGCAGAGGTGTTGCGGGGTTCGTCGGGCAGCACGGGAGGCTGTTTTC
rat_pomoter GCTTACGGACAGAG-----AGCCACACTCGGGCTTTCTCGAAGAGGTGAGTACTGCAATC
human_promoter GCTGGCTGG-----AGCCCGCTCG--CCCTAGGAGCTTCCTGCCTCCGTCCCCCAACCCG
rat_pomoter GTAGGCGCGGGTGCGACCCCCTAGTCCCCACTAAGCTTCCTGCTTCCCTCCCCCAACCCG
human_promoter GAACCGCCTTCCCCGACGCGGGCTCGGTGCCCACGCGC----------CCTGCTCGGAAG
rat_pomoter GAACCACCTCCGGGCTCGCTGGCTGGAAGATCCCGTGCGCTCCCTGCACCTGCTCGGAAG
human_promoter TTTTG--AGGGTCTTAGCTTTAGGTTAGCCTTCGCCCGGGGTCCCGGCCGGTGGCCGCGT
rat_pomoter TTTTGTGCGGGTCTCAGCTTTATAGCAGTCTTCGCCC-------CAGCCGCGGGCCGCAT
human_promoter GTCGACGCCCCTCCCCTCCCCCCGCGCGACACACACATATTCCAGTTCCTGGGAGTTTGC
rat_pomoter GTCTACACTGCCCCCCCACCCTCAG---------TCTTTTTCTAGTTCCTGGGAGTTTGC
human_promoter ATGGGGCTTGCCGCCATCCTTGCCTCTTCTGCCTCTGCCACATTGCATGGCCCGGGTCCC
rat_pomoter ACATTGCTTGCG---TGTCCTGCTTCTTCT-CACCACCGACCTTGCTTCTCCTCCCTCCT
human_promoter CCTCCTGGCGCGCCTCACCCCTCA----CAGCTATCGAGGCTCCTCGGGCTC--------
rat_pomoter CCGGATTGCCTTTGGCTCCACCCAGGATCAGCTCTTGAGTCTCTTCGGGCTTTTTTGGGA
human_promoter -------------
rat_pomoter CACTTTTTTGGGC

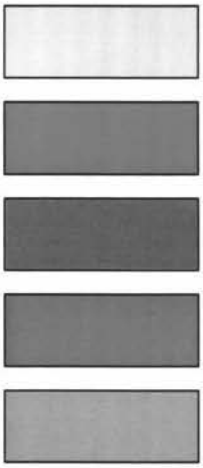
E. Sequence alignment for human, bovine and rat promoter region

```
human_promoter      TTTGTAGAGATGGGGTTTTGCCTTGTTGGTCTTGAACTCCTGACCTCAAGTGATCCCCCA
bovine_promoter     ------------------------------------------------------------A
rat_pomoter         ------------------------------------CCTGAATTTGAGCAACTTCATA
                                                                               *

human_promoter      CCTTGGCCTTCC--AAAGTGCTGGGATTACAGGTGT-GAGCCACCAAGACTGGCCACCCC
bovine_promoter     TCTGAGTA------GGGGTGGAGCTATTCCATGTAG-GAGTGG--AAGGCT------CTC
rat_pomoter         GAAGACATTTACTTAGGGAGAAGTGATCCCTCACAGAGATTTGGGTAGGTTCTAGGCCTT
                                    * *  *  **  *       **       **  *      *

human_promoter      CATTTTAGGACAAGAGAAGAGAGGTGATAAGGAACTGCTATTTCAAAAAGATGAATGGGA
bovine_promoter     TAT----GACCAACAGGAGAGAGGAGCTGAGGAAATGCTGTTTCAAAAAGATGCCTGTGA
rat_pomoter         TCTCTCAACTTCAGGAGCAAGGGGCACT-ATAAACTGTTATTTCTAAAGAATAAGTATGA
                      *         *      ** **   * *  ** ** * **** ***  **   *  **

human_promoter      GGGAAGGGACCAGAAAG-TGACATCATCAGGGAACACTTGAT--TCAGGCATGGGT--AG
bovine_promoter     GGGAAGGGACCAGAAAC-TGACATCATCAGGGATCACTTTGT--TCAGGCATGGCC--AA
rat_pomoter         GGGAAGGGACCAAAAAAATGACATCATCAAGGAGCACACGCTGGTCAAGCATGGGTGTAA
                    ************ ***  *********** *** ***    *  *** ******    *

human_promoter      CAAGTTCAGTTACCTTACTCCTGGCAGCAACAGGATTGGGGGAGGGGGTCTTGGAGAAGA
bovine_promoter     CAAGTGTA--------------------------TTGGGGGGAGAAGGATTTGGCCAGGT
rat_pomoter         CAAGTTCAGCCAGCCTTTGGAGGAGGGCTTGGGAGTGGGGGAGGGGGGCTTGGGAAAAGT
                    *****  *                          * ****  *  **  * **  * *

human_promoter      TTTTGTCTGCA--AATGTCGCAACACTGTTGCAGTGTGAGTATGACCCAAAGTGCTTGGC
bovine_promoter     TTTTGTGTACAGCAATGTGGTAGCACTGTTACAGTGTGA-------CCCCAGCGCCTGGC
rat_pomoter         GTTTGCCCAGGAGAGTGTTGCAACATTGTTGCAGGGAGCTTTTGACCCAGGGTTTTTGCC
                     ****        * *** * * ** **** *** * *        **   *    ** *

human_promoter      AGGGGGACAGGCCTGCTGC-----TTCCCTGCATTCTGGGCTGACCTCTTCACCTGAGTC
bovine_promoter     AGGAACACAGACATGTGGT-----CTGTTTGCACACTGCGCTGACCCTTTCACCTGAGCT
rat_pomoter         AGGGGCATAGCCATGCCACGCTGATTCTCTGCACTCTGGGCTGATG-CATCACCTGAGCC
                    ***   * ** * **          *   ****  *** *****     *********

human_promoter      GGTCAGTGGACGCTTTTGGCCTCCTTCCTGGAGCTAGACAGCTGTTAGGCTTAGGGAGCA
bovine_promoter     GCTCAGCGCATGAT-----CCTCCTTCCTGGAGCTAGACAGCTGTTAGGGTCAGGGAGCA
rat_pomoter         GATTAGAGGATGATTCTGGCCTCCTTCCTAGAGCTGGAAAATTGTTTGTTTCTGGGACCG
                    * * ** * * * *     ********** ***** ** *  **** *  *  **** *

human_promoter      GCTCCGGA--CCTAC-AGGGTTGGGGTGCAGGTAGGGAGGGAGGCTGCAGCATGTCAGAG
bovine_promoter     GCTCGAGAGACCTAG-AGGACTGGGATGGACATAGGAA----AGCTCCAGGATGTCTCAG
rat_pomoter         TCTGGGAAGACATACTGGGACTAAGGTACAAGCAAAAGAG--GCTTTCACTCCAGGGAAG
                     **    *  * **   **  *  * *  *   *          * **         **

human_promoter      TGGTAAATACATGCCT-GATTTCCAGGACTG----ATTTCACATATTCTGCCCCACAAGT
bovine_promoter     CAGTAAATCTATGTCTTGGTTTCTAGGACTGTCTGATTTTAAAAATTCTGTCCCACAAGT
rat_pomoter         CAGTAAATCGGAGTTCTGATTTCCAGGACGTTCTGATTTTATATATTCTGCTCCACAAGT
                      ******    *    * **** *****      **** * * ******  ********

human_promoter      TCACCAGCACCTGTCAGATTGGATGTCCCAGTTTTTGGTTTGGAAAATTCGATCTG--TC
bovine_promoter     TCACCAATACCTGTCAGACCAGGTGTCCCAGTTTTTGATTTGGAAAATGCACTCTG--TT
rat_pomoter         ---CGGGCACCTGTTAGACTGGGTGCTCCACTTTTGGATTTGGAAAATATGTTCTACCTC
                       *    ****** ***   * **  *** **** * **********    ***   *

human_promoter      TCA--GGTAATGGAGAAGGGGGAGACAGCAGTTCATTCATTCCAGT-GTCTAAGCCTGTG
bovine_promoter     TCCCCGGTAATGGAGAAGGGGAAGGCAGCAGTTCATTCATTCCTCTAGTCTAAGCGTGAG
rat_pomoter         TCATATGTAACAGAAAAGAAGAGGA-AGCTGTTCATTGCTTTCAGT-GTCTTAGTTTGAG
                    **    ****  ** ***  *  *  *** *******  ** *  * **** **  ** *

human_promoter      --GTTGGTGGGAACAAGA----------GACTGTCTGGGATGGCTCCAGCCCTTCGCTGG
bovine_promoter     --AT-AGTGGGGGCAGGGCGGAGAGCCCGAGCAGCAGAGATGGCCCCAGCCTTGCGCTAG
rat_pomoter         TTGTGGGAGGGAGCAAGAG---------GTTGGACAGGGATGGCCCCAGTGCTAGGTTTT
                       *  * ***  ** *           *     * * ****** ****   *  * *

human_promoter      AATCTCGCATTGAATTCCTAGCTCCGCCCTGATCCGTGCTGAGGCGAGCAGGTGAGTGGG
bovine_promoter     AATCTCTCATTGAATTCTCTGCTCTGTGCGCCCAGGTGAGTGGATGAGTGGTGGTGACTG
rat_pomoter         AATCTCAGAT-AAATGATCAGCTCCGCCCAGATCCGCGCTCAGGTGAGCCCGTAAGAG-G
                    ******  **  ***     **** *  *      * *    *  ***       *   *

human_promoter      ACGGACAGCGCTGTGCCTGCACCCTCCTAGCCCCACTTGCTTACCGCGGCCCCTGCCTTA
bovine_promoter     ACG--CGAGGTGGTGATTGACCCCTCCTAGCCCCACCTGCTCACGCGGTCCTCCGCCCTA
rat_pomoter         ACT--CGCGTTGGAACTGGCGTCCTTCTGGCCTCACTAGTTTACCTCTTCCTCCTATCTA
                    **   *      *     *   *** ** *** ***  * * **     ** *     **
```


