

Investigation of Molecular Regulatory Control of the Cardiac-enriched Acetyl-CoA Carboxylase

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DEDICATION

I dedicate this thesis to:

My mother, Dai, who has shown me the meaning of perserverence,

The memory of my father, Dhaya Bhai, for showing me the true meaning of dedication, diligence and unselfish service to others,

My sisters, Prashiela and Sharmila and brother, Rajesh for being better siblings than I deserve,

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If wrong in our hearts, our heads are right in vain
- Edward Young

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PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS STUDY

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ABBREVIATIONS

5'-UTR	5'-untranslated region
A	adenine
ACC	acetyl-CoA carboxylase
ACC α	acetyl-CoA carboxylase alpha isoform
ACC β	acetyl-CoA carboxylase beta isoform
ACS	acyl-CoA synthetase
Akt / PKB	protein kinase B
AMP	adenosine monophosphate
AMPK	5'-AMP-activated protein kinase
ANP	atrial natriuretic peptide
AP	adaptor primer
AP-1	activating protein
ATP	adenosine triphosphate
C	cytosine
CAT	carnitine-acetyl translocase
CoASH	coenzyme A
COUP-TF	chicken ovalbumin promoter transcription factor
CPT I	carnitine palmitoyl transferase I
CPT II	carnitine palmitoyl transferase II
CsCl	caesium chloride
CVD	cardiovascular disease
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulphoxide
DNA	deoxyribose nucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediamine tetra-acetic acid
ERK	extracellular responsive kinase
FABP	fatty acid binding protein
FAO	fatty acid oxidation
FARE	fatty acid response element
FAT/CD36	fatty acid translocase
FATP	fatty acid transport protein
G	guanosine
g	centrifugal force
g	gram
G-6-P	glucose-6-phosphate
GIK	glucose, insulin, potassium
GLUT1	glucose transporter 1
GLUT4	glucose transporter 4
GTF	general transcription factor
HIF1	hypoxia inducible factor
HLH	helix-loop-helix
LCFA	long chain fatty acid
ICPT 1	liver-type carnitine palmitoyl transferase 1
LVS	left ventricle and septum
LZ	leucine zipper
MAPK	mitogen-activated protein kinase
MCAD	medium chain acyl-CoA dehydrogenase
MCD	malonyl-CoA decarboxylase
mCPT 1	muscle-type carnitine palmitoyl transferase 1
MDF	muscle determination factor
MEF2C	myocyte enhancer factor-2C
mg	milligram
ml	millilitre
μ m	micron
μ l	microlitre
MRF	muscle regulatory factor
mRNA	messenger RNA
MW	molecular weight
n	number
NaOH	sodium hydroxide
NR	nuclear receptor
NRRE	nuclear receptor response element

°C	degree Celcius
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDH	pyruvate dehydrogenase complex
PK4	pyruvate dehydrogenase kinase 4
PGC-1	peroxisome proliferator-activated receptor- γ isoform coactivator 1
PKA	protein kinase A
PKB / LBK1	protein kinase B
PPAR α	peroxisome proliferator-activated receptor- α isoform
PPRE	PPAR response element
RAR	retinoic acid receptor
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RT	reverse transcription
RV	right ventricle
RXR	retinoid X receptor
SDS	sodium dodecyl sulphate
SEM	standard error of mean
SREBP	sterol regulatory element-binding protein
T	thymine
TBP	TATA-binding protein
TCA	tricarboxylic acid
TE	Tris ethylenediamine tetra-acetic acid
U	unit
UCP3	uncoupling protein 3
USF	upstream stimulatory factor
V	volt
v/v	volume for volume
VEGF	vascular endothelial growth factor

ABSTRACT

Collective data gathered from studies of different forms of heart disease indicate a common denominator among them, namely a detrimental change in energy homeostasis. This inference has led to increasing interest in metabolic interventions to treat the diseased myocardium, termed metabolic modulation. However, the underlying molecular mechanisms directing cardiac metabolism and the changes occurring during pathogenesis are poorly understood. The chronic modification of cardiac metabolism is a process known as metabolic remodeling, which is thought to be mediated by changes in gene expression patterns. This thesis focussed on the gene regulatory programmes directing the expression of metabolic genes during metabolic remodelling.

The current study was centred on the enzyme acetyl-CoA carboxylase (ACC) which catalyses the carboxylation of acetyl-CoA to generate malonyl-CoA, a central metabolite in the regulation of fatty acid oxidation (FAO). There are two isoforms of ACC, ACC α and ACC β , both of which are present in the heart. The two isoforms differ with respect to structure, subcellular localisation, tissue distribution and kinetic properties. The particular focal point of this work was the transcriptional regulation of cardiac acetyl-CoA carboxylase (ACC β), the precise regulatory role of this enzyme in the myocardium is unclear but it is thought to play a key role in cardiac FAO. The hypothesis was that the cardiac enriched isoform of ACC (ACC β) is distinct in its regulatory control of cardiac FAO compared to ACC α and that evaluation of the regulation of ACC β should provide further insight into its regulatory role in cardiac metabolic remodelling.

A rat model of hypobaric hypoxia was used; rats were subjected to one week of hypobaric hypoxia (11% ambient O₂). Morphological and haematological studies, as well as quantitative real-time PCR to assess changes in metabolic gene expression were conducted on heart tissue. Morphometric data and atrial natriuretic peptide (ANP) transcript levels indicated that hypoxia-induced right ventricular hypertrophy in experimental animals and a reversion to the foetal gene expression pattern in this ventricle. In both ventricles, there was a co-ordinated downward trend in the transcriptional regulation of several genes involved in mitochondrial fatty acid β -oxidation (PDK4, UCP3, mCPT1 and MCD), transcription of glucose transporters (GLUT1 and GLUT4) was not significantly altered.

Contrary to our expectations, ACC α and ACC β were similarly regulated in both ventricles, indicating that the expression of these isoforms are under the control of analogous regulatory mechanisms which are activated by a hypoxic rather than a hypertrophic stimulus. Transcript levels of both genes were decreased in the right ventricle (RV) and left ventricle and septum (LVS). This result was unexpected for two reasons, firstly the result suggested that both isoforms perform similar functions. Secondly, ACC transcript levels were regulated in a manner imitating that of genes involved in FAO indicating that in addition to regulating FAO via the synthesis of malonyl-CoA, ACC, particularly ACC β , which is the predominant isoform in the myocardium, may perform another function.

In order to further investigate ACC β transcriptional regulation, we isolated a 947 bp fragment in the 5'-flanking region of the rat ACC β gene. 5'-Rapid amplification of cDNA ends (5'-RACE) analyses showed that this fragment did not contain the known ACC β gene promoter. Sequence analysis of this region showed that it contained several putative promoter elements including TATA and CAAT boxes as well as response elements for several transcription factors implicated in the regulation of metabolism. Reporter constructs containing 764 bp, 600 bp and 257 bp of the novel sequence upstream of the translation start site of the gene, namely ACC764.luc, ACC600.luc and ACC257.luc, were synthesised. Transfection experiments using these constructs showed that this region could induce transcription in CV-1 fibroblast and H9c2

myoblasts. Transcriptional induction was almost three-fold higher in H9c2 myotubes suggesting a role for this region in developmental patterning. Induction of transcription was repressed in experiments with ACC600.luc, but this repression was not evident in experiments involving the other constructs indicating that there is a silencer in the region 600 to 258 bp upstream of the translation start site.

Co-transfection experiments using expression vectors for the transcription factors involved in the regulation of metabolic gene expression were conducted. The upstream stimulatory factors (USF), USF1 and USF2a showed an induction of transcription and the region involved was narrowed down to a 257 bp fragment containing two consensus *cis*-elements to which these factors bind. The nuclear receptors, peroxisome proliferator-activated receptor- α (PPAR α) and retinoid X receptor- α (RXR α), which are known regulators of cardiac metabolic gene expression also induced transcription from this region. Thus, the 5'-flanking region of the rat ACC β gene immediately upstream of the initiator ATG is able to drive transcription *in vitro* and may regulate ACC β gene expression in response to changes in substrate availability.

In summary, the data here presented delineate transcriptional modifications in cardiac metabolic gene expression induced by hypobaric hypoxia. There is a co-ordinate downregulation of genes involved in FAO. ACC α and ACC β were analogously regulated in response to hypoxia and hypoxia-induced hypertrophy indicating that these isoforms have similar regulatory functions. A novel 764 bp fragment in 5'-flanking region of the rat ACC β gene which may play a role in the transcription and/or post-transcriptional regulation of this gene in response to nutritional flux was isolated and characterised.

1.1. Cardiac Metabolism

Cardiac metabolism is the sum total of the reactions providing the heart with sufficient energy to perform its contractile function. These reactions are exquisitely regulated enabling the myocardium to respond swiftly to changing physiological conditions. Recently cardiac metabolism has become a largely neglected area of cardiology. Taegtmeyer [2] has even gone as far as to call it “the lost child of cardiology”.

Nevertheless, much has been learnt about metabolism in the myocardium since the link between cardiac metabolism and function was established by Langendorff in 1895 [3]. The metabolic substrates used by the heart were determined near the beginning of the last century and the biochemistry involved in these processes was investigated during the 1960s [4]. Experiments in the perfused heart led to elucidation of the interaction between glucose and fatty acid metabolism in the myocardium, the glucose-fatty acid cycle [5, 6] and myocardial preference for utilisation of fatty acids for oxidative purposes [7].

The emergence of the fields of molecular biology and genetics over the last forty years has provided tools for greater insight into cardiac metabolism at the gene and protein levels of regulation. Further insights into the interaction between glucose and fatty acid metabolism under different physiological and pathophysiological conditions have been gained. These new tools have revealed not only the complexity of the underlying causes of heart disease but have also underlined the importance of cardiac metabolism.

Cumulative data have led to one simplification; the proposal that a common denominator in different forms of heart disease is a detrimental change in energy homeostasis [8]. The resultant strengthening consensus of this inference is that contractile function of the dysfunctional heart could be improved by metabolic intervention. This alteration in cardiac energetics is known as metabolic modulation [9].

1.2. Metabolic Modulation

The idea of using metabolic interventions to treat the diseased myocardium is not new. As early as 1962, Sodi-Palleres proposed treating the ischaemic myocardium with an infusion of glucose, insulin and potassium (GIK) to replace lost potassium in the ischaemic region. Further information gathered from experimental research in the isolated perfused rat heart was instrumental in acquiring an understanding of cardiac metabolism and has complemented the potential application of metabolic modulation indicated by clinical observation.

Experimental data indicated that the myocardium readily utilised glucose and fatty acids for oxidative purposes, but fatty acids was the preferential substrate [5-7]. Proposed mechanisms for the benefits of glucose and glycolysis in ischaemia formed the basis of the Glucose hypothesis which suggested that glucose was advantageous to the ischaemic myocardium [10]. The first direct evidence of the beneficial effects of glucose on the ischaemic myocardium was derived from experiments using an isolated rat heart model [11].

De Leiris et al [11] found that glucose use during ischaemia resulted in less damage than fatty acid utilisation during the same period. Furthermore, when both glucose and fatty acids were present in the ischaemic heart, glucose diminished the damage caused by fatty acids. These results corresponded to the detrimental effects of high serum-free fatty acid levels observed in the human myocardium post-infarction [12].

Subsequent work confirmed that the availability of different substrates could profoundly influence the extent of ischaemic damage and recovery on reperfusion. Glycolytic ATP protected the ischaemic myocardium, decreasing the severity of ischaemic cell damage and preventing the occurrence of ventricular arrhythmias [13]. Moreover, glycolytic ATP was better able to prevent or delay ischaemic contracture than ATP derived from oxidative phosphorylation [14].

Lopaschuk et al [15] have suggested that interventions promoting glucose oxidation at reperfusion could also be beneficial to the myocardium. They proposed that the inhibition of glucose oxidation rather than glycolysis at reperfusion of ischaemic myocardium was harmful. Furthermore it was demonstrated that fatty acid oxidation in the reperfused myocardium caused an imbalance between glycolysis and glucose oxidation. The resultant accumulation of protons aggravated the ischaemic acidosis causing an ion imbalance including calcium overload. Consequently, ATP is used to correct the ionic imbalance rather than for contractile function, preventing recovery.

Recently, the fields of molecular biology and genetics have contributed greatly to our knowledge of cardiac metabolism. The results of work on animal models and human studies support a role for metabolic modulation in treating certain forms of heart disease. Cardiac-specific transgenic and knock-out animal models affecting metabolic genes have indicated that metabolic enzymes play a critical protective role in the myocardium. It has also been suggested that alterations in metabolic gene expression plays an important role in adaptation to chronic changes in environment [16-19].

Genetic investigations have revealed that a mutation in a gene coding for master metabolic modulator, 5'-AMP-activated protein kinase (AMPK) causes a form of familial hypertrophic cardiomyopathy [20]. Other significant findings such as the pyruvate-mediated improvement in contractile function of the failing myocardium [21] and the preliminary finding of improved cardiac function in chronic heart failure upon treatment with the inhibitor of mitochondrial fatty acid uptake, etomoxir [22] also implicate flawed metabolism in the development of various forms of heart disease. This finding has also resulted in a rapid increase in the number of putative metabolic targets for modulation.

To date, most research into the application of metabolic modulation of the myocardium has focussed on treating the ischaemic or reperfused ischaemic myocardium. Various studies using pharmacological agents promoting glucose utilisation at the expense of fatty acid use (reviewed in [9]) have shown that enhancing glycolysis and glucose oxidation limited ischaemic damage and promoted contractile recovery upon reperfusion. Hence mechanisms inhibiting or limiting fatty acid oxidation (FAO) are beneficial to the ischaemic myocardium.

The increasing data regarding the regulation of metabolic enzymes at the gene level has led to the prospect of targeting chronic forms of heart disease. For example, disorders with numerous causes, such as hypertrophy, could be targeted with the aim of preventing, reversing or stalling the development of pathological events [23, 24]. However, in order for the implementation of metabolic modulation and the identification of therapeutic agents, greater knowledge of the pathways affected is required.

Three questions arise when approaching this problem:

- 1) Which enzyme involved in a critical metabolic pathway controlling fuel substrate selection to choose for the investigation?
- 2) Which stage of regulation of this candidate to investigate?
- 3) Which physiological or pathophysiological model to use in order to study the regulation of this enzyme?

1.3. The Approach taken in this Study

The following sections of this chapter deal with the approach taken in this study to answer the above-mentioned questions and form the rationale behind this thesis. However, at this stage it is appropriate to contextualise this work in the broad field of cardiac metabolism. This topic covers a wide range of studies including the uptake, storage and utilisation of different energy sources, the enzyme pathways involved in the generation of ATP from fuels and the conversion of ATP into mechanical energy for contraction. In this study, the scope of cardiac metabolism has been narrowed and is concerned with the regulation of fuel substrate selection by the myocardium for the production of energy for contractile function.

In this section, I will explain the reasoning behind the selection of:

- 1) Transcriptional regulation as the stage of regulation examined
- 2) Acetyl-CoA carboxylase β (ACCB) as a candidate for study, and
- 3) The use of a rat hypobaric hypoxia model to delineate the transcriptional regulation of ACCB.

1.4. Which Stage of Regulation of this Candidate to Investigate?

There are several levels of regulation of eukaryotic genes, from the transcription of the gene to the active protein. This allows for tight regulation of proteins while still permitting mechanisms for rapid adaptation to changing environmental conditions. Thus, intervention at one or more levels of regulation could potentially be used in metabolic modulation. These levels of regulation include:

- 1) **Transcriptional:** Control of which genes are/not transcribed into RNA: this includes activation of the gene and recruitment of transcription factors which facilitate transcription
- 2) **Post-transcriptional:** Control of the processing of primary mRNA into a completed mature form and the transport of the mRNA from the nucleus into the cytoplasm,
- 3) **Translational:** Regulation of translation of mRNAs to polypeptides
- 4) **Post-translational:** Regulating the processing of polypeptides into active proteins
 - i) Processing of polypeptides into a protein eg. polypeptide folding
 - ii) Enzyme regulation by:
 - allosteric mechanisms including glycosylation and covalent changes such as phosphorylation and dephosphorylation of the polypeptide by kinases and phosphorylases, respectively.

In order to put the argument supporting the selection of transcriptional regulation into perspective, I have given:

- 1) a brief overview of the transcriptional process including the *cis* and *trans*-acting elements and regulatory mechanisms involved in transcription

- 2) a brief overview of the implications of transcriptional regulation in metabolic modulation and,
- 3) an example of the activity of the pivotal transcription factor, peroxisome proliferator-activated receptor α (PPAR α) in cardiac metabolism

1.4.1. Brief overview of Transcription

The process of transcription involves the interaction of DNA elements (*cis*-elements) and proteins (*trans*-acting) elements. Both these elements control transcription in a tissue- and developmental stage -specific manner and their activity may alter with changing physiological conditions.

The entire transcriptional process involves the following steps:

1) *DNA Activation and Initiation of transcription*

In order for transcription to occur, several events must take place including the unravelling of the chromatin (activating the DNA), the assembly and binding of general or basal transcription factors (GTFs) and RNA polymerase II (RNA pol II) to the promoter region of a gene to form an initiation complex [25], the melting of the DNA template and the formation of the first phosphodiester bond by RNA pol II. In the main, it is acknowledged that the key regulatory events controlling gene expression occur at this step [26].

2) *Elongation*

RNA pol II moves along the DNA template strand, synthesising a complementary RNA transcript. Recent work has shown that transcription elongation factors play an important role in the regulation of transcription [27].

3) *Termination of RNA synthesis.*

Transcription usually stops shortly after a terminator sequence which signals the dissociation of RNA pol II from the DNA template by several transcript cleavage factors [28].

This study has focussed on step 1 of this process, DNA activation and the initiation of transcription.

A) *Cis-acting Elements*

There are several distinct types of DNA elements (*cis*-elements) involved in controlling eukaryotic gene expression (Figure 1.1), these include:

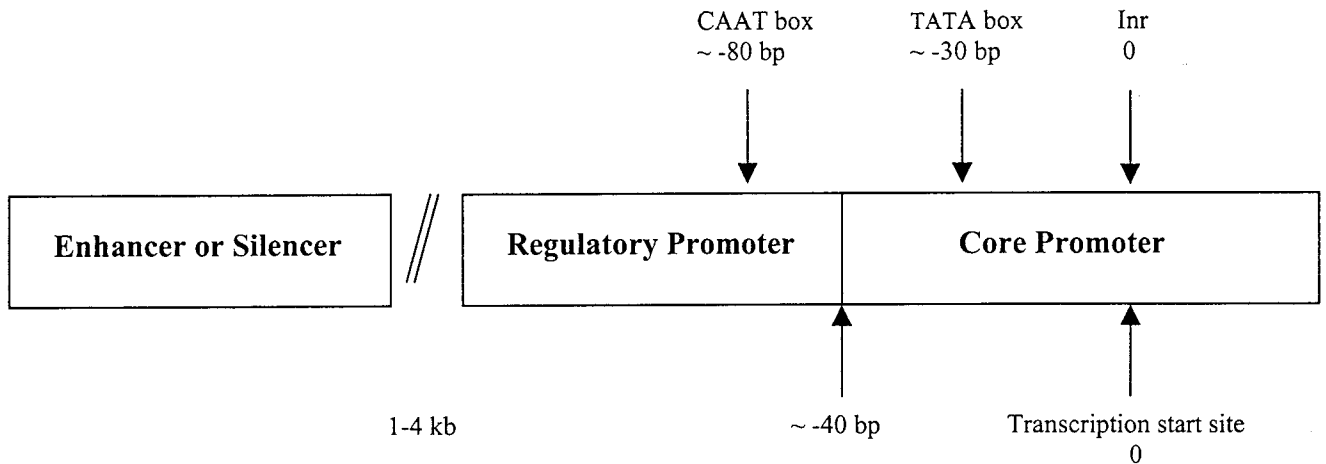


Figure 1.1. Typical *cis*-acting elements involved in the regulation of transcription.

The core promoter element spans the transcription start site and two common *cis*-elements located here are TATA boxes and initiator elements (Inr). A regulatory promoter is usually found upstream of a core promoter and contains response elements to upregulate transcriptional activity above basal levels and may contain a CAAT box. Enhancers and silencers may be positioned at great distances from the promoter, which they regulate. The positions are given relative to the transcription start site designated 0.

a) Core or basal promoter

The basal or core promoter is found in all protein-coding genes and is sufficient to drive the initiation of basal transcription. This is the DNA region that contains the transcription start site and is the sequence to which RNA pol II binds to, at the start of transcription. The region contains elements specific for the initiation of transcription and its sequence determines the basal or unregulated level of transcription and is sufficient to drive transcription *in vitro* and can also regulate gene expression levels [29-31].

The most common core promoter element is a TATA box which is present in 30-50% of promoters with the consensus sequence TATAAA. This response element is usually located 20 – 45 bp upstream of the transcription initiation site [32]. The GTP, TFIID, which binds to both the core promoter and RNA pol II is a complex of the TATA-binding protein (TBP) and 14 other GTFs called TBP-associated factors (TAFs). TBP recognizes and binds to the TATA box and functions as a border between DNA and GTFs which execute structural and enzymatic functions contributing to the initiation of transcription [33, 34].

Another common core promoter element is the initiator element (Inr). This element has the consensus sequence, YYAN(T/A)Y, where Y represents a pyrimidine (G/C) and N symbolizes any base. The Inr element can cover the transcription initiation site and recruit basal transcriptional machinery. A core promoter element can contain either, a TATA box or Inr element or both of these elements [31, 35].

b) Regulatory or Proximal Promoter Element

This is the region ~1 kb immediately upstream of the core promoter containing numerous transcription factor binding sites or response elements and is involved in raising transcription to normal (wild-type) levels [36].

A frequent promoter element is the CAAT box, consensus sequence (C/G)CAAT, that are usually located between 60 and 100 bp upstream of the start site of transcription ([37]. Several transcription factors containing a leucine zipper (LZ) motif are able to bind to this element (details below).

c) Enhancers

DNA regions containing gene regulatory elements upregulating promoter activity and increasing the levels of transcription above basal. Enhancers can be located at great distances from the gene promoter and can be situated upstream, downstream, or even within the gene they control and in either orientation. Enhancers may contain numerous response elements to which transcriptional activators can bind to form a large protein complex, an enhancosome, which is able to influence transcription [38].

d) Silencers

These are *cis*-elements involved in the repression of gene expression. Ogbourne and Antalis [26] grouped silencers into two groups according to the mechanism of repression in which they are involved:

i) Silencer Elements or Classical Silencers

Direct an active repression mechanism and their activity is independent of distance from the promoter and orientation.

ii) Negative Regulatory Elements (NREs)

These elements act in a position –dependent manner and repress transcription by means of a passive mechanism.

Insulators are a form of NREs, these are stretches of DNA (≥ 42 bp) located between the enhancer(s) and promoter or

silencer(s) and promoter of adjacent genes or clusters of adjacent genes. These elements prevent a gene from being influenced by the activation of an adjacent gene [39].

e) Response Elements

These are short stretches of DNA (10-20 bp), sufficiently conserved for recognition by a corresponding regulatory transcription factor. They serve to bind the transcription factor at the regulatory DNA region and may also play a role in the allosteric regulation of the protein. These sites are found primarily in enhancers and silencers but also in promoter regions. The significance of a response element can differ considerably depending on conditions such as cell type and physiological conditions. Several transcription factor binding sites or response elements have been associated with metabolic gene regulation, some examples are given below:

E-boxes, a DNA consensus sequence CANNTG, have been found to be involved in the regulation of several metabolic genes and are discussed later in this chapter.

Sp1 elements are binding sites for members of the stimulatory protein, Sp, family of transcription factors. These factors bind to several GC-rich consensus sequences including GCGTGT, TGCAC, TGC GC and GGCGG [40, 41]. Sp1 and Sp3 play a role in the transcriptional regulation of genes regulating fuel metabolism such as acetyl-CoA carboxylase- α isoform (ACC α) [42], β -enolase [43] and medium-chain acyl-CoA dehydrogenase (MCAD) [44].

The **Activating Protein-1 (AP-1) consensus sequence**, a binding site for AP-1, with the consensus sequence TGACTCA. AP-1 mediates the transcriptional regulation of numerous genes including the glucose transporter 1 (GLUT1) [45].

Nuclear Receptor Response Element (NRRE) are the DNA binding sites for members of the nuclear receptor (NR) superfamily of transcription factors. NRREs are comprised of two half sites with the AGAACA or AGGTCA sequence separated by 1 to 5 bases [46]. The NR factors involved in cardiac metabolism such as peroxisome proliferator-activated receptor α (PPAR α) and retinoid X receptor (RXR) recognise NRREs comprised of direct repeats of the AGGTCA sequence separated by one nucleotide. This sequence has also been called the fatty acid response element (FARE) or PPAR responsive element (PPRE). NRREs have also been implicated in the control of NR activity by exerting allosteric effects along with ligands to influence co-activator or co-repressor binding and thereby the transcriptional activity of the NR [46].

B) Transcription factors (*trans*-elements)

These are proteins which bind specific DNA sequences (response elements) in the regulatory regions of the gene, promoting or inhibiting expression of this gene. Activators are transcription factors which bind to promoter or enhancer sequences and upregulate transcription. Repressors are transcription factors which bind to promoter or silencer sequences and inhibit transcription. Co-activators and co-repressors are defined as proteins which act together with activators and repressors, respectively, to facilitate the function of the activators and repressors. These factors can control transcription in a manner dependent on (i) cell / tissue type, (ii) stage of development, and (iii) environmental / physiological conditions.

Transcription factors are usually modular and contain functional domains:

- i) Activation domain: region which interacts with other components of the transcription machinery such as RNA polymerase or other transcription factors. Transcription factors have been classified according to the type of activation domain they contain.
- ii) DNA binding domain: amino acids form a motif in the protein that recognise specific bases near the start of transcription. Transcription factors have been grouped according to their DNA-binding domains.

Described below are examples of two transcription factor families:

a) Basic Helix-Loop-Helix Transcription Factors

The **basic helix-loop-helix** (bHLH) motif is a highly conserved domain found in many transcription factors. Some proteins containing this motif are ubiquitously expressed such as upstream stimulatory factors (USFs) while others such as MyoD are expressed only in certain tissues. This motif contains a region of primarily basic amino acid residues involved in DNA binding and another containing hydrophobic residues which form the helix-loop-helix (HLH) domain. The helices are amphipathic and present a hydrophobic surface to the environment allowing the protein to dimerise by hydrophobic interaction with another factor presenting a similar surface [47]. The helices are connected by a loop of 12-28 amino acids (**Figure 1.2**).

bHLH transcription factors usually bind DNA at the inverted repeat, E-box with the sequence CANNTG, where N represents any base [48]. E-boxes are found in a wide range of promoters and enhancers prompting speculation about factors influencing the binding specificity at these sites. It has been established that the nucleotides flanking E-boxes can play an important role in determining the affinity with which a bHLH dimer binds to the E-box [49-51].

i) **Upstream Stimulatory Factors (USFs)**

Upstream Stimulatory Factors (USFs) are ubiquitously expressed transcription factors containing a bHLH motif, in addition these bHLH factors also contain a leucine zipper (LZ) motif in the N-terminal of the polypeptide. This subfamily of bHLH transactivators are referred to as bHLHLZ transcription factors. The LZ domain is involved in protein-protein interactions. It consists of a helix containing several leucine residues which are placed in such a way that they interact with similarly spaced leucine residues on another protein with a similar motif forming a “zipper” between them [52, 53].

These transcription factors have been found to regulate the transcription of a number of genes coding for proteins performing diverse functions. These include signalling proteins such as the protein tyrosine phosphatase, SHP-1 [54], the structural proteins, Type I collagen [55], cardiac myosin light chain-2 [56] and cardiac α -myosin heavy chain [57], the lipogenic proteins Spot 14 [58] the carboxyl ester lipase [59], the glycolytic enzymes: L-type pyruvate kinase [60, 61] and genes involved in FAO such as mCPT1 [62].

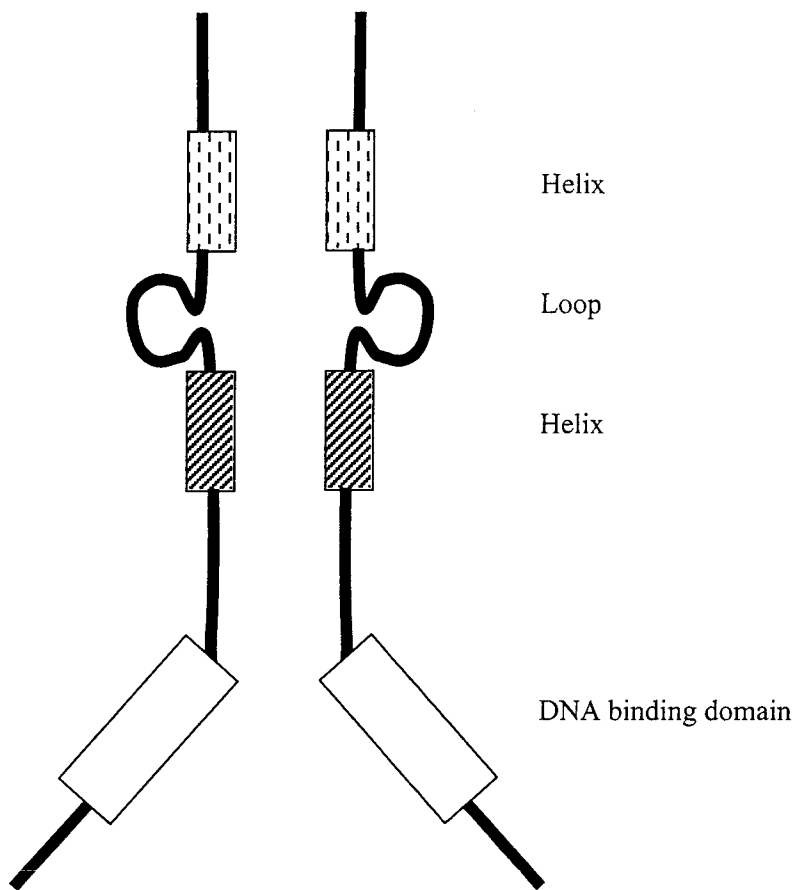


Figure 1.2. Schematic showing the domains of two basic helix-loop-helix transcription factors which formed a dimer. (Adapted from [1]).

USFs bind DNA as a dimer at the consensus sequence (E-box) but have also been found to bind response elements other than E-boxes. USF1 has been found to interact physically with an initiator element [63] and USF2 has been found to bind a response element with the sequence GAAAATATGATA [64]. USFs are able to form dimers that can be homo- or heterodimer of the two isoforms i.e. USF1 and USF2. USF1 and USF2 have distinct molecular weights, 43 kDa and 44 kDa, respectively [65]. There are also two splice variants of USF2, namely USF2a and USF2b. USF2a is the predominant variant *in vivo* [66]. USF1 and USF2 polypeptides have similar C-terminal regions, which contain the bHLH motif and therefore show identical dimerisation and DNA binding properties. In addition, USFs also contain a highly conserved domain called the USF-specific region (USR) which is upstream of the bHLH region which is thought to interact with co-activators and is essential for transcriptional activation by USFs [65].

Ratios of USF1 and USF2 vary in different cell types but USF1/USF2a heterodimers are the major species in most tissues, USF1 homodimers are less widespread while USF2a homodimers are rare. While the trans-activating mechanism of USFs have not yet been established, the basic binding domain of USFs has been found to exist as a bivalent homotetramer. This tetramer forms at physiological USF concentrations in the nucleus and depends on the integrity of the LZ motif. This attribute confers ability to bind simultaneously to two independent sites suggests DNA looping role in the *trans*-activating mechanism of USFs [53].

Although USFs are essential for the expression of certain genes, they have been found to be insufficient to drive high levels of gene expression and require other interacting factors or co-activators [59]. These interacting factors are also able to influence the involvement of USFs in the induction or inhibition of transcription of a particular gene [55]; [59]. Moore et al [62] have shown that USF2 can physically co-operate with peroxisome proliferator-activated receptor γ coactivator-1 (PGC-1), inhibiting the interaction of PGC-1 with the myocyte enhancer factor-2C (MEF2C) and inhibiting the synergy between PGC-1 and MEF2C, which activates transcription of the rat carnitine palmitoyl transferase1 β (CPT1 β) gene.

ii) **Muscle Regulatory Factors (MRFs)**

A group of muscle-related bHLHLZ transcription factors, called muscle regulatory factors (MRFs) or muscle determination factors (MDFs) have been identified. Four MRFs, namely, MyoD, Myf5, myogenin and MRF4 have been found to play central roles in myogenesis in skeletal muscle [67]. MRFs are thought to regulate different stages of the process and the functions of the different MRFs may overlap at the different stages of development. Myf5 and MyoD are involved in the first stage when cells are committed to a muscle cell fate forming myoblasts while myogenin and MRF4 are involved in terminal differentiation when myoblasts form myotubes. The role and regulation of MyoD has been relatively well investigated and is described below [68].