human_promoter TCCCA----CACTGGGAAGTATGCTCTGTTAGCCTCGGCTCAGCCTTGCCTC--ATCGGC
bovine_promoter TCCCATTTACAGTGGGAGGTCTCATCTATC--TCTGCTCTCGGCCTCGCTCC--ACCAGC
rat_pomoter TGTTC--TACGTCGGGCAGTCTGATTGTGTAGCGTGGTCATGTAGCTGATTCTAGTAAAT
human_promoter CCAGTGGGGAGAGTCCAGGGAGGAGAGAGGCTCCACGTGCTAAAGGAAAGCGGCCTG---
bovine_promoter TTAAGGAAGAGAGTCCA----------CGTGCAAATGGG----------
rat_pomoter TCTATGGGGTGGGTTTCAGGAGCGGAGAGGCGCCACGTGTGAAAGGAAAGCAGACGTAGC
human_promoter CCAGACACTGCTGCTTAAGGAGACTGTCTAAGCAATCGGATTCGGTGCGGCCGAGATGCA
bovine_promoter -CAAGCAGTCTTGC--AAAAAGTAGGTCTAAGCAACTGGGTTCGATGTGGCCGATTTCAA
rat_pomoter TTTCAAAAGCAGGTCTACCGCACTGGTTTGGTGTGGCGGCTTACAAACGCCAGAGATAAG
human_promoter GACGTCCGTTTCTCCCAAGGGAAGG-AGCAAGGGTACTCCAGGTCCC-AAAGCTGCGTTA
bovine_promoter GCTGCCAGTTTCTCCCAAAGCGAGATGGGAAGGAGACTCTGGGTCCCCAAGGCTGCGATA
rat_pomoter AACGTCAGTTGCTCCCAGGGGAGGG-AGGGAGGGGACGCCAGGTCCCGGGAGAAGTGCTA
human_promoter CAGCCAGTTGGGCTAAT--CCTACCCGCCCCTCCCCCAACTCCCCGAGTGGCAATCT--G
bovine_promoter CAGCCCATTCGGCCAACACCCCAGCCCCCACTCCCCAACACCCCATCGCAGCGGCTCTAG
rat_pomoter CTGCCTTTTGGGCCAAC-----AACC--CCCTCCCACAGCTACCCAACCGGC--------
human_promoter TCCAGCCCCTCAAGCGATCAGGCCACAACCCCCAGCACCGCGC----------GTTCT
bovine_promoter GCCCACCTCCTAAGCGATCAGGCCATGGCCCCCAGCCTCGTAA----------GCTCC
rat_pomoter -CCAGCCCCTGAAGCGATCAGGCCACAGCCCCCAGCCTCGTACAGCAGCTGGCCGGTTCT
human_promoter CCTCCCAGCCTAGCCCGGCCCATCTCCCCACCCG-GGCCCCGCCCCGGCCCC--------
bovine_promoter CCTCCCAGCGTAGCCCGGCCCATCTCCCCACCGGAGGCCCCGCCCCCGACCCCTCCCTGG
rat_pomoter CCTCCTAGAGCTGCCCCGCCCATCTCCCCATCCTAGGGCCGGCCCACGGCCC--------
human_promoter ---GGCAGGCTCCCAAGAGGCGGTG----------GATGGGCCAGCGGGA
bovine_promoter CTTGGCAGGCTCCGGAGAGGCGGTGCCCGCCAGCCGTCCGAGGCAGACGCACCAGCGGGA
rat_pomoter ---------CTCCCA----------CTTCCAAGA
human_promoter CTACAAGTCCCAGCAGGCCCCGGGGCCCGCCCTCGTGGGGCGGAGCCAAGCCAG-CAGCG
bovine_promoter CTACAAGTTCCAGCAAACCCTGGGGCCTGGCCTCG-GGGGCGGGGCTATATCAGGCAGCG
rat_pomoter CTACAAGTCCCGTCAGCCCC--GGGTCAGGCATGA--GGGCGGGGCCACCGCAG-CAGCG
human_promoter AATTGGGAGGAGCCCTGGCGCTCAGGCTAGGGAACGCGTGTGGCCAATCGCGGGGCCGTT
bovine_promoter AATTGGGAGGAGGTCTGGCGCTCAGGCTAGGGAATGCGTGTGGCCAATGGCCCGGCGGCT
rat_pomoter AATGGGGAGGAG-CCTAGAGCTCAGGCTAGGGAACGCGTGTGCCCAATCGCCAGGCTGCA
human_promoter CTCGCCGCGAGCCGATGGGGG-CGGGGAAAAGTCCGGCTGGGCCGGGACAAAAGCCGGAT
bovine_promoter CGCGCTGTGTGCCGATGGGGG-CGGGGAGAAGCCCGCCAGCGCCCGGACAAAAGCCAGGT
rat_pomoter TGGCCTGTGAGCTGATGGGGGGCGGGGAGGAGCCCGCTTGGGCCGGGACAAAAGCCGGAT
human_promoter CCCGGGAAGCTACCGGCTGCTGGGGTGCTCCGGATTCTGTGGAAGAA-----GCGCCGCG
bovine_promoter CTCCGGCGGCTGCGGGAGGCTGGAGCGCTCCTAGCAGTGGGCGGTGACTTGGGTTCTGTA
rat_pomoter CCCCGGCAGCTGCAGGCGGCTGGAGCGATCCGGGGAGT-TGGGGTAAGCTGGTGCTTACG
human_promoter CAC-GGACTTCG-GCAGAGGTGTTGCGGGGTTCGTCGGGCAGCACGGGAGGCTGTTTTCG
bovine_promoter GACTGAACGCCGCGCATAGACTTTCTCGGAGAGGT-GAGTGGC--CGACGGCTGTTTTCG
rat_pomoter GACAGAGAGCCACACTCGGGCTTTCTCGAAGAGGT-GAGTACT----GCAATCGTAGGCG
human_promoter CTGGCTGGAGCCCGCTCGCCCTAG-GAGCTTCCTGCCTCCGTCCCCCAACCCGGAACCGC
bovine_promoter CGAGATGGACCCCACCTGGTCCAG-GAGCTTCCTGCTTCCCTCCCCCAACCCGGAACCGC
rat_pomoter CGGGTGCGACCCCCTAGTCCCCACTAAGCTTCCTGCTTCCCTCCCCCAACCCGGAACCAC
human_promoter CTTCCCCGACGCGGGCTCGGTGCCCACGCGCCCTGCTCG----------GAAGTTTTGAG
bovine_promoter CTCCTTCGAAGCCAGCTTGGGGCTCACGCGCGCTCCCTGCACCTGCTT-GGAGTTTTGGG
rat_pomoter CTCCGGGCTCGCTGGCTGGAAGATCCCGTGCGCTCCCTGCACCTGCTCGGAAGTTTTGTG

```
human_promoter      --GGTCTTAGCTTTAGGTTAGCCTTCGCCCGGGGTCCCGGCCGGTGGCCGCGTGTCGACG
bovine_promoter     --GGTCTTAGTTTTAGGTTAGCCTTCGCT-GGGATCCCGGCCGGTGGCCTCGTGTCG---
rat_pomoter         CGGGTCTCAGCTTTATAGCAGTCTTCGCC-------CCAGCCGCGGGCCGCATGTCTA--
                      ***** ** ****    ** ******        ** ****  **** * ****

human_promoter      CCCCTCCCCTCCCCCCGCGCGACACACACATATTCCAGTTCCTGGGAGTTTGCATGGGGC
bovine_promoter     -CCCTCCCCTCCCCCGCCCCAACTCCTCCACATTCTAGTTCCCGG-AGTTTGCACAAGGC
rat_pomoter         -CACTGCCCCCCCACC------CTCAGTCTTTTTCTAGTTCCTGGGAGTTTGCACATTGC
                     * ** *** *** *       * *   *   *** ****** ** ********    **

human_promoter      TTGC-CGCCATCCTTGCCTCTTCTGCCTCTGCCACATTGCATGGCCCGGGTCCCCCTCCT
bovine_promoter     TGGC-CGCCGTTGTTGCCTGCCTTCCCTCCGCCGTATTACATCGCCC-------CTAACT
rat_pomoter         TTGCGTGTCCTGCTTCTTCTCACCACCGACCTTGCTTCTCCTCCCTCCTCCGGATTGCCT
                    * **  * * *  **          **         *  * *  * *           **

human_promoter      GGCGCGCCTCACCCCTCACAGCTATCGAGGCTCCTCGGGCTC------------------
bovine_promoter     GGCTCCCCTCATCCCCACCCCGGGACTAG--TCGCCGGGC--------------------
rat_pomoter         TTGGCTCCAC-CCAGGATCAGCTCTTGAGTCTCTTCGGGCTTTTTTGGGACACTTTTTTG
                        * ** *  *     *        **  **  *****
```