MyoD dimerises with ubiquitously expressed bHLH transcription factors, E12 or E47 to activate transcription during myogenesis [69, 70]. Muscle development is inhibited if MyoD, E12, or E47 are bound to the inhibitor of differentiation (Id) protein. Id contains the HLH motif required for dimerisation but not a basic region required for DNA binding. Dimerisation of Id with MyoD, E12, or E47 interferes with the ability of these proteins to bind DNA. Furthermore, the expression of Id in cells has been found to inhibit MyoD transcriptional activity [71]. The Id protein is synthesised in muscle cell precursors, myoblasts, but not during terminal differentiation of myoblasts into myotubes. Overexpression of Id in cultured myoblasts inhibits terminal differentiation of myoblasts [72]. MyoD has been found to interact with the myocyte enhancer factor-2 (MEF2) family of transcription factors to regulate myogenesis in skeletal muscle. It has been

established that MyoD can also bind to DNA elements which do not conform to the E-box sequence such as GCCTGTA [73].

iii) Sterol Regulatory Element-binding Proteins (SREBP)

There are three major isoforms of sterol regulatory element-binding proteins (SREBPs), namely SREBP1a, -1c and 2, which are coded for by two genes. SREBP1a and -1c are coded for by the same gene, which is under the control of two promoters producing products with distinct N-termini and which are differentially spliced. These factors have been identified as key regulators of nutritional homeostasis. This is a unique member of the bHLH family of transcription factors binding the inverted repeat, E-box, as well as the direct repeat sterol regulatory element (SRE), which has the consensus sequence, TCACNCCAC. This flexibility is due to the presence of a tyrosine residue in the basic domain of the bHLH motif which allows this domain to slightly change conformation and bind to the SRE [74]. SREBP transcription factors have been implicated in the upregulation of at least 30 genes involved in the synthesis and uptake of cholesterol, fatty acids, triglycerides and phospholipids including ACC α [75, 76].

iv) Nuclear Receptor Superfamily of Transcription Factors

Nuclear receptors form a superfamily of ligand-binding transcription factors involved in the regulation of gene expression during development as well as in the regulation of energetic homeostasis [77]. Members of this family include the peroxisome proliferator-activated receptors (PPARs), retinoid X receptors (RXR) and retinoic acid receptors (RAR). These DNA-binding proteins are modular in structure and contain 3 functional regions (**Figure 1.3**).

The three function domains are:

a) N-terminal domain (NTD)

This domain is poorly conserved and the length and amino acid sequences in this region vary greatly between members of this family of transcription factors. It contains sequences termed activator function 1 (AF1) which are important in protein-protein interactions involved in NR transactivation [78].

b) DNA binding domain (DBD)

This is the most highly conserved domain in the NR and plays a vital role in the correct binding of the NR to its corresponding DNA sequence. It also plays a role in the nuclear localization of the NR and its interaction with other transcription factors and co-activators or co-repressors. This domain is folded into a globular shape with two helices at right angles to each other forming a hydrophobic core.

b) Ligand-binding Domain (LBD)

This domain is situated at the carboxy-terminal of the NR polypeptide. It plays a role in sequence recognition and dimerisation through protein/DNA and/or protein-protein interactions.

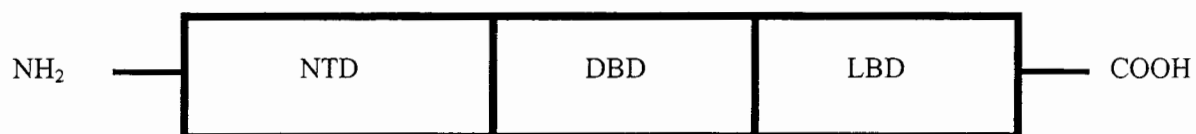


Figure 1.3. Schematic representation depicting the three functional domains of nuclear receptors.

The N-terminal domain (NTD) of nuclear receptors contains sequences termed activator function important in protein-protein interactions involved in NR *trans*-activation. The DNA binding domain (DBD) plays a critical role in the correct binding of the NR to its corresponding DNA sequence, in the nuclear localisation of the NR and its interaction with other transcription factors and co-activators or co-repressors. The ligand-binding domain (LBD) plays a role in sequence recognition and dimerisation.

C) Mechanisms of Activation and Repression

Although a large number of transcription factors have been identified and their structures and roles have been delineated, the precise regulatory mechanisms of most of these factors, including those examined in this study, have as yet not been definitively elucidated. Several general mechanisms in which transcription can be activated or repressed are described below.

a) **Mechanisms of Activator Activity**

Activation usually involves the binding of transcriptional activators to their response elements where they enlist and control the activities of chromatin –remodelling factors and transcriptional machinery. A number of the mechanisms employed by activators are:

i) Recruitment of Chromatin-remodelling factors

Activator proteins play an important role in the regulation of transcription by controlling the access of chromatin – remodeling factors to promoters [79]. These factors regulate the activation and priming of supercoiled DNA. Supercoiled DNA is a tightly packaged nucleoprotein complex consisting of DNA coiled around proteins including histone proteins, which is then folded into fibers to form chromatin. Activators can act on histone proteins in several ways including acetylation, phosphorylation, methylation and ubiquitination [46, 80], to alter chromatin structure enabling the assembly of a transcriptional complex.

Acetylation of lysine residues at the N-terminal of histone proteins by the chromatin remodelling enzymes, histone acetyltransferases (HATs) such as CBP and p300, is the best characterised mechanism of histone modification. The reaction results in a lower affinity of the histone protein for the DNA molecule and giving components of transcriptional activation access to gene regulatory sequences. For example, MyoD is able to activate transcription of muscle specific genes by remodelling chromatin at enhancer regions through working co-operatively with the HAT protein, p300 [73, 81]. Recent data indicate that transcriptional activation mechanisms of a number of NRs involve histone modifications resulting in the relaxation of the chromatin structure and can also generate co-activator binding sites [82].

ii) Recruiting and Stabilising Components of the Transcriptional Initiation Complex

Activators can bind to both *cis*-elements and components of the RNA pol II initiation complex and thereby play an important role in the assembly of the transcriptional complex. For example, Sp1 physically interacts with the GTF complex, TFIID, through TAFs and facilitates the binding of TFIID to the promoter [83]. The assembly of various transcriptional complexes are currently under intense investigation and emerging evidence indicates that this is an ordered process whereby factors are recruited in a precise sequence [28].

iii) Controlling the activity of the Transcriptional Machinery

Trans-activators along with their co-activators can stimulate RNA pol II activity by increasing the rate of elongation through the recruitment of transcriptional elongation factors [27]. They can also facilitate the reinitiation of transcription

by advancing promoter clearance of the transcription start site and recycling parts of the initiation complex for multiple rounds of transcription [80, 84].

b) Mechanisms of Repressor Activity

The mechanisms repressing gene expression are also of critical importance. This is illustrated by the involvement of aberrant transcriptional repression in several genetic disorders [85]. Transcriptional repression can be general or gene-specific. A general repression mechanism downregulates all transcription through the modification of components of the initiation complex by a repressor and/or co-repressor and preventing transcription. Gene-specific repression entails the repression of a specific gene by a particular repressor or co-repressor [86]. Repressors can act in two ways to inhibit transcription, namely the remodelling of chromatin and the inhibition of activators.

i) Recruitment of Chromatin-remodelling factors

Transcriptional repressors are able to act by associating with and activating histone deacetylases (HDACs) and are involved in the deacetylation of lysine residues in histone proteins. In the deacetylated state, the positively charged histones interact with the negatively charged DNA backbone and direct the packaging of DNA into condensed, compacted chromatin rendering *cis*-elements involved in transcriptional initiation inaccessible to activators [81]. For example, myocyte enhancer-2 (MEF2) work together with HDACs to inhibit muscle-specific gene expression [87].

ii) Inhibiting Activator Action

Repressors can act by inhibiting the binding of GTFs and preventing the formation of the transcriptional initiation. Transcriptional repressors also compete with activators for binding sites or binding to the activators. For instance, the chicken ovalbumin protein transcription factor (COUP-TF) binds the PPAR α response element in the foetus thus inhibiting PPAR α activity [88]. Prevention of activator binding can also occur via a passive mechanism, where DNA elements such as insulators prevent a gene from being influenced by the activation of an adjacent gene by virtue of their position in the genome [39]. Another mechanism inhibiting activators is the recruitment of co-repressors to the promoter by repressors. Co-repressors can interact with DNA binding factors preventing activators from remodelling chromatin or working with GTFs [80].

1.4.2. Implications of Transcriptional Regulation in Metabolic Modulation

Taegtmeyer et al [89] categorised the different mechanisms used by the heart to adapt to changing conditions according to the type of change i.e. acute or sustained. Immediate or acute changes result in co-ordinated activation or inactivation of enzymes at the post-translational level. Chronic or sustained changes were proposed to lead to adjustments in metabolic gene expression i.e. at the transcriptional level.

Physiological and pathophysiological conditions, which chronically modify substrate (oxygen, glucose, fatty acids) availability alter cardiac metabolism, a process called metabolic remodelling [90]. It has been suggested that in adjusting to chronic changes, metabolism forms the link between gene expression and contractile function of the heart as metabolic intermediates induce the translocation of transcription factors regulating gene expression [89].

Changes occurring in cardiac energy metabolism during mammalian development have been well described [91,92], however, information regarding the transcriptional regulation of cardiac metabolic genes during this period is only now emerging. During the perinatal period, there is a shift in energy substrate utilisation from glucose and lactate being the primary energy sources in the foetal heart to fatty acids in the adult heart [93]. This is due to the changes in environment and substrate availability. The foetal heart is relatively hypoxic, hence ATP is synthesised from oxygen-conserving pathways of glycolysis and lactate oxidation. In the postnatal heart, there is an alteration in oxygen availability along with a change to high-fat content maternal milk becoming the primary nutritional source. Recent data has shown that this switch in energy substrate utilisation is accompanied by modifications in the gene expression involved in these oxidative pathways [94].

During the neonatal period in the heart, there is a co-ordinated upregulation of genes involved in cardiac fatty acid utilisation. One mechanism regulating these changes involves the transcription factor, PPAR α , which is a pivotal transcriptional modulator directing elevated postnatal FAO [95]. This transcription factor is described here to illustrate the role of transcriptional regulation in cardiac metabolism under physiological and pathophysiological conditions, as well as the molecular mechanisms regulating gene expression.

1.4.3. The Role and Regulation of PPAR α in Cardiac Gene Expression

PPAR α belongs to the subfamily of PPAR which is part of the nuclear hormone receptor superfamily [96]. There are three PPAR isoforms, namely α , β/δ and γ [97]. These isoforms differ with respect to tissue distribution and expression at various stages of development. PPAR α is most abundant in the tissues with high mitochondrial FAO capacities, such as the heart. The regulation of this transcription factor during normal development as well as with the progress of pathogenesis has been the subject of much investigation.

PPAR α is upregulated during the neonatal period and has been implicated in induction of several genes associated with fatty acid uptake and activation, mitochondrial importation and β -oxidation (**Figure 1.4**) [95]. Recent advances also suggest that the deactivation of PPAR α -mediated gene expression may be responsible for the switch to a foetal pattern of gene expression in the hypertrophic heart [93].

The molecular mechanisms through which PPAR α regulates gene expression has been the subject of extensive research [78]. At least four steps are required for PPAR α -mediated transcription (**Figure 1.5**). These four steps are (a) the ligand activation of PPAR or its dimerisation partner, retinoid X receptor (RXR), (b) the interaction of PPAR α with RXR to form a heterodimer, (c) binding of the PPAR α /RXR heterodimer to the DNA consensus sequence in the regulatory region of the PPAR α -target gene and (d) the recruitment of co-factors to form a PPAR α transcriptional complex. Each step permits additional levels at which PPAR α activity can be controlled.

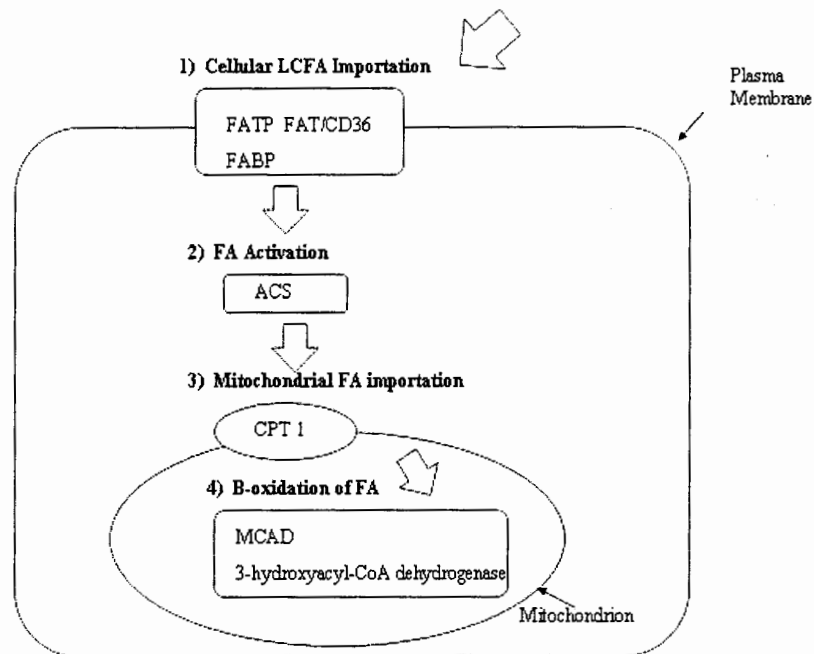


Figure 1.4. Schematic showing a number of PPAR α -target genes in the mitochondrial fatty acid oxidative pathway.

There are four stages in the fatty acid oxidative pathway at which PPAR α regulates gene expression, some of the PPAR α -targets at each stage of this pathway are given in the figure. 1) Cellular fatty acid (FA) uptake and transport: fatty acid transport protein (FATP) and fatty acid translocase (FAT/CD36) facilitate the uptake of long chain fatty acids (LCFA); fatty acid binding protein (FABP) transports LCFA within the cell. 2) FA Activation: acyl-CoA synthetase (ACS) activates LCFA to form acyl-CoA. 3) Mitochondrial fatty acid uptake: CPT1 is part of the carnitine shuttle system and regulates the rate-limiting step of this pathway. 4) β -oxidation: medium-chain acyl-CoA dehydrogenase (MCAD). The large arrows show the path of LCFAs and their derivatives within the cell (Adapted from [94]).

1) *Ligand activation of PPAR α or its dimerisation partner, retinoid X receptor (RXR)*

Ligand-binding may initiate or prevent allosteric changes in PPAR α or RXR to induce or inhibit the recruitment of co-factors and the ensuing induction or inhibition of target gene transcription. Ligands can bind to one or both of these transcription factors for activation. Although the endogenous ligands of PPAR α have not been conclusively defined, several putative ligands, such as fenofibrate, have been identified. Gottlicher et al [98] have shown that fatty acids can activate PPAR α -mediated gene expression. In contrast, the binding of fatty acyl-CoAs to PPAR α have been found to change its conformation and consequently prevent the binding of activating ligands [99, 100].

2) *Binding of PPAR α to RXR to form a heterodimer*

Upon ligand activation, PPAR α binds to RXR, if either transcription factor has been ligand-activated, to form a heterodimer [101]. The availability of RXR can regulate PPAR α transcriptional activity. For example, Huss et al [102] found that there was a decrease in RXR α levels under hypoxic conditions. They proposed that diminished RXR α levels resulted in decreased PPAR α /RXR α heterodimerisation and the consequent decrease in PPAR α -mediated gene expression. RXR also binds other nuclear receptor proteins, thus limiting the RXR available for heterodimerisation with PPAR α .

3) *Binding of the PPAR α /RXR heterodimer to the DNA consensus sequence in the regulatory region of the PPAR α -target gene*

PPAR α usually binds to a consensus *cis*-regulatory DNA region in the promoters or enhancer regions of PPAR α -target genes called the PPRE or FARE. PPRE consists of a direct repeat of six nucleotides separated by one nucleotide. The consensus sequence is TGACCT N TGACCT. PPAR α binds to one repeat while RXR binds to the other.

Other nuclear receptor proteins such as chicken ovalbumin promoter transcription factor (COUP-TF) also bind to PPRE inhibiting PPAR α activity [88]. In agreement, Sack et al. [44] have proposed that the increased levels of COUP-TF paralleling foetal levels observed during the development of hypertrophy is a mechanism for the observed decreased expression of several PPAR α -target genes. Furthermore, it was suggested that this mechanism eventually results in the reversion to the foetal gene expression pattern.

4) *Recruitment of co-factors to form a PPAR α transcriptional complex*

The binding of the PPAR α /RXR heterodimer to the PPRE allows for the recruitment of co-factors. Co-factors may be co-activators or co-repressors which induce or inhibit gene transcription. For example, PGC-1 is found at relatively high levels in the heart, has been the focus of much study [103]. PGC-1 has been found to activate PPAR α . Furthermore, it was suggested that such transcriptional mechanisms contributes to the reversion to the foetal gene expression pattern with the development of cardiac pathology, such as cardiac hypertrophy [104]

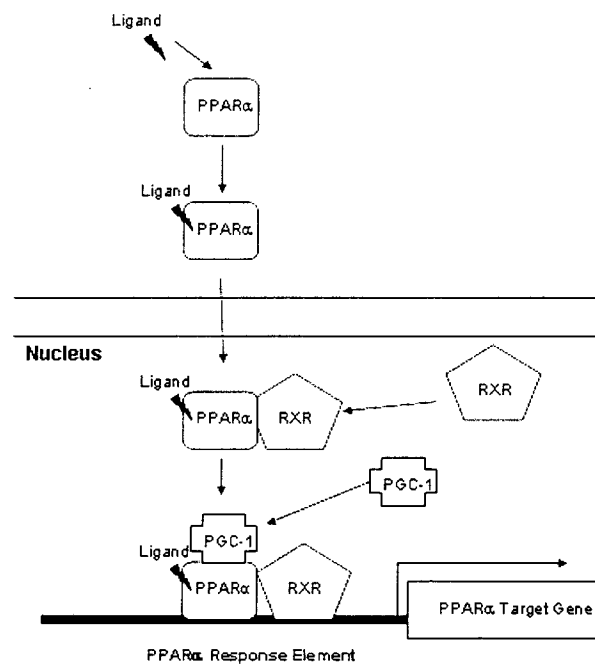


Figure 1.5. The regulation of PPARα activity.

PPARα activity is regulated at a number of stages including ligand activation, heterodimerisation with RXR, binding to the PPARα response element in the regulatory region of PPARα target genes and the recruitment of cofactors, such as PGC-1, to form a transcriptional complex.

Conformational changes to PPAR α form additional mechanism regulating its activity. The mitogen-activated protein kinase (MAPK) family acts on PPAR α [105]. Extracellular responsive kinase (ERK) phosphorylation inhibits PPAR α [94]. In contrast, PPAR α is activated by p38 MAPK [106].

The derangements in cardiac metabolism observed in studies on PPAR α -null mice [107, 108] and cardiac-specific PPAR α overexpressing mice [109, 110] have further emphasised the importance of PPAR α in regulating cardiac metabolism. FAO is downregulated in PPAR α knock-out mice while glucose use is upregulated [108]. On the other hand, PPAR α overexpressing mice exhibit an upregulation of fatty acid utilisation at the expense of glucose [109]. This abnormal pattern of cardiac metabolism observed in the latter study is similar to that seen in the diabetic phenotype, which eventually leads to the development of cardiac hypertrophy. Hence, the complexity of PPAR α regulation and abnormalities seen in PPAR α -null and -cardiac specific overexpressing mice stresses the importance of cardiac metabolic gene expression and its regulation.

Investigating the transcriptional regulation of genes is therefore being considered as a novel target for metabolic modulation in chronic conditions leading to maladaptive metabolic remodelling. Such innovative interventions may play a critical role in the prevention of chronic cardiovascular diseases (CVD), especially in light of global studies showing an increasing incidence of CVDs due to changes in the lifestyle associated with industrialisation and urbanisation [111, 112]. This burden of CVD is of particular concern in developing countries such as South Africa, where the effects of industrialisation and urbanisation are only now beginning to be realised. Hence in order to effectively manage this rapidly emerging problem, new therapeutic approaches such as metabolic modulation are needed to cope with the burden of the increasing incidence of CVDs. For these reasons, we investigated the transcriptional regulation of ACC β .

1.6. Which Candidate Metabolic Enzyme to Select for Investigation?

As mentioned above, the mechanisms regulating fatty acid oxidation (FAO) are of great interest as potential targets of metabolic modulation. Therefore, we have investigated the enzyme pathway regulating the rate-limiting step of FAO in the heart. Fatty acid metabolism (**Figure 1.6**) in the myocardium can be divided into four steps:

- 1) Transport of fatty acids into the cardiomyocyte
- 2) Activation of fatty acids in the cytoplasm
- 3) Transport of activated fatty acids from the cytoplasm into the mitochondrion (carnitine shuttle system)
- 4) Fatty acid β -oxidation (FAO) in the mitochondrion

The regulation of fatty acid import into the mitochondrion (step 3) was considered in this study as it is a critical juncture in this pathway. The transport of activated LCFAs into the mitochondrion is required for FAO (see **Figure 1.7**). The rate-limiting step in the import of LCFA into the mitochondrion is controlled by CPT I.

CPT I is part of the carnitine shuttle system and is located on the outer mitochondrial membrane. This enzyme converts fatty acyl-CoA to fatty acyl carnitine, which can be transported into the mitochondrion, a critical step in FAO since the inner mitochondrial membrane is impermeable to fatty acyl-CoA. The fatty acyl carnitine in the mitochondrion is converted back into fatty acyl-CoA by CPT II [113].

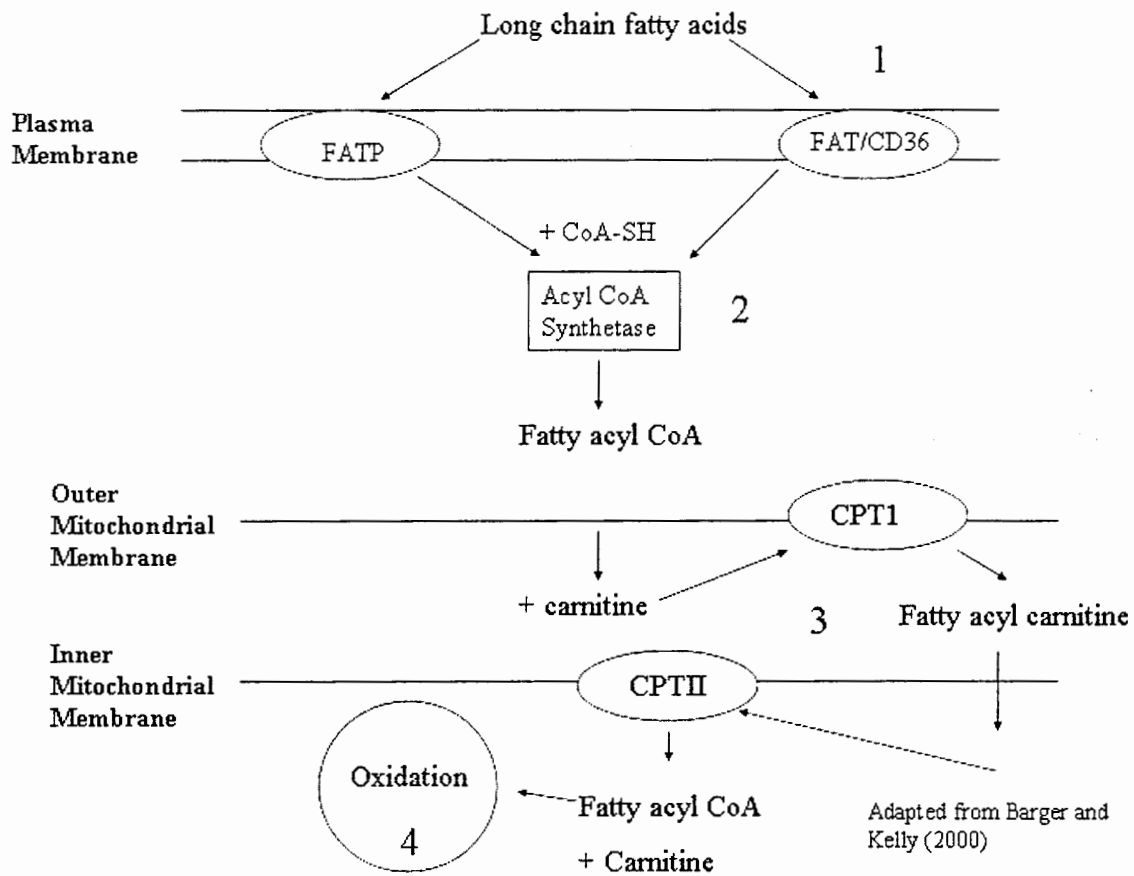


Figure 1.6. Fatty acid metabolism in the heart.

Long chain fatty acids are imported into the cells (1) by fatty acid transport protein (FATP) and fatty acid translocase (FAT/CD36). The fatty acids are then activated by acyl-CoA synthetase (2) to form fatty acyl-CoA. Fatty acyl-CoA then enters the carnitine shuttle (3) where it is converted to fatty acyl carnitine by the carnitine palmitoyl transferase 1 (CPT 1), translocated across the inner mitochondrial membrane and reconverted to fatty acyl-CoA by CPT II. Fatty acyl-CoA in the mitochondrion enters the β -oxidation cycle (4). (Adapted from [93]).

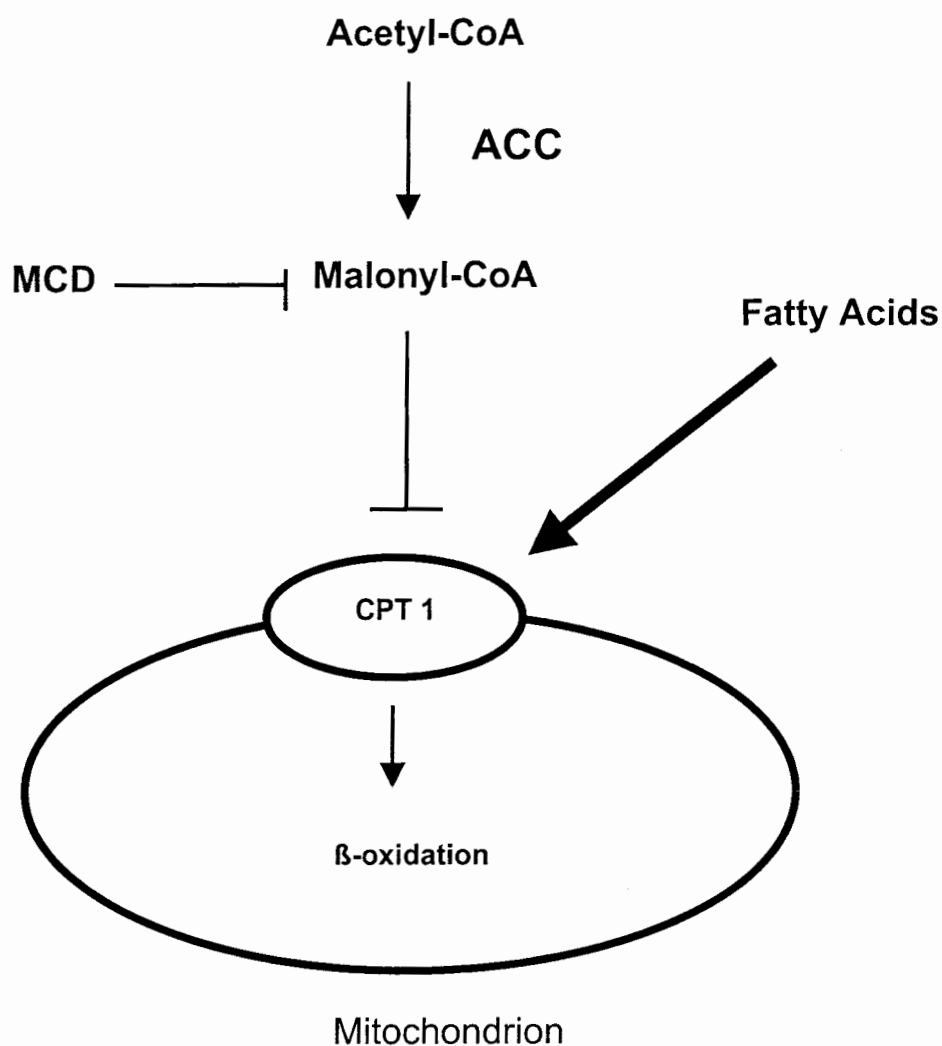


Figure 1.7. The regulation of mitochondrial fatty acid importation.

The rate-limiting step in the importation of long chain fatty acids into the mitochondrion is regulated by carnitine palmitoyl transferase (CPT 1). CPT 1 is allosterically inhibited by malonyl-CoA. Malonyl-CoA is generated by the carboxylation of acetyl-CoA in a reaction catalysed by acetyl-CoA carboxylase (ACC), and degraded by malonyl-CoA decarboxylase (MCD).

There are two isoforms of CPT 1, liver-type (lCPT 1) and skeletal muscle-type (mCPT 1); 88 kDa and 82 kDa, respectively. Both isoforms are present in the heart [114] with mCPT1 the predominant isoform and lCPT 1 only accounting for 2% of the total CPT 1 in the rat heart [115]. The two isoforms differ with respect to sensitivity to malonyl-CoA, the allosteric inhibitor of CPT 1; mCPT 1 being highly sensitive to this metabolite. The mean ($\pm$ SEM) concentration of malonyl-CoA required to inhibit 50% of CPT 1 activity (IC_{50}) in the adult rat heart is $0.1 (\pm 0.01) \mu\text{M}$. This is 27-fold less than that required for the same level of suppression in the adult rat liver ($2.7 \pm 0.4 \mu\text{M}$) [116, 117]. Other studies have shown that the IC_{50} of mCPT 1 is as low as 50 nM [118]. This greater sensitivity has been attributed to the tightness of the binding of malonyl-CoA to high affinity sites on the mCPT 1 enzyme [119].

The greater sensitivity of mCPT 1 to malonyl-CoA suggests that the levels of malonyl-CoA in the heart regulates the rate of FAO [120-122] and in this way provides a mechanism for glucose suppression of FAO when glucose use is upregulated [123]. Furthermore, it is not mCPT 1 sensitivity to malonyl-CoA that changes with altering environmental conditions but rather the levels of malonyl-CoA. Thus, malonyl-CoA is a key metabolite in the regulation of FAO and it is essential to unravel the mechanisms controlling the levels of malonyl-CoA in the heart.

There are two enzymes in the heart which regulate the levels of malonyl-CoA:

- 1) Acetyl-CoA carboxylase (ACC) which regulates its production. The focus of this thesis is the regulation of ACC in the heart and is discussed in detail in the following section.
- 2) Malonyl-CoA decarboxylase (MCD) which regulates its degradation. This enzyme catalyses the decarboxylation of malonyl-CoA to form acetyl-CoA.

1.5.1. Acetyl-CoA Carboxylase (ACC)

ACC catalyses the ATP-dependent carboxylation of acetyl-CoA to generate malonyl-CoA, a central metabolite in the regulation of FAO (**Figure 1.8**). The overall reaction is a two-step procedure entailing the ATP-dependent formation of carboxybiotin followed by the transfer of the carboxyl moiety to acetyl-CoA to form malonyl-CoA. There are two isoforms of ACC, ACC α and ACC β , which catalyse the same reaction. The two isoforms differ with respect to structure, subcellular localisation, tissue distribution and kinetic properties. Both isoforms are present in the heart [124]. ACC α has a relative molecular weight of 260 kDa while that of ACC β is 280 kDa. In the human genome, the isoforms are encoded by separate genes, ACC α has been mapped to chromosome 17 while ACC β is located on chromosome 12 [125, 126]. The cDNAs encoding the rat and human ACC α and ACC β have been isolated and characterised [125, 127-132]. The predicted amino acid sequence of human and rat ACC β showed 80% homology [127], the nucleotide and predicted amino acid sequences of the isoforms had 60 and 80% identity, respectively. Not all of the phosphorylation sites found in ACC α have been conserved in ACC β . However, the two critical serine residues required for phosphorylation and inactivation of ACC α are conserved in ACC β . The sequences differ primarily at the N-terminus, there are an additional 136 amino acid residues in ACC β which account for the different molecular weights of the two isoforms. The N-terminus is conserved across species [133].