Keys to appendix 1:



E boxes

Glucocorticoid response element (GRE)

CCAAT boxes

GC boxes

Sterol regulatory element (SRE)

Appendix 2 – CLUSTAL W for Sequenced PCR Deletion Construct

A. CLUSTAL W for Bovine promoter and the 1513 constructs

```
bovine_promoter             ------------------------------------------------
sp6_reverse_complement      ------------------------------------------------
T7_sequence                 AMMYYKTTTTATATCGMGTCGMTGCTCCGGCCGCCATGGCCGCGGGATTA

bovine_promoter             ------------------------------------------------AT
sp6_reverse_complement      ------------------------------------------------
T7_sequence                 TGGTACCGAGAGGTAGTCCTTCTCCAAGATGSGTKCTATTTGGGGAGAAT

bovine_promoter             CTGAGTAGGGGTGGAGCT-ATTCCATGTAGGAGTGGAAGGCTCTCTATGA
sp6_reverse_complement      ------------------------------------------------
T7_sequence                 CTGAGTAGGGGTGGAGCTCATTCCATGTAGGAGTGGAAGGCTCTCTATGA

bovine_promoter             CCAACAGGAGAGAGGAGCTGAGGAAATGCTGTTTCAAAAAGATGCCTGTG
sp6_reverse_complement      ------------------------------------------------
T7_sequence                 CCAACAGGAGAGAGGAGCTGAGGAAATGCTGTTTCAAAAAGATGCCTGTG

bovine_promoter             AGGGAAGGGACCAGAAACTGACATCATCAGGGATCACTTTGTTCAGGCAT
sp6_reverse_complement      ------------------------------------------------
T7_sequence                 AGGGAAGGGACCAGAAACTGACATCATCAGGGATCACTTTGTTCAGGCAT

bovine_promoter             GGCCAACAAGTGTATTGGGGGGAGAAGGATTTGGCCAGGTTTTTGTGTAC
sp6_reverse_complement      ------------------------------------------------
T7_sequence                 GGCCAACAAGTGTATTGGGGGGAGAAGGATTTGGCCAGGTTTTTGTGTAC

bovine_promoter             AGCAATGTGGTAGCACTGTTACAGTGTGACCCCAGCGCCTGGCAGGAACA
sp6_reverse_complement      ------------------------------------------------
T7_sequence                 AGCAATGTGGTAGCACTGTTACAGTGTGACCCCAGCGCCTGGCAGGAACA

bovine_promoter             CAGACATGTGGTCTGTTTGCACACTGCGCTGACCCTTTCACCTGAGCTGC
sp6_reverse_complement      ------------------------------------------------
T7_sequence                 CAGACATGTGGTCTGTTTGCACACTGCGCTGACCCTTTCACCTGAGCTGC

bovine_promoter             TCAGCGCATGATCCTCCTTCCTGGAGCTAGACAGCTGTTAGGGTCAGGG
sp6_reverse_complement      -------------CTYCTCYKGGRACTAGACAGCTGTTAGGGTCAGGG
T7_sequence                 TCAGCGCATGATCCTCCTTCCTGGAGCTAGACAGCTGTTAGGGTCAGGG
                                        **  **  **********************

bovine_promoter             AGCAGCTCGAGAGACCTAGAGGACTGGGATGGACATAGGAAAGCTCCA
sp6_reverse_complement      AGCAGCTCGAGAGACCTAGAGGACTGGGATGGACATAGGAAAAGTCCA
T7_sequence                 AGCAGCTCGAGAGACCTAGAGGACTGGGATGGACATAGGAAAGCTCCA
                            ************************************************

bovine_promoter             GGATGTCTCAGCAGTAAATCTATGTCTTGGTTTCTAGGACTGTCTGATTT
sp6_reverse_complement      GGATGTCTCAGCAGTAAATCTATGTCTTGGTTTCTAGGACTGTCTGATTT
T7_sequence                 GGATGTCTCAGCAGTAAATCTATGTCTTGGTTTCTAGGACTGTCTGATTT
                            ************************************************

bovine_promoter             TAAAAATTCTGTCCCACAAGTTCACCAATACCTGTCAGACCAGGTGTCC
sp6_reverse_complement      TAAAAATTCTGTCCCACAAGTTCACCAATACCTGTCAGACCAGGTGTCC
T7_sequence                 TAAAAATTCTGTCCCACAAGTTCACCAATACCTGTCAGACCAGGTGTCC
                            ************************************************

bovine_promoter             CAGTTTTTGATTTGGAAAATGCACTCTGTTTCCCCGGTAATGGAGAAGGG
sp6_reverse_complement      CAGTTTTTGATTTGGAAAATGCACTCTGTTTCCCCGGTAATGGAGAAGGG
T7_sequence                 CAGTTTTTGATTTGGAAAATGCACTCTGTTTCCCCGGTAATGGAGAAGGG
                            ************************************************
```

```
bovine_promoter             GAAGGCAGCAGTTCATTCATTCCTCTAGTCTAAGCGTGAGATAGTGGGGG
sp6_reverse_complement      GAAGGCAGCAGTTCATTCATTCCTCTAGTYTAAGCGTGAGATAGTGGGGG
T7_sequence                 GAAGGCAGCAGTTCATTCATTCCTCTAGTCTAAGCGTGAGATAGTGGGGG
                            ****************************** ********************

bovine_promoter             CAGGGCGGAGAGCCCGAGCAGCAGAGATGGCCCCAGCCTTGCGCTAGAAT
sp6_reverse_complement      CAGGGCGGAGAGCCCGAGCAGCAGAGATGGCCCCAGCCTTGCGCTAGAAT
T7_sequence                 CAGGGCGGAGAGCCCGAGCAGCAGAGATGGCCCCAGCCTTGCGCTAGAAT
                            **************************************************

bovine_promoter             CTCTCATTGAATTCTCTGCTCTGTGCGCCCAGGTGAGTGGATGAGTGGTG
sp6_reverse_complement      CTCTCATTGAATTCTCTGCTCTGTGCGCCCAGGTGAGTGGATGAGTGGTG
T7_sequence                 CTCTCATTGAATTCTCTGCTCTGTGCGCCCAGGTGAGTGGATGAGTGGTG
                            **************************************************

bovine_promoter             GTGACTGACGCGAGGTGGTGATTGACCCCTCCTAGCCCCACCTGCTCACG
sp6_reverse_complement      GTGACTGACGCGAGGTGGTGATTGACCCCTCCTAGCCCCACCTGCTCACG
T7_sequence                 GTGACTGACGCGAGGTGGTGATTGACCCCTCCTAGCCCCACCTGCTCACG
                            **************************************************

bovine_promoter             CGGTCCTCCGCCCTATCCCATTTACAGTGGGAGGTCTCATCTATCTCTGC
sp6_reverse_complement      CGGTCCTCCGCCCTATCCCATTTACAGTGGGAGGTCTCATCTATCTCTGC
T7_sequence                 CGGTCCTCCGCCCTATCCCATTTACAGTGGGAGGTCTCATCTATCTCTGC
                            **************************************************

bovine_promoter             TCTCGGCCTCGCTCCACCAGCTTAAGGAAGAGAGTCCACGTGCAAATGGG
sp6_reverse_complement      TCTCGGCCTCGCTCCACCAGCTTAAGGAAGAGAGTCCACGTGCAAATGGG
T7_sequence                 TCTCGGCCTCGCTCCACCAGCTTAAGGAAGAGAGTCCACGTGCAAATGGG
                            **************************************************

bovine_promoter             CAAGCAGTCTTGCAAAAAGTAGGTCTAAGCAACTGGGTTCGATGTGGCCG
sp6_reverse_complement      CAAGCAGTCTTGCAAAAAGTAGGTCTAAGCAACTGGGTTCGATGTGGCCG
T7_sequence                 CAAGCAGTCTTGCAAAAAGTAGGTCTAAGCAACTGGGTTCGATGTGGCCG
                            **************************************************

bovine_promoter             ATTTCAAGCTGCCAGTTTCTCCCAAAGCGAGATGGGAAGGAGACTCTGGG
sp6_reverse_complement      ATTTCAAGCTGCCAGTTTCTCCCAAAGCGAGATGGGAAGGAGACTCTGGG
T7_sequence                 ATTTCA-GCTGCCAGTTTCTCCCAAAGCGAGATGGGAAGGAGACTCTGGG
                            ****** ***************************************

bovine_promoter             TCCCCAAGGCTGCGATACAGCCCATTCGGCCAACACCCCAGCCCCCACTC
sp6_reverse_complement      TCCCCAAGGCTGCGATACAGCCCATTCGGCCAACACCCCAGCCCCCACTC
T7_sequence                 TCCCCAAGGCTGCGATACAGCCCATTCGGCCAACACCCCAGCCCCCACTC
                            **************************************************

bovine_promoter             CCCAACACCCCATCGCAGCGGCTCTAGGCCCACCTCCTAAGCGATCAGGC
sp6_reverse_complement      CCCAACACCCCATCGCAGCGGCTCTAGGCCCACCTCCTAAGCGATCAGGC
T7_sequence                 CCCAACACCCCATCGCAGCGGCTCTAGGCCCACCTCCTAAGCGATCAGGC
                            **************************************************

bovine_promoter             CATGGCCCCCAGCCTCGTAAGCTCCCCTCCC-------------------
sp6_reverse_complement      CATGGCCCCCAGCCTCGTAAGCTCCCCTCCC-------------------
T7_sequence                 CATGGCCCCCAGCCTCGTAAGCTCCCCTCCCGCGTAGCCGGTCATYCTCC
                            ********************************

bovine_promoter             -AGCGTAGCCCGGCCCATCTCCCCACCGGAGGCCCCGCCCCCGACCCCTC
sp6_reverse_complement      -AGCGTAGCCCGGCCCATCTCCCCACCGGAGGCCCCGCCCCCGACCCCTC
T7_sequence                 CACCGGAGGCCGGCCCATCTCCCCACCGGAGGCCCCGCCCCCGACCCCTC
                             * ** ** ****************************************

bovine_promoter             CCTGGCTTGGCAGGCTCCGGAGAGGCGGTGCCCGCCAGCCGTCCGAGGCA
sp6_reverse_complement      CCTGGCTTGGCAGGCTCCGGAGAGGCGGTGCCCGCCAGCCGTCCGAGGCA
T7_sequence                 CCTGGCTTGGCAGGCTCCGGAGAGGCGGTGCCCGCCAGCCGTCCGAGGCA
                            **************************************************

bovine_promoter             GACGCACCAGCGGGACTACAAGTTCCAGCAAACCCTGGGGCCTGGCCTCG
sp6_reverse_complement      GACGCACCAGCGGGACTACAAGTTCCAGCAAACCCTGGGGCCTGGCCTCG
T7_sequence                 GACGCACCAGCGGGACTACAAGTTCCAGCAAACCCTGGGGCCTGGCCTCG
                            **************************************************

bovine_promoter             GGGGCGGGGCTATATCAGGCAGCGAATTGGGAGGAGGTCTGGCGCTCAGG
sp6_reverse_complement      GGGGCGGGGCTATATCAGGCAGCGAATTGGGAGGAGGTCTGGCGCTCAGG
T7_sequence                 GGGGCGGGGCTATATCAGGCAGCGAATTGGGAGGAGGTCTGGCGCTCAGG
                            **************************************************
```

```
bovine_promoter           CTAGGGAATGCGTGTGGCCAATGGCCCGGCGGCTCGCGCTGTGTGCCGAT
sp6_reverse_complement    CTAGGGAATGCGTGTGGCCAATGGCCCGGCGGCTCGCGCTGTGTGCCGAT
T7_sequence               CTAGGGAATGCGTGTGGCCAATGGCCCGGCGGCTCGCGCTGTGTGCCGAT
                          **************************************************

bovine_promoter           GGGGGCGGGGAGAAGCCCGCCAGCGCCCGGACAAAAGCCAGGTCTCCGGC
sp6_reverse_complement    GGGGGCGGGGAGAAGCCCGCCAGCGCCCGGACAAAAGCCAGGTCTCCGGC
T7_sequence               GGGGGCGGGGAGAAGCCCGCCAGCGCCCGGACAAAAGCCAGGTCTCCGGC
                          **************************************************

bovine_promoter           GGCTGCGGGAGGCTGGAGCGCTCCTAGCAGTGGGCGGTGACTTGGGTTCT
sp6_reverse_complement    GGCTGCGGGAGGCTGGAGCGCTCCTAGCAGTGGGCGGTGACTTGGGTTCT
T7_sequence               GGCTGCGGGAGGCTGGAGCGCTCCTAGCAGTGGGCGGTGACTTGGGTTCT
                          **************************************************

bovine_promoter           GTAGACTGAACGCCGCGCATAGACTTTCTCGGAGAGGTGAGTGGCCGA-C
sp6_reverse_complement    GTAGACTGAACGCCGCGCATAGACTTTCTCGGAGAGGRAGCTTATTAAAT
T7_sequence               GTAGACTGAACGCCGCGCATAGACTTTCTCGGAGAGGRAGCTTATTAAAT
                          ************************************    *     *
bovine_promoter           GGCTGTTTTCGCGAGATGGACCCC-ACCTGGTCCAGGAGCTTCCTGCTTC
sp6_reverse_complement    CACTAGTGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAAC--G
T7_sequence               CACTAGTGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAAC--G
                            **  *   **    ** *   * ***   *    ***** **  *

bovine_promoter           CCTCCCCCAACCCGGAACCGCCTCCTTCGAAGCCAGCTTGGGGCTCACGC
sp6_reverse_complement    CGWRGAKCWGKGCAAAWRC------------------------------
T7_sequence               CGWRGAKCWGKGCAAAWRC------------------------------
                          *      *    *  *  *

bovine_promoter           GCGCTCCCTGCACCTGCTTGGAGTTTTGGGGGTCTTAGTTTTAGGTTAGC
sp6_reverse_complement    ---------------------------------------------------
T7_sequence               ---------------------------------------------------

bovine_promoter           CTTCGCTGGGATCCCGGCCGGTGGCCTCGTGTCGCCCTCCCCTCCCCCGC
sp6_reverse_complement    ---------------------------------------------------
T7_sequence               ---------------------------------------------------

bovine_promoter           CCCAACTCCTCCACATTCTAGTTCCCGGAGTTTGCACAAGGCTGGCCGCC
sp6_reverse_complement    ---------------------------------------------------
T7_sequence               ---------------------------------------------------

bovine_promoter           GTTGTTGCCTGCCTTCCCTCCGCCGTATTACATCGCCCCTAACTGGCTCC
sp6_reverse_complement    ---------------------------------------------------
T7_sequence               ---------------------------------------------------

bovine_promoter           CCTCATCCCCACCCCGGGACTAGTCGCCGGGC
sp6_reverse_complement    --------------------------------
T7_sequence               --------------------------------
```