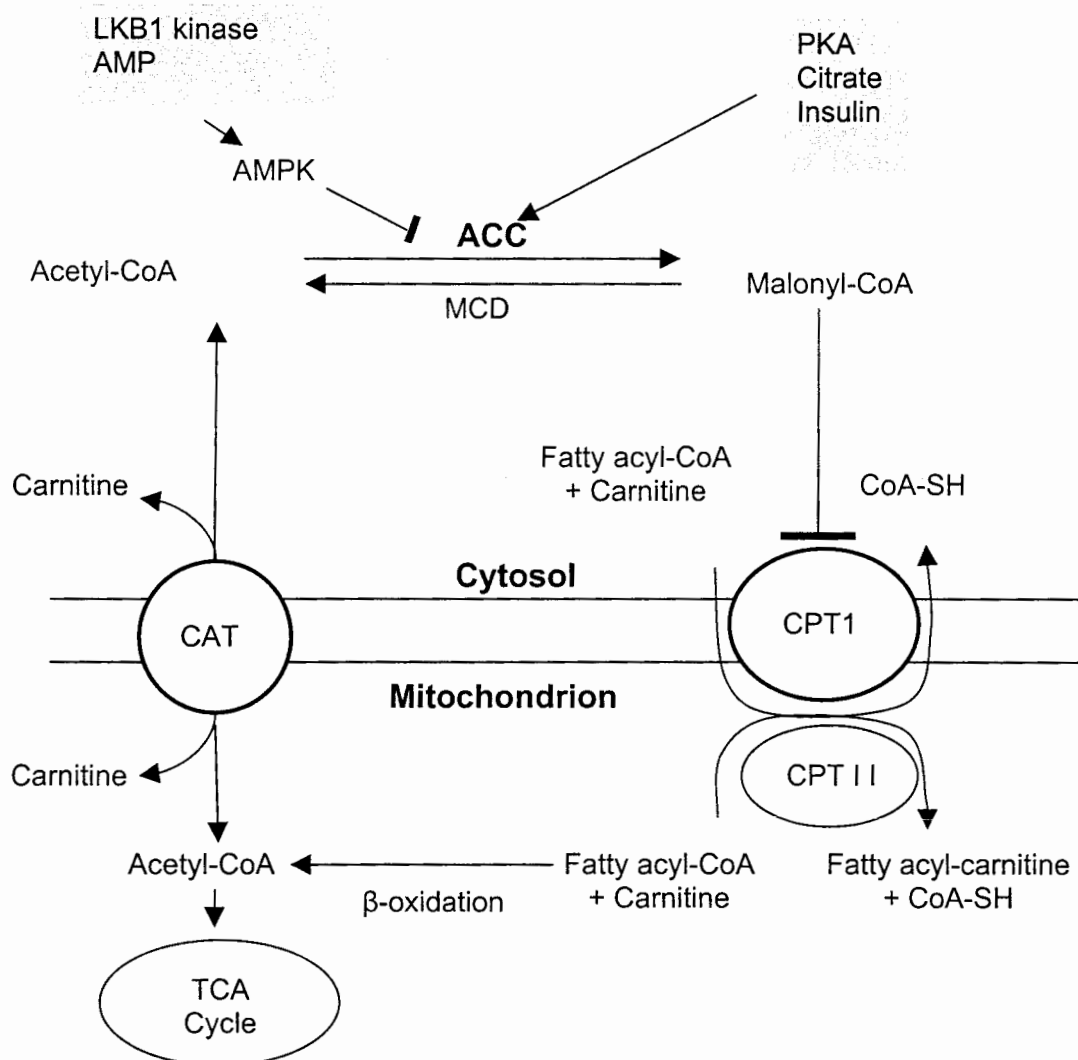


Figure 1.8. The role of ACC in the regulation of cardiac fatty acid oxidation.

ACC catalyses the reaction converting acetyl-CoA to malonyl-CoA. The substrate, in this reaction is derived from the decarboxylation of malonyl-CoA by malonyl-CoA decarboxylase (MCD). Mitochondrial oxidation of glucose and fatty acids results in an increase in the mitochondrial acetyl-CoA/ coenzyme A (CoASH) ratio. The carnitine-acetyl translocase (CAT) allows acetyl-carnitine to exit the mitochondrion. The cytosolic acetyl-carnitine is broken down into carnitine and acetyl-CoA, the latter is the substrate of ACC to synthesise malonyl-CoA. Malonyl-CoA inhibits mCPT 1 and consequently mitochondrial fatty acid uptake for β -oxidation. The activity of ACC is inhibited by phosphorylation by AMPK, mitigating the inhibition of β -oxidation. AMPK is activated by LKB1 kinase and AMP under conditions of metabolic stress. ACC is also activated by insulin, protein kinase A (PKA) and citrate (Adapted from [134])

Investigations into the subcellular localisations of the ACC isoforms in mammals have shown that ACC α is a cytosolic protein while ACC β is associated with the mitochondrial membrane [133]. The differences in the N-termini of the isoforms are thought to account for the dissimilar subcellular locales. One hundred and fourteen amino acid residues at the N-terminus of ACC β form a unique peptide, which is hydrophobic in nature, particularly the first 20 amino acid residues which are thought to be involved in the insertion of ACC β within the mitochondrial membrane. The association of ACC β with the mitochondrial membrane suggests that it is involved in the regulation of FAO. These distinct subcellular locations have also supported the argument that the ACC isoforms play differing roles in the regulation of fatty acid metabolism.

This suggestion was supported by the different kinetic properties of ACC α and ACC β . ACC α has a higher activation constant for citrate and a higher K_m for acetyl-CoA. The implications of the different functions of the ACC isoforms were first alluded to after the surprising discovery of ACC in the heart [124, 135]. This finding was initially puzzling since malonyl-CoA was thought to function solely as a primer for fatty acid synthesis and would therefore not be synthesised by ACC in the heart, which was not considered to be a site of fatty acid synthesis. The malonyl-CoA found in the myocardium was considered to be the product of propionyl-CoA carboxylase in the mitochondrion. In order to determine whether the ACC isoforms perform distinct functions, their regulation must be elucidated.

Conjecture about different roles of the isoforms is also due to their dissimilar tissue distributions. ACC α is highly expressed in lipogenic tissues such as the liver and white adipose tissue while ACC β is the predominant isoform in the heart, brown adipose tissue and skeletal muscle, which have a high oxidative capacity for fatty acids [127, 129, 131]. These distinct tissue distributions as well as the differing subcellular localisations of the ACC isoforms led to the suggestion that ACC α is involved in fatty acid synthesis while ACC β is involved in the regulation of FAO. The idea that malonyl-CoA is compartmentalised within the cell with the malonyl-CoA generated by ACC α regulating FA synthesis while that generated by ACC β is involved in FAO, has been put forward [133].

The following section describes the regulatory mechanisms controlling ACC isoforms. The section emphasises regulatory mechanisms in the rat and human, especially those controlling ACC β .

1.5.2. Regulation of ACC Isoforms

a) ACC α Regulation

ACC α and its regulation in the rat has been relatively well described and is quite complex including several short-term and long-term mechanisms.

i) Short-term mechanisms:

The short-term regulatory mechanisms controlling ACC α change the kinetic properties of the enzyme altering its sensitivity to cellular metabolites. These control mechanisms are at the post-translational level of regulation entailing allosteric and covalent means to direct ACC α activity rapidly in response to changing conditions such as changing hormonal levels with fasting and refeeding [136, 137].

The rat ACC α also contains at least 8 phosphorylation residues, these are Ser23, Ser25, Ser29, Ser77, Ser79 and Ser95, Ser1200 and Ser1215 [128, 138]. Several kinases are known to reversibly phosphorylate ACC α including cAMP-dependent protein kinase (PKA), 5'-AMP-activated protein kinase (AMPK), protein kinase C, casein kinase II and calmodulin-dependent protein kinase [139]. Phosphorylation does not always correlate with inactivation of ACC α ; AMPK is able to phosphorylate ACC α at three sites, namely Ser79, Ser1200 and Ser1215 but only phosphorylation of Ser79 results in inactivation [138, 140]. Furthermore, not all phosphorylation sites are specific to a particular kinase, for example Ser1200 can be phosphorylated by PKA. Prior phosphorylation at one site can by one kinase can prevent the phosphorylation activity of another kinase at an alternate phosphorylation site [138, 141]. ACC α is known to be dephosphorylated by phosphatases of type 2A which are activated by changes in physiological conditions such as increased insulin levels [142].

Citrate and acyl-CoA are known to activate and inhibit ACC α , respectively through allosteric alteration [139]. Allosteric and covalent regulatory mechanisms interact to activate ACC; only ACC α in a dephosphorylated state is sensitive to allosteric modification by physiological citrate levels [143].

ii) Long-term mechanisms:

Long-term mechanisms controlling ACC α include control of transcription and post-transcriptional modifications influenced by interacting hormonal and dietary factors. Five primary transcripts of the rat ACC α have been isolated, characterised. These transcripts result from selective use of two promoters, called PI and PII, and differential splicing [144].

The two promoters have been sequenced and characterised. PI flanks the 5'-end of exon 1 while PII is located within intron 1. Transcription from PI generates the majority of ACC α transcripts and contains TATA and CAAT boxes near the transcription initiation site as well as response elements for activators and repressors. The activity of PI varies in different tissues and with nutritional states and expression from this promoter is low under normal conditions. However, it is inducible under different physiological conditions such as refeeding after fasting [145, 146] and thyroid state [147]. A number of *cis*-elements and *trans*-elements have been identified and their interactions characterised, such as the induction of transcription from this promoter by the binding of the CCAAT/enhancer binding protein (C/EBP) to the CAAT box [146]

PII on the other hand is constitutively active, is expressed at similar levels in all tissues and contains no TATA or CAAT boxes but does contain *cis*-elements [145]. Although PII was not found to be affected by nutritional status such as fasting and refeeding [145], it is sensitive to insulin, cAMP and glucose [142]. For example, in the presence of high glucose levels Sp1 is able to bind to Sp-1 sites within PII and induce transcription from this promoter [42, 148].

The transcripts from the two promoters have distinct 5'-UTRs and these regions affect translational efficiency *in vitro* and may control the half lives of the different mRNA species. The 5' UTR heterogeneity in the ACC α mRNA may be involved in post-transcriptional control, at the level of translation, of the ACC gene expression [149]. It has been proposed that the different types of ACC α mRNA maybe a way for the cell to meet its requirements for FA. The mRNAs generated by the constitutively active PII may be used to meet the basic cellular needs such as for membrane phospholipids while those from PI can be generated under conditions promoting the storage of energy sources [145].

The ACC α promoters of several other species including the chicken [150] and bovine [151] have been isolated and characterised. Most recently, the human ACC α gene has been sequenced and described [152]. The gene is regulated by three promoters, namely PI, PII, and PIII. PI is a constitutively active promoter and lacks homology with the PI sequences of other mammalian ACC. PII is hormonally regulated while PIII is expressed in a tissue-specific manner. The mRNA transcripts are differentially spliced and it has been speculated that this could contribute to general ACC α activity either by controlling the mRNA stability and translational efficiency or by increasing the stability and specific activity of the ACC α enzyme.

b) ACC β Regulation

Until recently, little was known about the regulation of ACC β . However, the development ACC β -null mice and the finding that these mice do not become obese even with higher food intake due to elevated FAO levels [153] has instigated much investigation into the regulation of ACC β . Investigation was further intensified with the finding that ACC β -null mice are protected against developing type 2 diabetes, when placed on an obesity-inducing diet [154, 155]. New preliminary data emerging from a study in humans have supported the idea of involvement of ACC β in the development of diabetes. Debard and co-workers found a 2.5-fold increase in ACC β gene expression in the skeletal muscle of type 2 diabetic patients [156]. Most of this data emerged after this study was initiated in 1999.

i) Short-term mechanisms

ACC β also requires citrate for activation, but has a lower activation constant for citrate [124, 135, 157]. The current interest in ACC β as a target for anti-obesity and anti-diabetic drugs has brought about the identification of specific inhibitors of ACC β such as members of the cyclohexanedione class of herbicides which compete with acetyl-CoA, the substrate of ACC, to bind to ACC β [158].

ACC β is also a phosphoprotein, but this covalent regulatory mechanism has not been extensively investigated. Initial analysis showed that not all of the phosphorylation sites found in the human ACC α have been conserved in ACC β [125]. The phosphorylation sites on the rat ACC β protein have not been definitively identified. However, it has been ascertained that PKA [159] and AMPK can phosphorylate ACC β in rats [159-163]. These studies have also shown that covalent phosphorylation and dephosphorylation of ACC β is a primary short-term regulatory mechanism controlling this enzyme in skeletal and heart muscle. Similar findings have also been found in human skeletal [164] and cardiac [165] muscle.

Ex vivo studies have shown that ACC β is involved in the regulation of cardiac FAO during development [166, 167]; the activity levels of this enzyme are high during the foetal stage when glucose and lactate form the primary cardiac energy sources. The mechanisms regulating ACC β are, however, still relatively unclear compared to those of ACC α , which have been well described. The primary regulatory mechanism of skeletal and cardiac ACC β activity involves the serine threonine kinase, AMPK. AMPK phosphorylates ACC β , inactivating it and thereby reducing the amount of malonyl-CoA available to inhibit mCPT 1 and consequently FAO (**Figure 1.8**).

AMPK has come to be regarded as a “sensor of cellular energy status” (reviewed [140]). It is activated by metabolic stresses and is also regulated by hormones. This kinase is sensitive to the cellular ATP:ADP and ATP:AMP ratios, gauges of the cell’s energetic status. It is allosterically activated by 5’-AMP and through phosphorylation by upstream kinases including the recently identified LKB1 kinase [168, 169]. Insulin has also been found to inhibit AMPK [167, 170] via the

antiapoptotic kinase, Akt or PKB [171]. Hence, the regulation of ACC β by AMPK holds important implications for the regulation of cardiac FAO during development as well as during periods of metabolic stress.

During the neonatal period, there is an increase in AMPK activity in the heart with a consequent decrease in ACC β activity [167]. This contributes to the switch from glycolysis and lactate utilisation in the foetus to FAO in the adult heart. The observation has been attributed to the reduced postnatal insulin plasma levels and subsequent decrease in the inhibition of AMPK.

The AMPK-ACC-Malonyl-CoA axis has also been implicated in the detrimental effects of FAO in reperfused ischemic heart. AMPK activity is increased at the end of ischaemia resulting in a decrease in ACC activity and malonyl-CoA levels upon reperfusion [170, 172]. This results in increased FAO at the expense of glucose oxidation during the reperfusion period, proposed to result in harmful consequences [15].

ii) Long-term mechanisms

The transcriptional and post-transcriptional regulation of cardiac ACC β is less well understood. Two types of human ACC β transcripts have been found, and a human ACC β promoter, called PII β , has been identified [173]. In the same study, another putative human ACC β gene promoter was also identified, but did not show any transcriptional activity in the various cell lines tested. PII β exhibited high transcriptional activity in a myoblast cell line and has been further investigated [174]. Several *cis*-elements in PII β were identified and implicated in its regulation in myocytes. The TGAAA near the transcription initiation site is involved in basal regulation of gene expression as this promoter lacks a TATA box and Inr element. Several E-boxes as well as a GCCTGTCA sequence in the 5'-flanking region were found and it was established that these response elements interacted with MRFs to induce transcription. The E-boxes bind MyoD in the form of heterodimers with E-proteins while the novel *cis*-element has a strong affinity for homodimers of the MRFs, MyoD, Myf4 and Myf6.

Further investigation has revealed that MRF regulation of PII β in myocytes is controlled by retinoic acids and this regulation is dependent of the stage of development of the myocyte [174]. Transcriptional activation by MyoD in undifferentiated myoblasts was inhibited by the retinoic acid receptors, retinoic acid receptor α (RAR α) and retinoic receptor α (RXR α) while ACC β levels in myotubes were significantly increased by exposure to retinoids. Since PII β does not contain binding sites for the nuclear receptors, RXR α and RAR α and it has been proposed that these proteins interact with MRFs to regulate ACC β expression.

PII β is also the major human ACC β promoter in the liver and is regulated by nutritional status mediated by the transcription factor SREBP-1 [76]. SREBP-1, most likely SREBP-1c, binding to two SRE in PII β was increased with refeeding a high carbohydrate diet after fasting. Moreover, Sp1 binding to a specific element within PII β was involved with SREBP-1 activation but the mechanism has not yet been explained.

While this study was underway, two rat ACC β gene promoter sequences were deposited into Genbank (www.ncbi.nlm.nih.gov/entrez) including an ACC β muscle-specific (Genbank accession number AY217098) and a liver-specific promoter sequence (Genbank accession number AY217099). To date no further information regarding the activity and regulation of these promoters have been published.

1.6. Which Physiological or Pathophysiological Model to use to Study the Regulation of a Target for Metabolic Modulation?

In order to study and contextualise the transcriptional regulation of ACC β , we used a rat model of hypobaric hypoxia [175]. In this section, I will briefly describe this model in the myocardium. Thereafter, I will give a short overview of the metabolic pathways examined here, paying particular attention to the enzymes encoded by the genes examined in this study.

1.6.1. A Model of Hypobaric Hypoxia in the Rat

We used a rodent model of hypobaric hypoxia. Hypobaric hypoxia is a stress experienced by organisms at high altitudes resulting from low barometric pressure, and also in hypoxic disease as a result of low arterial oxygen tension. Under these conditions, organisms must adapt to decreased oxygen availability relative to sea-level, while still maintaining aerobic physiological processes.

Various physiological modifications against constant hypoxia have been established in animal models. These include increased haematocrit to elevate the oxygen-carrying capacity of blood [176]. Changes in metabolism have also been detected; chronic hypoxia induces two mechanisms to improve energy production. Firstly there is an increase in anaerobic metabolism and secondly an improved effectiveness in the use of oxygen in aerobic ATP synthesis.

The early phase of hypoxic heart disease is thought to induce adaptive or protective mechanisms to sustain normal organ function in response to diminished oxygen availability. Hence, people indigenous to high altitudes have been investigated to elucidate the protective metabolic responses to chronic hypoxia by the normal healthy heart. Studies have examined the adaptation to high altitude hypoxia in humans through comparison of individuals indigenous to sea-level with those native to high altitudes. Two groups of highlanders have been extensively studied, the Himalayan Sherpa and Andean Quechua populations.

Investigation of cardiac metabolism in these individuals has revealed significantly raised cardiac glucose preference in the high-altitude inhabitant relative to a subject indigenous to the sea-level environment [176]. Furthermore, the phosphocreatine (PCr) : ATP ratio, a reflection of cardiac energy status, was altered in the Sherpa compared to that of the individual at sea-level [177]. The lower cardiac PCr : ATP ratio in the Sherpa was similar to that observed in the diseased human myocardium [178] and thought to be a consequence of altered substrate utilisation by the myocardium. The upregulation of cardiac glucose utilisation was considered to be an adaptive response to chronic hypoxia as glucose usage is a relatively oxygen-sparing means of synthesising ATP compared to fatty acid use.

The molecular mechanisms underlying cardiac metabolic adaptation to hypobaria have not been identified. Therefore, we have examined the gene expression pattern of several modulators of metabolism in the myocardium. The rodent model used in this study has been extensively studied and described as a model of low arterial oxygen tension as seen in human hypoxic disease and exposure to high altitude [179, 180]. Rats were placed inside a hypoxic chamber, which was then sealed and the atmospheric pressure was thereafter gradually reduced. In order to remove animals, the pressure in the chamber was gradually increased to atmospheric levels before the chamber was opened.

Some of the effects of hypobaric hypoxia on the heart using this model have been described previously [179]. The chronic exposure to the hypobaric hypoxic stimulus results in the development of right ventricular hypertrophy. Subject animals show an abnormally rapid growth of the right ventricle (RV). There is an increase in muscle fibre size and total muscle mass, but no change in cell number, indicating hypertrophy rather than hyperplasia (**Figure 1.9**).

The onset of right ventricular (RV) hypertrophy is considered to be a result of the maladaptation of a compensatory mechanism used by the lung to adjust to damage in small sections of the lung. In brief, this mechanism results in the constriction of pulmonary vasculature to parts of the lung, which are damaged and poorly ventilated in this way decreasing the flow of blood to these regions. Blood is diverted to better ventilated regions improving the efficiency with which it is oxygenated. However, this mechanism becomes maladaptive under chronic hypoxic conditions when the entire lung is affected and all the pulmonary vasculature constricts. This phenomenon is known as hypoxic pulmonary vasoconstriction, which results in the RV of the heart having to pump with a greater force to overcome the increased vascular resistance i.e. an increased afterload on the RV. Consequently, the RV adapts to this stimulus by hypertrophying.

An important characteristic of this model is that the systemic blood pressure is unchanged and consequently the left ventricle and septum (LVS) of the heart does not hypertrophy but is nevertheless subjected to hypoxia. Therefore, this model is ideal to examine the effects of both hypoxia-induced hypertrophy as observed by changes in the RV and the effects of hypoxia on cardiac gene expression, in the absence of hypertrophy, can be studied by examining the left ventricle. In light of this, we have characterised the changes in the gene expression of metabolic enzymes regulating the metabolic pathways of the two major fuels used by the heart, glucose and FA.

No animal model is, however, ideal for the study of human physiological and pathophysiological conditions. The main advantage of this model is that it is a non-invasive model of pulmonary vasoconstriction which does not entail surgical interventions often needed by other *in vivo* models which place additional stress on the animals. Moreover, up to 12 animals could be simultaneously housed in a sealed chamber, allowing for controlled conditions eliminating variation in administration of treatment which may occur if animals are handled individually. A disadvantage of the model was that the chamber had to be opened every 48 hours to change cages and replenish food and water, this was not ideal as animals were not continually exposed to hypoxia and there were intermittent periods of acclimatisation and declimatisation.

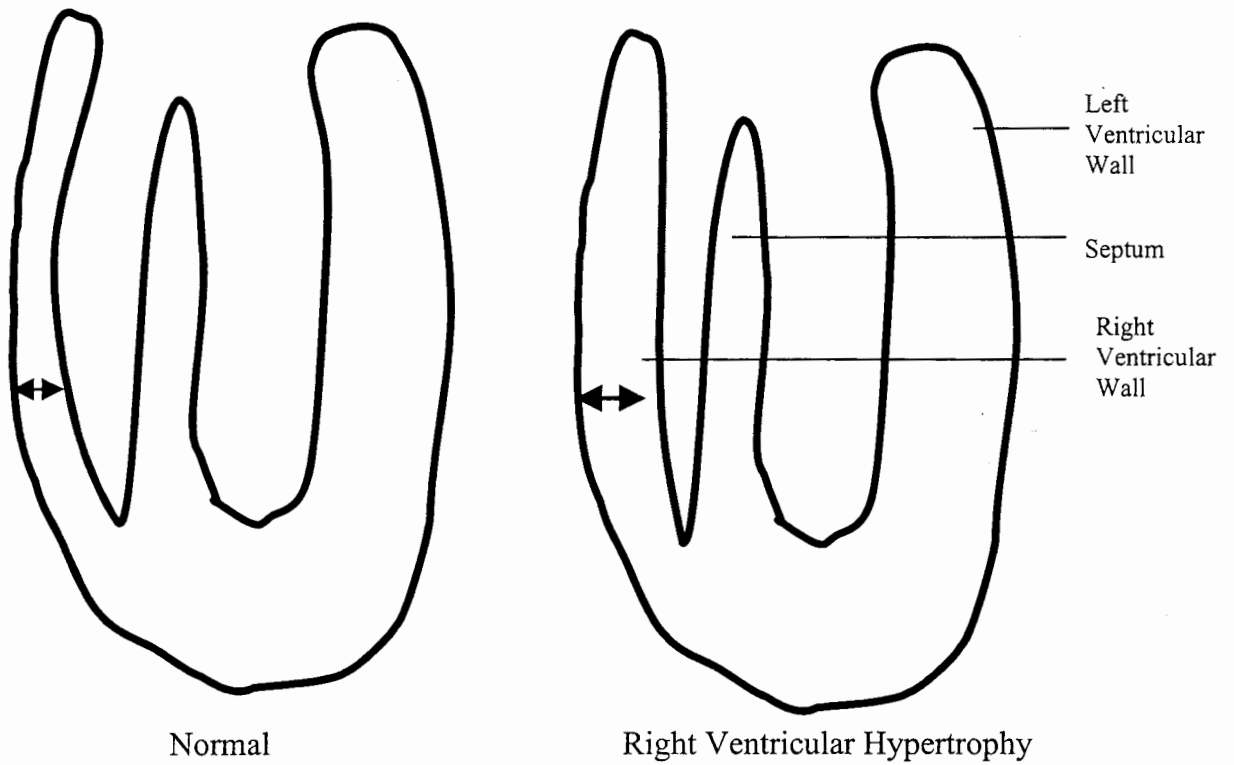


Figure 1.9. The development of right ventricular hypertrophy.

Exposure to chronic hypobaric hypoxia results in the thickening of the right ventricular walls, as indicated by the arrows. There is an increase in muscle fibre size and total muscle mass in this ventricle indicating hypertrophy, signified by the arrows.

1.6.2. Cardiac Glucose Metabolism

Glucose is the primary fuel in the foetal heart. The adult heart reverts to a foetal pattern of metabolism under pathogenic conditions such as those inducing the development of hypertrophy. For the purposes of this work, the glycogen component of glucose metabolism was not covered.

Glucose is catabolised in the heart along an enzyme pathway that involves 3 steps:

- 1) Glucose uptake
- 2) Glycolysis
- 3) Glucose oxidation

Briefly, glucose uptake into the cardiomyocyte is regulated by the transmembrane glucose gradient, glucose concentration and the activity of the glucose transporters (GLUT), predominantly by the constitutively expressed GLUT1 isoform and insulin-responsive GLUT4 isoform. GLUTs transport glucose by facilitated diffusion down glucose concentration gradients. Intracellular glucose can enter the glycolytic pathway, which results in the generation of two molecules of pyruvate from one molecule of glucose. The pyruvate generated from glycolysis can be anaerobically converted to lactate in anaerobic glycolysis. Alternately, pyruvate enters the mitochondrion where it is further oxidised. In the mitochondrion, pyruvate is irreversibly converted to acetyl-CoA by the pyruvate dehydrogenase complex (PDH) and is further oxidised (glucose oxidation) in the tricarboxylic acid (TCA) cycle and the electron transport chain to synthesise mitochondrial ATP. PDH regulates the rate-limiting step in glucose oxidation [134]. It can be inhibited by the concentration of end-products of FAO, such as acetyl-CoA and NADH, and by phosphorylation via the ATP-dependent PDH-kinase complex (PDK) phosphorylation (**Figure1.10**).

In the present study, the gene expression of three regulators of glucose utilisation were examined: GLUT1, GLUT4 and PDK4. The GLUTs regulate the rate-limiting step in myocardial glucose utilisation [181, 182]. Of the two cardiac enriched isoforms, GLUT1 is considered to be a constitutive transporter mediating basal uptake of glucose [183, 184]. GLUT4 protein levels are upregulated in the adult heart and it is more sensitive to insulin. The GLUTs are stored in intracellular vesicles [185] and when stimulated by insulin or another activator, the vesicles move and fuse with the cell membrane, consequently increasing the rate of glucose uptake by the cell.

PDH catalyses the irreversible step in glucose oxidation and its regulation is therefore critical to myocardial metabolism. PDK phosphorylates PDH, inactivating the complex. There are four distinct isoforms of PDK, which have distinct tissue distributions and kinetic properties. Three isoforms of PDK, namely PDK1,-2 and -4 are found in the heart [186]. In this study, we examined PDK4, the isoform implicated in long-term regulation of PDH in rat skeletal [187] and cardiac [188] muscle in response to starvation and diabetes. It is considered to be the PDK isoform active in the switch from glucose to fatty acid utilisation in the neonatal heart. PDK 4 has recently been regarded as a representative PPAR α -target gene [189, 190] and is involved in regulating PDH activity to changing environmental conditions in the adult heart. PDK activity is regulated by intermediates of glucose and fatty acid metabolism [190].

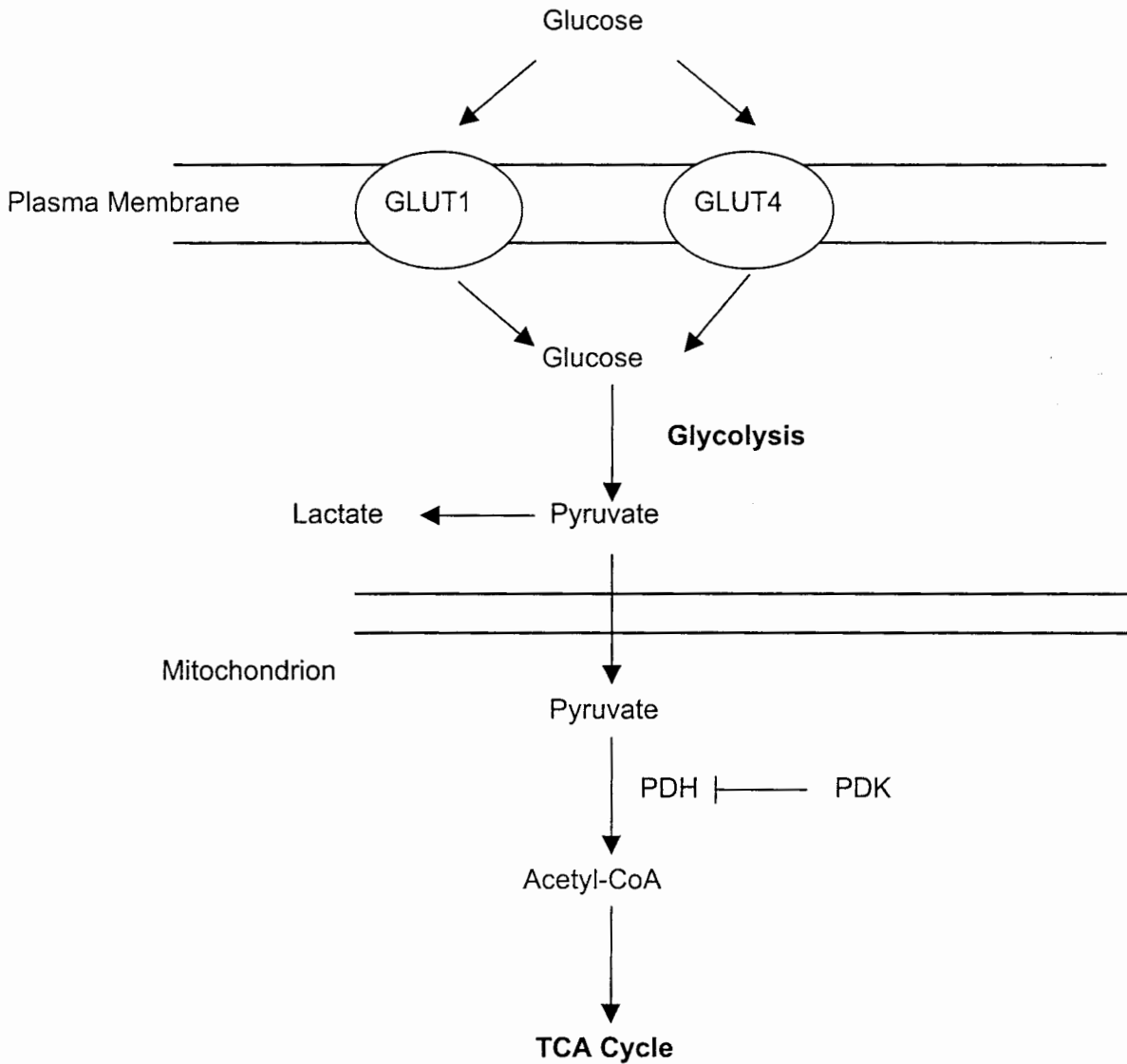


Figure 1.10. Glucose utilisation in the cardiomyocyte.

Glucose enters the cardiomyocyte via facilitative glucose transporters, GLUT1 and GLUT 4. The intracellular glucose is phosphorylated and can continue to move through the glycolytic pathway producing pyruvate. Pyruvate can be reversibly converted to lactate under anaerobic conditions or irreversibly converted to acetyl-CoA by the pyruvate dehydrogenase complex (PDH) in the mitochondrion; this reaction is inhibited by PDH-kinase complex (PDK) phosphorylation of PDH. Acetyl-CoA is oxidised in the tricarboxylic acid (TCA) cycle.

1.6.3. Cardiac Fatty Acid Oxidation

Fatty acids can account for the majority of the adult mammalian heart's energy requirements under fasting conditions. Nevertheless, relatively little is known about the regulation of cardiac fatty acid utilisation compared to that of glucose. The growing importance of this pathway due to its involvement in the increased incidence of chronic forms of heart disease therefore necessitates investigation of the mechanisms regulating cardiac FAO. The cardiac fatty acid oxidative pathway and its regulation have been outlined previously (**Figure 1.6**). We have assessed the expression patterns of five proteins regulating various stages of this process:

- a) PPAR α , as described in a previous section of this chapter, is a transcription factor regulating the expression of many genes involved in fatty acid utilisation in the myocardium.
- b) Malonyl-CoA decarboxylase (MCD) is an enzyme catalysing the decarboxylation of malonyl-CoA to form acetyl-CoA. It is therefore involved in the regulation of a rate-limiting step of mitochondrial fatty acid importation.
- c) Muscle carnitine palmitoyl transferase 1 (mCPT 1) catalyses the rate limiting step of mitochondrial fatty acid importation,
- d) Medium chain acyl-CoA dehydrogenase (MCAD) is a critical enzyme in the β -oxidation spiral catalyzing the first step of medium chain fatty acid oxidation,
- e) Uncoupling protein 3 (UCP3), this protein is present in the inner mitochondrial membrane and its precise function of this protein is unknown. Uncoupling proteins were commonly thought to uncouple the oxidation of fuels via the electron transport chain from ATP synthesis, squandering energy as heat. There is, however, increasing evidence that UCP3 plays a critical role in energy homeostasis in muscle [191, 192]. This putative role is that under conditions of high FAO, mitochondrial acyl-CoA is converted to fatty acid anions and CoASH. The anions are transported out of the mitochondrion and the CoASH retained; this mechanism allows for the maintenance of CoASH levels for participation in the TCA cycle and mitochondrial FAO [191]. The mechanism is analogous to that of carnitine acetyl translocase (CAT) which exports mitochondrial acetyl-carnitine into the cytosol when the mitochondrial acetyl-CoA / CoASH ratio increases with glucose and FAO (**Figure 1.3**). This role may provide a way of protecting the mitochondrion from the accumulation of LCFA in the mitochondrial matrix, which could disrupt membranes [192]. UCP3 is expressed in cardiac tissue [193] and its expression has been linked to plasma free fatty acid levels [194]. UCP3 expression is also thought to be regulated by PPAR α [195, 196].