B. CLUSTAL W for Bovine promoter and the 513 constructs

```
bovine_promoter     ATCTGAGTAGGGGTGGAGCTATTCCATGTAGGAGTGGAAGGCTCTCTATGACCAACAGGA
513                 ------------------------------------------------------------

bovine_promoter     GAGAGGAGCTGAGGAAATGCTGTTTCAAAAAGATGCCTGTGAGGGAAGGGACCAGAAACT
513                 ------------------------------------------------------------

bovine_promoter     GACATCATCAGGGATCACTTTGTTCAGGCATGGCCAACAAGTGTATTGGGGGGAGAAGGA
513                 ------------------------------------------------------------

bovine_promoter     TTTGGCCAGGTTTTTGTGTACAGCAATGTGGTAGCACTGTTACAGTGTGACCCCAGCGCC
513                 ------------------------------------------------------------

bovine_promoter     TGGCAGGAACACAGACATGTGGTCTGTTTGCACACTGCGCTGACCCTTTCACCTGAGCTG
513                 ------------------------------------------------------------

bovine_promoter     CTCAGCGCATGATCCTCCTTCCTGGAGCTAGACAGCTGTTAGGGTCAGGGAGCAGCTCGA
513                 ------------------------------------------------------------

bovine_promoter     GAGACCTAGAGGACTGGGATGGACATAGGAAAGCTCCAGGATGTCTCAGCAGTAAATCTA
513                 ------------------------------------------------------------

bovine_promoter     TGTCTTGGTTTCTAGGACTGTCTGATTTTAAAAATTCTGTCCCACAAGTTCACCAATACC
513                 ------------------------------------------------------------

bovine_promoter     TGTCAGACCAGGTGTCCCAGTTTTTGATTTGGAAAATGCACTCTGTTTCCCCGGTAATGG
513                 ------------------------------------------------------------

bovine_promoter     AGAAGGGGAAGGCAGCAGTTCATTCATTCCTCTAGTCTAAGCGTGAGATAGTGGGGGCAG
513                 ------------------------------------------------------------

bovine_promoter     GGCGGAGAGCCCGAGCAGCAGAGATGGCCCCAGCCTTGCGCTAGAATCTCTCATTGAATT
513                 ------------------------------------------------------------

bovine_promoter     CTCTGCTCTGTGCGCCCAGGTGAGTGGATGAGTGGTGGTGACTGACGCGAGGTGGTGATT
513                 ------------------------------------------------------------

bovine_promoter     GACCCCTCCTAGCCCCACCTGCTCACGCGGTCCTCCGCCCTATCCCATTTACAGTGGGAG
513                 ------------------------------------------------------------

bovine_promoter     GTCTCATCTATCTCTGCTCTCGGCCTCGCTCCACCAGCTTAAGGAAGAGAGTCCACGTGC
513                 ------------------------------------------------------------

bovine_promoter     AAATGGGCAAGCAGTCTTGCAAAAAGTAGGTCTAAGCAACTGGGTTCGATGTGGCCGATT
513                 ------------------------------------------------------------

bovine_promoter     TCAAGCTGCCAGTTTCTCCCAAAGCGAGATGGGAAGGAGACTCTGGGTCCCCAAGGCTGC
513                 ------------------------------------------------------------TGC
                                                                                ***

bovine_promoter     GATACAGCCCATTCGGCCAACACCCCAGCCCCCACTCCCCAACACCCCATCGCAGCGGCT
513                 GATACAGCCCATTCGGCCAACACCCCAGCCCCCACTCCCCAACACCCCATCGCAGCGGCT
                    ************************************************************

bovine_promoter     CTAGGCCCACCTCCTAAGCGATCAGGCCATGGCCCCCAGCCTCGTAAGCTCCCCTCCCAG
513                 CTAGGCCCACCTTCTAAGCGATCAGGCCATGGCCCCCAGCCTCGTAAGCTCCCCTCCCAG
                    ************ ********************************************
```

```
bovine_promoter     CGTAGCCCGGCCCATCTCCCCACCGGAGGCCCCGCCCCCGACCCCTCCCTGGCTTGGCAG
513                 CGTAGCCCGGCCCATCTCCCCACCGGAGGCCCCGCCCCCGACCCCTCCCTGGCTTGGCAG
                    ************************************************************

bovine_promoter     GCTCCGGAGAGGCGGTGCCCGCCAGCCGTCCGAGGCAGACGCACCAGCGGGACTACAAGT
513                 GCTCCGGAGAGGCGGTGCCCGCCAGCCGTCCGAGGCAGACGCACCAGCGGGACTACAAGT
                    ************************************************************

bovine_promoter     TCCAGCAAACCCTGGGGCCTGGCCTCGGGGGCGGGGCTATATCAGGCAGCGAATTGGGAG
513                 TCCAGCAAACCCTGGGGCCTGGCCTCGGGGGCGGGGCTATATCAGGCAGCGAATTGGGAG
                    ************************************************************

bovine_promoter     GAGGTCTGGCGCTCAGGCTAGGGAATGCGTGTGGCCAATGGCCCGGCGGCTCGCGCTGTG
513                 GAGGTCTGGCGCTCAGGCTAGGGAATGCGTGTGGCCAATGGCCCGGCGGCTCGCGCTGTG
                    ************************************************************

bovine_promoter     TGCCGATGGGGGCGGGGAGAAGCCCGCCAGCGCCCGGACAAAAGCCAGGTCTCCGGCGGC
513                 TGCCGATGGGGGCGGGGAGAAGCCCGCCAGCGCCCGGACAAAAGCCAGGTCTCCGGCGGC
                    ************************************************************

bovine_promoter     TGCGGGAGGCTGGAGCGCTCCTAGCAGTGGGCGGTGACTTGGGTTCTGTAGACTGAACGC
513                 TGCGGGAGGCTGGAGCGCTCCTAGCAGTGGGCGGTGACTTGGGTTCTGTAGACTGAACGC
                    ************************************************************

bovine_promoter     CGCGCATAGACTTTCTCGGAGAGGTGAGTGGCCGACGGCTGTTTTCGCGAGATGGACCCC
513                 CGCGCATAGACTTTCTCGGAGAGGTGAGTG-----------------------------
                    ******************************

bovine_promoter     ACCTGGTCCAGGAGCTTCCTGCTTCCCTCCCCCAACCCGGAACCGCCTCCTTCGAAGCCA
513                 ------------------------------------------------------------

bovine_promoter     GCTTGGGGCTCACGCGCGCTCCCTGCACCTGCTTGGAGTTTTGGGGGTCTTAGTTTTAGG
513                 ------------------------------------------------------------

bovine_promoter     TTAGCCTTCGCTGGGATCCCGGCCGGTGGCCTCGTGTCGCCCTCCCCTCCCCCGCCCCAA
513                 ------------------------------------------------------------

bovine_promoter     CTCCTCCACATTCTAGTTCCCGGAGTTTGCACAAGGCTGGCCGCCGTTGTTGCCTGCCTT
513                 ------------------------------------------------------------

bovine_promoter     CCCTCCGCCGTATTACATCGCCCCTAACTGGCTCCCCTCATCCCCACCCCGGGACTAGTC
513                 ------------------------------------------------------------

bovine_promoter     GCCGGGC
513                 -------
```