1.7. Aims of this project

ACC plays an important role in cardiac fuel selection and more information is required about its regulation. The findings outlined above suggest that ACC α and $-\beta$ perform different functions. Therefore, based on this premise, **it is the hypothesis of this study that the cardiac enriched isoform of acetyl-CoA carboxylase (ACC β) is distinct in its regulatory control of cardiac fatty acid oxidation compared to acetyl-CoA carboxylase α -isoform. Evaluation of the regulation of ACC β should therefore provide further insight into its regulatory role in cardiac metabolic remodelling.**

The aims of this study were:

- 1) Outline the metabolic gene expression pattern in cardiac tissue isolated from rats exposed to hypobaric hypoxia,
- 2) Assess the cardiac gene expression of both ACC isoforms, ACC α and ACC β , under hypobaric hypoxic conditions,
- 3) Identify and delineate molecular mechanisms regulating ACC β gene expression.

During the course of this investigation, data from other studies compelled us to re-examine the impact of this new information on the aims and relevance of this study as well as our long-term goals. It has been discussed that the human ACC β gene promoters were identified in 2001 [173] and that two years later, sequences purported to be the rat ACC β gene promoter sequences were deposited into Genbank. At both of these points, the question arose as to whether it was relevant to continue this study which had commenced in 1999. It was decided to continue with this investigation after the identification of the human ACC β promoters in 2001 since our long-term aim is to test the relevance of ACC β manipulation for the purposes of metabolic modulation. A suitable animal model is required for this purpose as it is necessary to examine the regulation of ACC β gene expression in the disease setting *in vivo* so that it can be observed how this gene is regulated as disease progresses and to delineate factors influencing its progression. Hence, it is essential to generate a suitable animal model in which ACC β is regulated in a similar manner to the human ACC β isoform. Therefore, we continued to examine the regulation of ACC β gene expression in the rat to compare it with the emerging data from studies on the human ACC β promoter. This in turn allowed us to determine the suitability of a rat model for this purpose. The study was continued after the rat ACC β sequences were deposited into Genbank in 2003, as we had isolated a novel sequence in the 5'-flanking region of the rat ACC β gene which we considered to be an important putative gene regulatory region which could be involved in the regulation of rat ACC β gene expression.

2.1 Characterisation of the Metabolic Gene Regulation in Response to Hypobaric Hypoxia in the Rat

2.1.5. *In vivo* Exposure to Hypobaric Hypoxia

Male Wistar rats (160 - 220 g body weight) were kept under controlled conditions (12:12 hour light-dark cycle) with access to laboratory chow and water *ad libitum*. For hypoxia experiments, rats were housed in a lexan hypobaric chamber (SciTech, Cape Town, South Africa) as described by Smith et al [175]. Rats were exposed to the hypobaric stimulus (45 kPa- 11% ambient oxygen) for 48 hours or 1 week, thereafter animals were sacrificed using CO₂. All rats were weighed on sacrifice. The hearts were removed and weighed. The ventricles were dissected out separately, weighed and stored at -80°C until RNA analyses were performed. The pressure was returned to atmospheric levels every 48 hours for no more than 20 minutes to replenish bedding, food and water. Decompression and recompression periods of 15 minutes were performed before opening and after closing the chamber. The appropriate age and sex matched normoxic controls were used for experiments. The University of Cape Town's Animal Research Ethics Committee approved all animal experiments (Ref No. 00/23N).

2.1.6. Determination of Haematocrit Levels

Upon dissection, blood was collected by cardiac puncture for measurement of haematocrit. This was achieved by collecting blood in heparinised capillary tubes and centrifuging at 3 500 rpm for 3 minutes in a haemofuge to separate serum and red blood cells. Haematocrit levels were determined as a percentage of red blood cells in whole blood using the following formula:

$$(\text{Red blood cells} / \text{Whole blood}) \times 100\%$$

2.1.7. RNA Extraction and Quantitation

Total RNA was extracted from 100-200 mg frozen tissue. All samples were kept on ice during the procedure to prevent RNA degradation. The tissue was completely homogenised in 2 ml of Total RNA Isolation Reagent (TRIR; Abgene House) to prevent enzymatic degradation of RNA. In order to remove fatty acids and proteins, 1 ml of chloroform was added to the homogenate and vortexed for 15 seconds, incubated on ice for 5 minutes. The organic and aqueous layers were separated by centrifugation (11,500 g for 15 minutes at 4°C) and the aqueous phase was transferred to a fresh sterile tube and the chloroform extraction repeated. The aqueous phase was transferred to a fresh sterile tube, an equal volume of isopropanol was added, the sample was vortexed to mix and stored overnight at -20°C to precipitate the RNA. On the following day, the samples were centrifuged at 11,500 g for 15 minutes at 4°C to pellet the RNA. The supernatant was removed and the pellet was resuspended in 750 µl of 75% v/v ethanol to remove salts precipitated by the isopropanol and the RNA was pelleted again by centrifugation at 11,500 g for 10 minutes at 4°C. The ethanol was carefully removed, the pellet was air-dried for 30 seconds and the RNA was resuspended in 15-20 µl v/v 0.01% DEPC-treated water and stored at -80°C. In order to quantitate the RNA, 4 µl RNA was diluted 996 µl in sterile distilled water and the absorbance at 260 nm (A₂₆₀) measured using a spectrophotometer. The RNA concentration ([RNA]) was calculated using the following formula:

$$[\text{RNA}] (\mu\text{g/ml}) = A_{260} \times \text{dilution factor} (250) \times 40 \mu\text{g/ml}$$

The value obtained was divided by 1000 to determine the [RNA] in $\mu\text{g}/\mu\text{l}$. The absorbance at 280 nm (A_{280}) was also measured and the ratio of A_{260} : A_{280} calculated to establish the degree of protein contamination within the RNA samples. Pure RNA has a A_{260} : A_{280} ratio of 2; samples were only used for northern blot analysis if A_{260} : A_{280} ratio was >1.7 .

2.1.8. Northern Blot Hybridisation

The protocol used in this study is based on that of Sambrook et. al. [197].

2.1.8.1. RNA Gel Electrophoresis and transfer to Nylon Membrane

A denaturing 1.2% agarose gel for RNA separation was prepared by dissolving 2.2 g agarose (Hispanagar) in 132 ml of sterile distilled water, by heating (100°C). A pre-warmed solution (60°C) of 37% v/v formaldehyde (29 ml) and 10X MOPS (18ml) were added to the warm agarose and the solution was poured into a gel casting apparatus (BIORAD, USA). Appendix A contains the recipes for solutions used.

RNA samples were prepared for gel electrophoresis as follows: 19.3 μl of buffer containing 12.5 μl de-ionised formamide, 4 μl 37% v/v formaldehyde, 2.5 μl 10X MOPS solution and 0.3 μl of 10 mg/ml ethidium bromide, was added to 20 μg of total RNA diluted in 0.1% v/v DEPC-treated water to a volume of 6 μl . The samples were incubated at 60°C for 15 minutes and then placed on ice for 2 minutes. Loading dye (2.5 μl) was added to each sample and briefly vortexed. A gel prepared beforehand was immersed in 1 X MOPS and the samples were loaded into the gel wells. The RNA was separated electrophoretically at 80 V for four hours. Equal RNA loading was confirmed by ultraviolet (UV) light, the intensity of the fluorescent bands produced by the binding of ethidium bromide to RNA is proportional to [RNA]. The integrity of RNA was established by visualising the ratio of the 28S to 18S ribosomal RNA bands (>2) and the lack of smearing, an indication of degradation. The electrophoretically separated RNA was transferred onto a nylon membrane (Hybond-N; Amersham Life Sciences, USA) by means of capillary transfer overnight. On the following day, the RNA was fixed to the membrane using a crosslinker (UniEquip); $1.2 \times 10^5 \mu\text{J} / \text{cm}^2$ for 25 seconds.

2.1.8.2. Generation of Probe for Northern Blot Hybridisation

The cDNA fragment used as a probe for northern blot analysis was generated as follows:

Two oligonucleotides were designed using the open reading frame sequence of the rat ACC β ([127]; Genbank Accession no. AB004329):

ACC-cDNA_{fwd}: 5'- TGG ACG GAG GAC AGC CAG CAT -3' (+ 1219 to + 1239) and

ACC-cDNA_{rev}: 5'- GGC TTA AAG GCC TCG TCT GGG TTT -3' (+ 1933 to + 1956).

The GeneAmp® EZ *rTth* RNA PCR kit (Perkin Elmer) was used to carry out reverse transcription and PCR as instructed by the manufacturer. The PCR product was purified using the Qiaquick gel extraction kit (Qiagen), ligated with the shuttle vector, pGEM®-T Easy (Promega Corporation) and subsequently used to transform JM109 cells. Transformed cells were

selected and cloned. Plasmids were isolated from the cells using the High Pure Plasmid Isolation Kit (Roche) and the presence of the correct insert fragment (737 bp) was verified by sequencing.

2.1.8.3. Probing of Northern Blot

The cDNA fragment described above was used for Northern blot analysis. The cDNA fragment was radiolabelled with α -P³²-dCTP, using the Random Primed DNA Labelling Kit (Boehringer Mannheim) as instructed by the manufacturer. Unincorporated α -P³²-dCTP was removed by passing the reaction mixture through Push Column Beta Shield Device and NucTrap® Probe Purification Columns (Stratagene) as instructed by the manufacturer. Stratagene Quickhyb solution (Stratagene) was used for prehybridisation and hybridisation steps. Eleven millilitres of the Stratagene Quickhyb solution was preheated to 65°C and the nylon membrane was then prehybridised for 1 hr in the preheated solution. The radiolabelled probe and preboiled, and 100 μ l of sonicated salmon sperm cDNA were denatured for 10 min at 100°C and then placed on ice for 2 minutes before it was added to the prehybridisation solution. The membrane was allowed to hybridise for one hour at 65 °C, whereafter it was washed in 50 ml of 2 X SSC at room temperature for 5 min and the solution was discarded. This step was repeated and the membrane was then finally washed in 50 ml of prewarmed 1 X SSC/ 1% SDS at 65 °C for 10 min. The membrane was then placed in a plastic folder

2.1.9. Quantitative Real- Time PCR

Quantitative real-time PCR was used as an alternate method to northern blot analysis, this procedure was carried out by our collaborators, Prof. H. Taegtmeier, Drs P. Razeghi and M.Young at the University of Texas-Houston Medical School, (Houston, Texas USA), who carried out the real-time PCR experiments. Basically, the method used by our collaborators involved the isolation of RNA from cardiac tissue, which was then used as a template for reverse transcription and PCR (RT-PCR). RT-PCR was carried out using a gene-specific primer to generate cDNA. The cDNA was used as a template in real-time PCR. Real-time PCR is based on the detection and quantitation of a fluorescent reporter. The increase in fluorescent signal is directly proportional to the amount of PCR product in the reaction. The amount of fluorescence was measured at each cycle permitting the detection of PCR product in “real time”. Standard curves were established for each cDNA and used to quantitate the amount of template. The endogenous 18S rRNA was measured and used to normalise results in order to control for variations in RNA concentration between samples [198].

We carried out the animal experiments in Cape Town, South Africa and performed the morphological as well as the haematological analyses. The heart tissue was harvested and sent to our collaborators in Houston, Texas, USA where RNA was isolated, reverse transcribed and the quantitative real-time PCR was performed for 18S rRNA, GLUT1, GLUT4, mCPT 1, MCD, MCAD, PDK4, UCP3, PPAR α , ACC α , ACC β , ANP and VEGF. The protocols have been previously described [198]. We analysed the data obtained from these experiments. The transcript for the constitutive gene product, 18S rRNA, was used to normalise data, the data is presented as the number of transcripts per number of 18S rRNA molecules.

2.2 Isolation and Characterisation of a 5'-flanking Region of the Rat ACC β Gene

2.2.1. Isolation of 5'- flanking of the Rat ACC β cDNA

In order to delineate molecular mechanisms regulating ACC β gene expression in the rat myocardium, we used the Rat Genome Walker kit (Clontech) to isolate the 5'-flanking region of the rat ACC β gene. This kit allows for the isolation of genomic sequences upstream of a known sequence. Contained in the kit were four “libraries” of rat genomic DNA. Each library had been commercially constructed by restricting genomic DNA with one of four restriction enzymes, namely *EcoRV*, *DraI*, *PvuII* and *SspI*. An oligonucleotide with a known sequence, an adaptor, was ligated to the 5'-ends of the digested DNA fragments.

In this laboratory, PCR was carried out using the libraries as templates and a forward primer annealing to the oligonucleotide ligated to the 5'-end of the genomic DNA fragments and a reverse primer binding to the rat ACC β coding region. The product obtained from this reaction was used as a template in a second round of PCR using nested primers, these are primers which partially overlap with the primers used in the first round of PCR thus amplifying a region within the PCR product of the initial round of PCR (Figure 2.1).

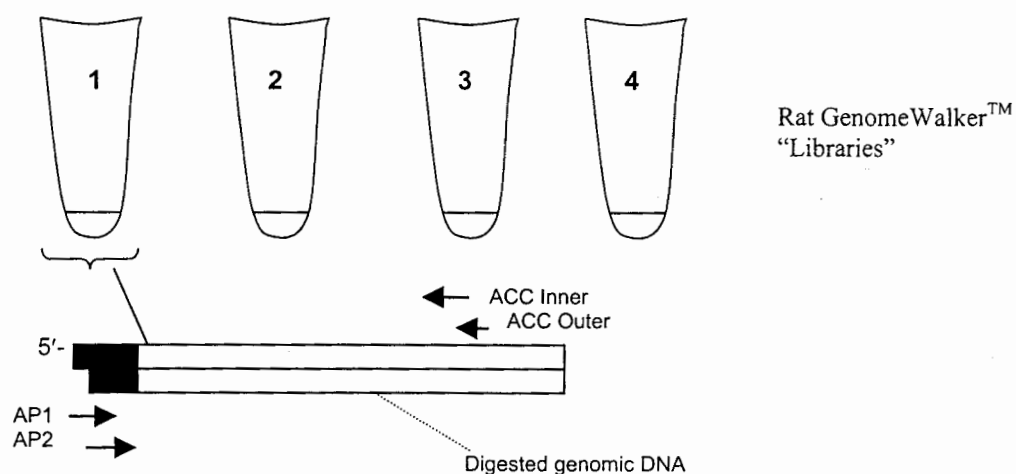
PCR was carried out using two adaptor primers (AP1 and AP2, Clontech) and two primers designed using the cDNA sequence of the rat ACC β (Abe et al 1998; Genbank Accession no. AB004329):

Primer 1 (ACC Outer): 5' - TGC TCG GAA GGA GGC TTG CAT CCA CCT TA - 3' (+133 to +105) and

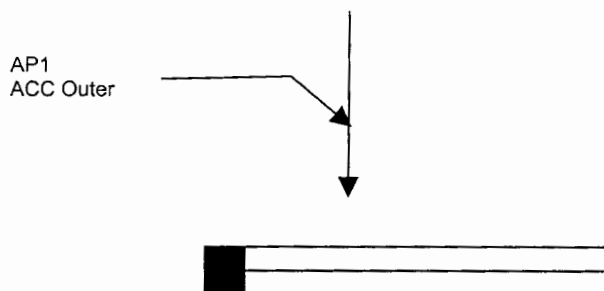
Primer 2 (ACC Inner): 5' - CTG TTG CTG AGC GGC CTC GAG TCT GT - 3' (+104 to +79).

The PCR mix was set-up according to the manufacturer's instructions, including the appropriate controls and a final concentration 30% dimethyl sulphoxide (DMSO; v/v), an enhancing agent. A primary reaction was cycled as follows: 94 °C for 2 seconds, 67 °C for 3 minutes for 7 cycles, 94 °C for 2 seconds, 65 °C for 3 minutes for 40 cycles, a final extension of 65 °C for 4 minutes. The products of a primary PCR reaction using AP1 and ACC Outer primer were diluted 50-fold and used as templates for a secondary reaction using the primers AP2 and ACC Inner. The secondary reaction was cycled as follows: 94 °C for 2 seconds, 72 °C for 3 minutes for 7 cycles, 94 °C for 2 seconds, 67 °C for 3 minutes for 22 cycles, a final extension of 67 °C for 4 minutes. The reactions were carried out in a GeneAmp PCR System 2400 (Applied Biosystems). Five microlitres of PCR product were separated electrophoretically at 100 V for 30 minutes in a 1.5% agarose TAE gel to confirm the presence of PCR product.

1) Four Rat GenomeWalker™ “Libraries” commercially constructed by restricting genomic DNA with one of four restriction enzymes, namely *EcoRV*, *DraI*, *PvuII* and *SspI*. An oligonucleotide with a known sequence, an adaptor, was ligated to the 5'-ends of the digested DNA fragments which contain consensus sequences for the binding of adaptor primers (AP1 and AP2) for nested PCR. In order to isolate the 5'-flanking region of the rat ACC β gene, the primers, ACC Outer and ACC Inner containing sequences of the rat ACC β cDNA were used.



2) Primary PCR using Rat GenomeWalker™ “Libraries” as templates and the primers, AP1 and ACC Outer for amplification.