C. CLUSTAL W for Bovine promoter and the 213 constructs

```
bovine_promoter     ATCTGAGTAGGGGTGGAGCTATTCCATGTAGGAGTGGAAGGCTCTCTATGACCAACAGGA
213                 ------------------------------------------------------------

bovine_promoter     GAGAGGAGCTGAGGAAATGCTGTTTCAAAAAGATGCCTGTGAGGGAAGGGACCAGAAACT
213                 ------------------------------------------------------------

bovine_promoter     GACATCATCAGGGATCACTTTGTTCAGGCATGGCCAACAAGTGTATTGGGGGGAGAAGGA
213                 ------------------------------------------------------------

bovine_promoter     TTTGGCCAGGTTTTTGTGTACAGCAATGTGGTAGCACTGTTACAGTGTGACCCCAGCGCC
213                 ------------------------------------------------------------

bovine_promoter     TGGCAGGAACACAGACATGTGGTCTGTTTGCACACTGCGCTGACCCTTTCACCTGAGCTG
213                 ------------------------------------------------------------

bovine_promoter     CTCAGCGCATGATCCTCCTTCCTGGAGCTAGACAGCTGTTAGGGTCAGGGAGCAGCTCGA
213                 ------------------------------------------------------------

bovine_promoter     GAGACCTAGAGGACTGGGATGGACATAGGAAAGCTCCAGGATGTCTCAGCAGTAAATCTA
213                 ------------------------------------------------------------

bovine_promoter     TGTCTTGGTTTCTAGGACTGTCTGATTTTAAAAATTCTGTCCCACAAGTTCACCAATACC
213                 ------------------------------------------------------------

bovine_promoter     TGTCAGACCAGGTGTCCCAGTTTTTGATTTGGAAAATGCACTCTGTTTCCCCGGTAATGG
213                 ------------------------------------------------------------

bovine_promoter     AGAAGGGGAAGGCAGCAGTTCATTCATTCCTCTAGTCTAAGCGTGAGATAGTGGGGGCAG
213                 ------------------------------------------------------------

bovine_promoter     GGCGGAGAGCCCGAGCAGCAGAGATGGCCCCAGCCTTGCGCTAGAATCTCTCATTGAATT
213                 ------------------------------------------------------------

bovine_promoter     CTCTGCTCTGTGCGCCCAGGTGAGTGGATGAGTGGTGGTGACTGACGCGAGGTGGTGATT
213                 ------------------------------------------------------------

bovine_promoter     GACCCCTCCTAGCCCCACCTGCTCACGCGGTCCTCCGCCCTATCCCATTTACAGTGGGAG
213                 ------------------------------------------------------------

bovine_promoter     GTCTCATCTATCTCTGCTCTCGGCCTCGCTCCACCAGCTTAAGGAAGAGAGTCCACGTGC
213                 ------------------------------------------------------------

bovine_promoter     AAATGGGCAAGCAGTCTTGCAAAAAGTAGGTCTAAGCAACTGGGTTCGATGTGGCCGATT
213                 ------------------------------------------------------------

bovine_promoter     TCAAGCTGCCAGTTTCTCCCAAAGCGAGATGGGAAGGAGACTCTGGGTCCCCAAGGCTGC
213                 ------------------------------------------------------------

bovine_promoter     GATACAGCCCATTCGGCCAACACCCCAGCCCCCACTCCCCAACACCCCATCGCAGCGGCT
213                 ------------------------------------------------------------

bovine_promoter     CTAGGCCCACCTCCTAAGCGATCAGGCCATGGCCCCCAGCCTCGTAAGCTCCCCTCCCAG
213                 ------------------------------------------------------------
```

```
bovine_promoter      CGTAGCCCGGCCCATCTCCCCACCGGAGGCCCCGCCCCCGACCCCTCCCTGGCTTGGCAG
213                  ------------------------------------------------------------


bovine_promoter      GCTCCGGAGAGGCGGTGCCCGCCAGCCGTCCGAGGCAGACGCACCAGCGGGACTACAAGT
213                  ------------------------------------------------------------


bovine_promoter      TCCAGCAAACCCTGGGGCCTGGCCTCGGGGGCGGGGCTATATCAGGCAGCGAATTGGGAG
213                  ------------------------------------------------------------GAG
                                                                                   ***

bovine_promoter      GAGGTCTGGCGCTCAGGCTAGGGAATGCGTGTGGCCAATGGCCCGGCGGCTCGCGCTGTG
213                  GAGGTCTGGCGCTCAGGCTAGGGAATGCGTGTGGCCAATGGCCCGGCGGCTCGCGCTGTG
                     ************************************************************

bovine_promoter      TGCCGATGGGGGCGGGGAGAAGCCCGCCAGCGCCCGGACAAAAGCCAGGTCTCCGGCGGC
213                  TGCCGATGGGGGCGGGGAGAAGCCCGCCAGCGCCCGGACAAAAGCCAGGTCTCCGGCGGC
                     ************************************************************

bovine_promoter      TGCGGGAGGCTGGAGCGCTCCTAGCAGTGGGCGGTGACTTGGGTTCTGTAGACTGAACGC
213                  TGCGGGAGGCTGGAGCGCTCCTAGCAGTGGGCGGTGACTTGGGTTCTGTAGACTGAACGC
                     ************************************************************

bovine_promoter      CGCGCATAGACTTTCTCGGAGAGGTGAGTGGCCGACGGCTGTTTTCGCGAGATGGACCCC
213                  CGCGCATAGACTTTCTCGGAGAGGTGAGTG----------------------------
                     ****************************

bovine_promoter      ACCTGGTCCAGGAGCTTCCTGCTTCCCTCCCCCAACCCGGAACCGCCTCCTTCGAAGCCA
213                  ------------------------------------------------------------


bovine_promoter      GCTTGGGGCTCACGCGCGCTCCCTGCACCTGCTTGGAGTTTTGGGGGTCTTAGTTTTAGG
213                  ------------------------------------------------------------


bovine_promoter      TTAGCCTTCGCTGGGATCCCGGCCGGTGGCCTCGTGTCGCCCTCCCCTCCCCCGCCCCAA
213                  ------------------------------------------------------------


bovine_promoter      CTCCTCCACATTCTAGTTCCCGGAGTTTGCACAAGGCTGGCCGCCGTTGTTGCCTGCCTT
213                  ------------------------------------------------------------


bovine_promoter      CCCTCCGCCGTATTACATCGCCCCTAACTGGCTCCCCTCATCCCCACCCCGGGACTAGTC
213                  ------------------------------------------------------------


bovine_promoter      GCCGGGC
213                  -------
```

Appendix 3 – Luciferase Assays Raw Data

A. Lucifrease assay data 1

LUCIFERASE MAXIMA

cmvSPORT	pGL3B	WT -1513	513	-213	blank
28	167	3727	2952	162	523
43	43	3358	2015	152	123
50	117	6497	2867	281	89
					245

B-Gal RESULTS

cmvSPORT	pGL3B	1513	513	213	blank
0.295	0.078	0.135	0.139	0.132	0.066
0.289	0.077	0.127	0.141	0.138	0.069
0.221	0.74	0.157	0.169	0.136	0.068
					0.067667

luciferase minus blank

-217	-78	3482	2707	-83
-202	-202	3113	1770	-93
-195	-128	6252	2622	36

cmvSPORT	pGL3B	1513	513	213
		B-Gal minus blank		
0.227333	0.010333	0.067333	0.071333	0.064333
0.221333	0.009333	0.059333	0.073333	0.070333
0.153333	0.672333	0.089333	0.101333	0.068333

corrected: luciferase/B-gal

-954.545	-7548.39	51712.87	37948.6	-1290.155
-912.651	-21642.9	52466.29	24136.36	-1322.275
-1271.74	-190.382	69985.07	25875	-526.8293
averages				
-1046.31	-9793.88	58054.75	29319.99	-695.2004

		corrected average relative to -1513		
cmvSPORT	pGL3B	1513	513	213
-1.80228	-16.8701	100	50.50403	-1.197491
		Average deviation from the mean		
150.2849	7899.321	7953.552	5752.407	814.6864
percentage error				
-14.3633	-80.6557	13.70009	19.61941	-117.1873

B. Luciferase assay data 2

LUCIFERASE MAXIMA

cmvSPORT	pGL3B	WT -1513	-513	-213	blank
76	92	27143	10676	336	213
34	205	17119	9759	313	18
50	82	23283	14364	282	37

B-Gal RESULTS

cmvSPORT	pGL3B	WT -1513	-513	-213	blank
0.099	0.071	0.171	0.134	0.153	0.062
0.079	0.072	0.132	0.135	0.152	0.065
0.9	0.07	0.162	0.163	0.176	0.067

luciferase minus blank

76	92	27143	10676	336
34	205	17119	9759	313
50	82	23283	14364	282

cmvSPORT	pGL3B	WT -1513	-513	-213
		B-Gal minus blank		
0.099	0.071	0.171	0.134	0.153
0.079	0.072	0.132	0.135	0.152
0.9	0.07	0.162	0.163	0.176

corrected: luciferase/B-gal

767.6768	1295.775	158731	79671.64	2196.078
430.3797	2847.222	129689.4	72288.89	2059.211
55.55556	1171.429	143722.2	88122.7	1602.273
averages				
417.8707	1771.475	144047.5	80027.74	1952.521

corrected average relative to -1513

cmvSPORT	pGL3B	WT -1513	-513	-213
0.290092	1.229785	100	55.55648	1.35547

Average deviation from the mean

241.5434	717.1647	9788.972	5396.637	233.4986

percentage error

57.80339	40.48404	6.795654	6.743458	11.95883

C. Luciferase assay data 3

LUCIFERASE MAXIMA

cmvSPORT	pGL3B	WT -1513	-513	-213	blank
208	55	35733	10569	160	10
100	181	17882	11950	98	58
56	87	19046	18988	136	61
					43

B-Gal RESULTS

cmvSPORT	pGL3B	WT -1513	-513	-213	blank
0.093	0.08	0.278	0.222	0.179	0.071
0.078	0.0867	0.215	0.227	0.18	0.071
0.081	0.086	0.218	0.247	0.175	0.071
					0.071

luciferase minus blank

165	12	35690	10526	117
57	138	17839	11907	55
13	44	19003	18945	93

cmvSPORT	pGL3B	WT -1513	-513	-213
		B-Gal minus blank		
0.022	0.009	0.207	0.151	0.108
0.007	0.0157	0.144	0.156	0.109
0.01	0.015	0.147	0.176	0.104

corrected: luciferase/B-gal

7500	1333.333	172415.5	69708.61	1083.333
8142.857	8789.809	123881.9	76326.92	504.5872
1300	2933.333	129272.1	107642	894.2308
averages				
5647.619	4352.159	141856.5	84559.19	827.3838

corrected average relative to -1513

cmvSPORT	pGL3B	WT -1513	-1228	-1066
3.98122	3.068001	100	59.60896	0.583254

Average deviation from the mean

2898.413	2958.434	20372.64	15388.57	215.1977

percentage error

51.32097	67.97624	14.36144	18.19858	26.00942

D. Collated data from the 3 luciferase assays

cmvSPORT	pGL3B	-1513	-513	-213
Expt 1		**corrected: luciferase/B-gal**		
123.1672	16161.29	55351.49	41383.18	2518.135
194.2771	4607.143	56595.51	27477.27	2161.137
326.087	174.0208	72727.61	28292.76	4112.195
AVG				
214.5104	6980.818	61558.2	32384.4	2930.489
		relative to wt %		
0.348468	11.34019	100	52.60778	4.760518
AVG DEV				
74.38437	6120.315	7446.274	5999.182	787.804
		% error		
34.67634	87.67332	12.09632	18.52491	26.88302
Expt 2		**corrected: luciferase/B-gal**		
7500	1333.333	172415.5	69708.61	1083.333
8142.857	8789.809	123881.9	76326.92	504.5872
1300	2933.333	129272.1	107642	894.2308
AVG				
5647.619	4352.159	141856.5	84559.19	827.3838
		relative to wt %		
3.98122	3.068001	100	59.60896	0.583254
AVG DEV				
2898.413	2958.434	20372.64	15388.57	215.1977
		% error		
51.32097	67.97624	14.36144	18.19858	26.00942
Expt 3		**corrected: luciferase/B-gal**		
767.6768	1295.775	158731	79671.64	2196.078
430.3797	2847.222	129689.4	72288.89	2059.211
55.55556	1171.429	143722.2	88122.7	1602.273
AVG				
417.8707	1771.475	144047.5	80027.74	1952.521

relative to wt %

0.290092	1.229785	100	55.55648	1.35547

AVG DEV

241.5434	717.1647	9788.972	5396.637	233.4986

% error

57.80339	40.48404	6.795654	6.743458	11.95883

SUMMARY OF ALL EXPTS THIS SERIES

cmvSPORT	pGL3B	-1513	-513	-213	
0.348468	11.34019	100	52.60778	4.760518	**Expt 1**
3.98122	3.068001	100	59.60896	0.583254	**Expt 2**
0.290092	1.229785	100	55.55648	1.35547	**Expt 3**

AVG(graphed)

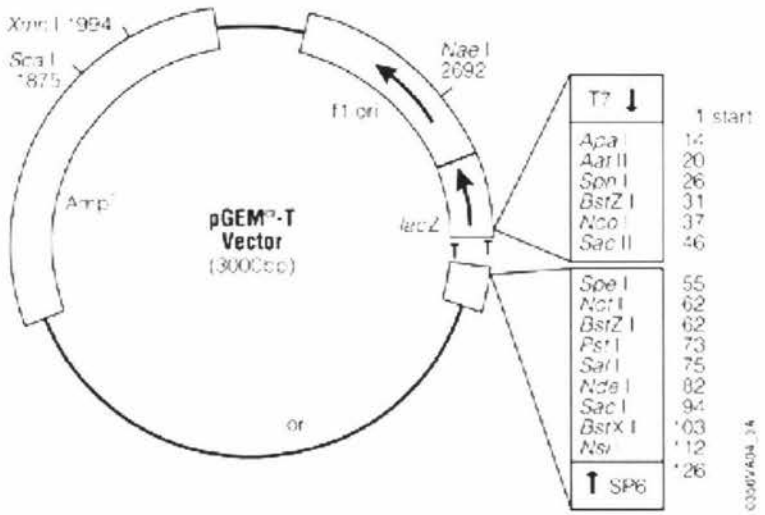
1.539927	5.212659	100	55.92441	2.23308	**Overall**

% error(graphed)

1.627529	4.085022	0	2.45637	1.684958	**Overall**

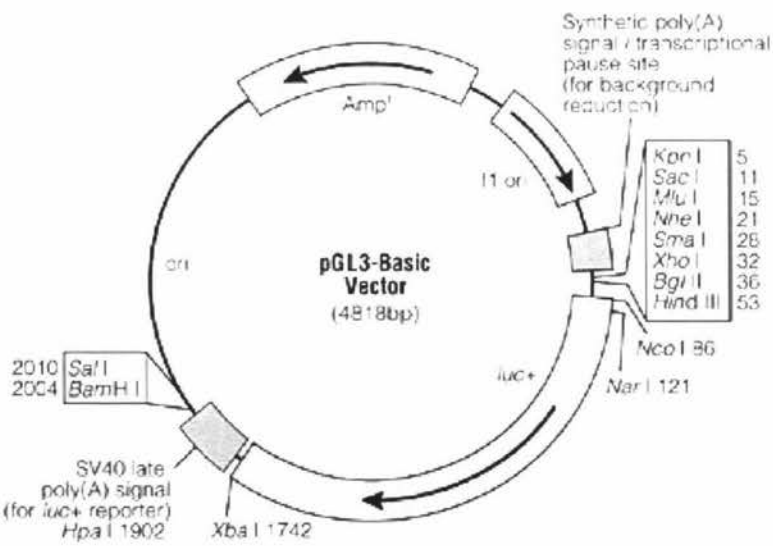
Appendix 4 – Plasmid Vectors

A. pGEM®-T Vector



pGEM®-T vector
Containing the T over hangs, flanked by the multiple cloning sites located in the lac Z gene. The ligation of the insert will disrupt the lac Z gene that produces the blue colour and therefore will produce white colonies.

B. pGL3 - Basic Vector



pGL3 - Basic vector
The pGL3 – Basic vector contains the *Luc*$^+$ gene for the expression of reporter gene luciferase, multiple cloning site for directional cloning and ampicillin resistance gene for bacterial selection.

Appendix 5 – PCR Primers

Bt_ACLY_1513_F:

5' – TAATGGTACCGAGAGGTAGTCCTTCTCCAAGATGGGTGCT - 3'

Bt_ACLY_513_F:

5' – TAATGGTACCTGCGATACAGCCCATTCGGCCAACACCC - 3'

Bt_ACLY_213_F:

5' – TAATGGTACCGAGGAGGTCTGGCGCTCAGGCTAGGGAATGCG - 3'

Bt_ACLY_R:

5' – TAATAAGCTTCCTCTCCGAGAAAGTCTATGCGCGGCGTTC - 3'