3) Secondary or “nested” PCR using the product of the primary reaction as template and the primers, AP2 and ACC Inner for amplification. The products of this reaction were isolated and sequenced.

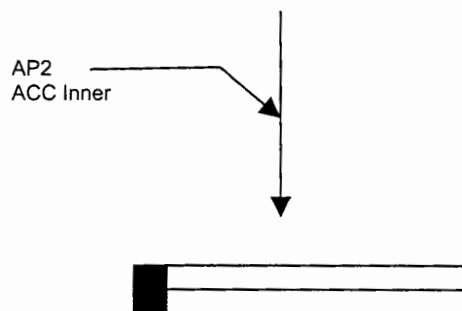


Figure 2.1. Schematic showing the premise of the Rat GenomeWalker™ kit (Clontech)

Three rat genomic libraries (DL-*EcoRV*, DL-*DraI* and DL-*PvuII*) yielded fragments, which were cloned into pGEM-T Easy vector (Promega) and sequenced using a Perkin Elmer 377 automated sequencer. Sequencing in the 5' to 3' direction using the ACC Inner primer did not yield good results due to the presence of a polyadenosine sequence in the region which interfered with the sequencing reaction, consequently sequencing was carried out in the 3' to 5' direction using the AP2 primer provided with Rat Genome WalkerTM kit (Clontech). Two additional sense primers were also used to sequence further upstream:

Primer 3: 5'- ACG AAG CAG GAA AAC ATG GCA-3' (-296 to -316) and

Primer 4: 5'- TGG TCA GCC TTG AAA ACA CAT -3' (-861 to -881).

Sequence analyses to identify potential *cis*-acting elements in this sequence were performed with the Transcription Element Search System (TESS) program on the transcription factor database, TRANSFAC (<http://transfac.gbf.de/TRANSFAC> or <http://www.bionet.nsc.ru/TRRD>) [40] and WWW Signal Scan IMD Search Service (<http://www.dna.affrc.go.jp/htdocs/PLACE/>) [41].

2.2.2. 5'-Rapid Amplification of cDNA Ends (5'-RACE)

A 5'-rapid amplification of cDNA ends (5'-RACE) protocol was used to isolate the transcription start site of the rat ACC β gene after the initial unsuccessful application of primer extension analysis (methodology and results are given in Appendix B). We successfully used two 5'-RACE protocols. First, we used the GeneRacerTM Kit (Invitrogen), which required the use of RNA isolated in the laboratory. Briefly, it involved the selection and isolation of complete mRNA molecules, followed by the ligation of an oligonucleotide to the 5'-end of mRNA molecules. Reverse transcription using a primer annealing to the rat ACC β coding region generated a cDNA molecule containing the 5'-flanking region of this transcript and the cDNA molecule was amplified by PCR. The second 5'-RACE kit used was the Marathon-ReadyTM cDNA kit (Clontech) containing commercially prepared cDNA with an oligonucleotide ligated to the 5'-ends. We had previously found that RNA isolated in our laboratory was partially degraded most likely caused by the presence of RNases and shearing due to handling, despite stringent precautions. The use of commercially prepared cDNA provided in the Marathon-ReadyTM cDNA kit was used to increase the likelihood of isolating transcripts present at low levels in the rat myocardium.

In brief, the protocol used for the GeneRacerTM kit (Invitrogen) was as follows: total rat cardiac RNA was treated with calf intestinal phosphatase to remove 5'-phosphates in order to remove truncated mRNA and other RNA. The dephosphorylated mRNA was treated with tobacco acid pyrophosphatase to remove the 5'-cap. An oligonucleotide, GeneRacerTM RNA Oligo, was ligated using T4 RNA ligase. The ligated mRNA was reverse transcribed using the ACC Inner primer. Nested PCR using the ACC Outer and GeneRacerTM 5'-Primers in the primary reaction and ACC Inner and GeneRacerTM 5'-Nested primers in the secondary reaction. One PCR product was obtained and purified using S.N.A.P.TM columns (Invitrogen), cloned into pCR $\oplus$ -TOPO vector (Invitrogen) and sequenced.

5'-RACE was repeated using the Marathon-ReadyTM cDNA kit (Clontech) which contained a rat heart cDNA library. Briefly, PCR was carried out using two adaptor primers (AP1 and AP2, Clontech) and the primers; ACC Outer and ACC Inner, used in the isolation of the fragment. The PCR mix was set-up according to the manufacturer's instructions, including the appropriate controls. Nested PCR was carried out, the primary reaction was cycled as follows: 94°C for 5

seconds, 72°C for 4 minutes for 5 cycles, 94 °C for 5 seconds, 70 °C for 4 minutes for 5 cycles, 94 °C for 5 seconds, 68 °C for 4 minutes for 25 cycles and an incubation at 4 °C. The products of a primary PCR reaction using AP1 and ACC Outer primer was diluted 50-fold and used as templates for a secondary reaction using the primers: AP2 and ACC Inner. The primary reaction was cycled as follows: 94°C for 5 seconds, 74 °C for 2.5 minutes for 5 cycles, 94 °C for 5 seconds, 72 °C for 2.5 minutes for 5 cycles, 94 °C for 5 seconds, 70 °C for 2.5 minutes for 20 cycles and incubated at 4°C. The reactions were carried out in a GeneAmp PCR System 2400 (Applied Biosystems). The PCR product was gel purified, cloned into pGEM-T Easy vector (Promega) and sequenced in the 5' to 3' direction using the ACC Inner primer using a Perkin Elmer 377 automated sequencer.

2.2.3. Cell Culture

An African Green monkey kidney cell line, CV-1 (ATCC no. CCL70; ECCAC 87032605) was maintained in Dulbecco's Modified Eagles Medium (Sigma) supplemented with 10% foetal calf serum (Delta Bioproducts) and glutamine (Biowhittaker) as well as the antibiotics, penicillin and streptomycin. Fresh media was added every two to three days.

The embryonic rat heart derived cell line, H9c2 (ECACC ref no. 88092904) was maintained as myoblasts in Dulbecco's Modified Eagles Medium (Sigma) supplemented with 10% foetal calf serum (Delta Bioproducts) and glutamine (Biowhittaker) as well as the antibiotics, penicillin and streptomycin. For differentiation, cells at about 73-80% confluence were grown in differentiation media consisting of Dulbecco's Modified Eagles Medium (Sigma) supplemented with 1% horse serum (Sigma) and glutamine (Biowhittaker), penicillin and streptomycin for 10 days. Fresh media was added every two to three days.

Cells were grown at 37 °C in a humidified incubator at 5% CO₂. Cells were manipulated in a laminar flow tissue culture hood. Media and additives which were not purchased as sterile solutions were sterile filtered using .22µm filters or autoclaved before use.

2.2.4. Gene Constructs and Cloning

The following plasmids were used as controls in transfection experiments:

2.2.4.1. Control Plasmids

pGL2-Control: Positive control vector for luciferase expression, containing a firefly luciferase coding region under the control of SV40 promoter and enhancer sequences (Promega Corporation).

pGL2-Basic: Negative control vector for background luciferase activity, containing a firefly luciferase coding region but lacking a promoter or enhancer sequences (Promega Corporation). Regions of the 5'-flanking region of the rat ACCβ were cloned into this plasmid upstream of the luciferase coding region.

pRL-CMV: The reporter vector contains the coding region of the sea pansy (*Renilla*) luciferase gene under the control of the CMV promoter region (Promega Corporation). This plasmid was used as an internal control to determine transfection efficiency.

2.2.4.2. Generation of ACC β 5'-flanking Region Reporter Constructs

Due to the lack of restriction sites and the AT-rich nature of the ACC β 5'-flanking region, from -947 to -764 bp upstream of the translation start site, the transcriptional activity of the entire ACC β 5'-flanking region isolated could not be tested.

ACC β 5'-flanking region reporter constructs were cloned as follows:

ACC764.luc

The cloned fragments obtained during the isolation of the 5'-flanking region of the rat ACC β gene were used as a template in PCR using primers with *Xho*I and *Hind*III recognition sites at the 5' ends:

Forward ACC764:

5'- AAGG **CTCGAG** TTG GAT AAC CAG TTG GCA CG-3' (-764 to -745)

***Xho*I recognition site**

Reverse:

5'- AAGG **AAGCTT** TCA GAC AAC CTG TCA AGG GAA G-3' (-22 to -1).

***Hind*III recognition site**

This reaction yielded a fragment containing the -764 to -1 region of the 5'- flanking region of the ACC β gene with *Hind*III and *Xho*I recognition sites at the 5' and 3' ends, respectively. The PCR product was purified using the Qiaquick gel extraction kit (Qiagen), ligated with the shuttle vector, pGEM®-T Easy (Promega Corporation) and subsequently used to transform JM109 cells. Initially, an effort was made to insert these sequences directly into pGL2-Basic, but these efforts did not succeed, most likely due to primer design, the restriction sites may have been placed too close to the ends of the PCR products for digestion. Transformed cells were selected and cloned. Plasmids were isolated from the cells using the High Pure Plasmid Isolation Kit (Roche) and digested with the enzymes, *Xho*I and *Hind*III. The digested fragment with "sticky ends" was gel purified and ligated with pGL2-Basic (Promega), which had been digested with the enzymes, *Xho*I and *Hind*III, and gel purified (**Figure 2.2**).

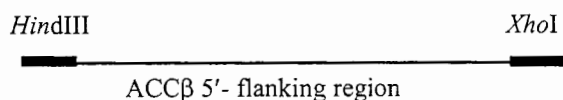
ACC600.luc

This plasmid was constructed using the same method as described above with the same reverse primer, but using the forward primer:

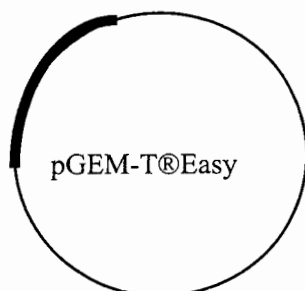
5'-AAGG **CTCGAG** GTG TAC CAC ATG TAT GCC CAG-3' (-600 to -580)

***Xho*I recognition site**

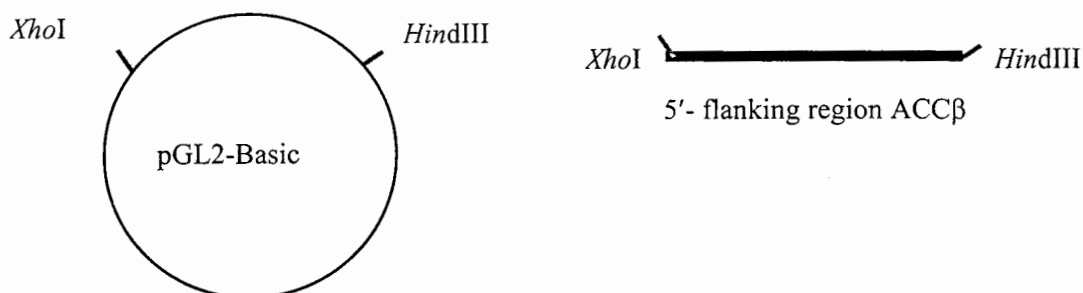
1. Primers for PCR designed to clone segments of the ACC β 5'-flanking region with adjacent *Xho*I and *Hind*III recognition sites at the 5' and 3' ends, respectively.



2. The PCR products containing the promoter region were inserted into the shuttle vector, pGEM-T® Easy (Promega Corporation) to produce a promoter-pGEM-T®-T Easy construct



3. The pGL2-Basic and the promoter-pGEM-T®-T Easy construct were digested with *Xho*I and *Hind*III to generate "sticky ends", the digested fragments were eluted.



4. Ligated digested pGL2-Basic and ACC β promoter fragment to generate the ACC β promoter reporter constructs.

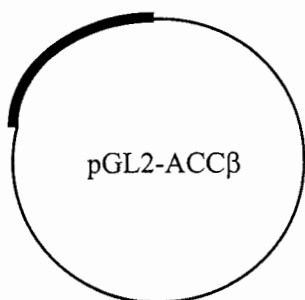


Figure 2.2. Cloning of ACC β 5'-flanking region reporter constructs.

ACC257.luc

The plasmid, ACC257.luc was constructed using the same method as for ACC764.luc and ACC600.luc, but using the forward primer:

5'-AAGG **CTCGAG** CTT CCT GGC ACC GTT TCA-3' (-257 to -240)

*Xho*I recognition site

2.2.4.3. Expression Vectors

pUSF1: The USF-SR α expression vector coding for USF1 was a kind gift of T. Kamataki, Hokkaido University, Japan and has been previously described [199]. This plasmid will be referred to as pUSF1.

pUSF2: The expression vector, psv-USF2 [200] was the kind gift of M. Sawadogo, MD Andersen Cancer Center, University of Texas, USA. This plasmid will be referred to as pUSF2.

pPPAR α : The pSG5rPPAR-SR α expression vector coding for PPAR α was a kind gift of L. Michalik, University of Lausanne. This plasmid will be referred to as pPPAR α .

pRXR α : The expression vector, pBOS-RXR α [200] was the kind gift of D. Kelly, Washington University in St Louis, USA and has been previously described in [201]. This plasmid will be referred to as pRXR α .

2.2.4.4. Plasmid DNA Preparation and Purification

Plasmid DNA used for transfections was prepared and purified using the Maxi Plasmid kit (Qiagen) or by the alkaline lysis maxipreparation procedure and ultracentrifugation in a caesium chloride (CsCl) gradient as described in Sambrook et al. [197] with slight modifications. The alkaline lysis maxipreparation procedure and ultracentrifugation in a caesium chloride (CsCl) gradient involved the preparation of a starter culture by inoculating 6 ml Luria Broth (LB) supplemented with ampicillin (100 μ g/ml) with frozen stocks of *E. coli* carrying the plasmid of interest and incubated for 12-16 hours in a shaking incubator at 37 °C. One millilitre of this starter culture was used to inoculate 500 ml of LB with ampicillin (100 μ g/ml) which was incubated for 12-16 hours in a shaking incubator at 37°C. The culture was centrifuged to pellet bacteria, the pellet was resuspended in 40 ml of a sterile filtered solution of 50 mM glucose/ 10mM EDTA/ 25mM Tris-HCl (Solution 1). Cells were lysed by the addition of 80 ml of 200 mM NaOH/ 1% SDS (Solution 2) and followed by the adding 45 ml of autoclaved Solution 3 (3 M potassium acetate, 11.5% glacial acetic acid). The cell debris was pelleted by centrifugation at 7000 rpm for 10 min at 4 °C and the supernatant containing the plasmid was filtered through gauze. Ninety milliliters of isopropanol was added and mixed with the supernatant. This solution was centrifuged at 7000 rpm for 10 min at 4 °C and the pellet was discarded. The pellets were air dried and then resuspended in 2 ml H₂O.

A CsCl gradient was set up by mixing 4.8 g CsCl, 2.1 ml H₂O, 200 μ l ethidium bromide (10 mg/ml) and the resuspended DNA pellet into a centrifugation tube. The mixture was centrifuged at 3000 rpm for 10 min at room temperature. The supernatant was transferred to fresh ultracentrifugation tubes, which were sealed and ultracentrifuged at 53,000 rpm overnight at 20 °C. On the following day, the band containing the plasmid DNA was extracted from the ultracentrifugation tube by puncturing the wall of the tube with a syringe and extracting the band containing plasmid DNA. Ethidium bromide was removed by the addition of an equal volume of isobutanol and vigorously shaking this solution, allowing the solution to settle with the ethidium bromide settling in the top layer of isobutanol, which was removed and discarded. This step was repeated until no ethidium bromide was visible in the isobutanol layer. DNA was precipitated in

2.5 X volume of isopropanol, washed with 70% ethanol, air dried and resuspended in TE buffer. The plasmid DNA was quantitated and its purity assessed using a spectrophotometer. Its identity was confirmed by restriction enzyme digestion.

2.2.5. Transient Transfections

2.2.5.1. Transient Transfections of CV-1 Cells

Initially, we attempted to transiently transfect CV-1 cells using variations of the calcium phosphate method, but this method did not prove to be optimal (Please refer to Appendix B for methodology and discussion). In light of this, we used the liposomal transfection reagent, FuGENE 6 Transfection Reagent (Roche), and established the following protocol which was used in this study.

One day before transfections were carried out, CV-1 cells were plated at a density of 3.375×10^4 cells/well in 24-well cell culture plates (15.6mm, Costar). On the following day, when the cells were 50-60% confluent, the media on the cells was replaced with fresh media. The cells were transfected with 0.6 μ g ACC β 5'-flanking region reporter construct and 10 ng pRL-CMV using FuGENE 6 Transfection Reagent (Roche). On the day that the cells were transfected, FuGENE 6 diluted in serum-free medium was added to the plasmid DNA so that the ratio of FuGENE 6 to plasmid DNA was 5 μ l FuGENE 6: 3 μ g DNA. A FuGENE 6:DNA complex was formed by allowing this mixture to incubate at room temperature for 15 minutes. Thereafter, it was added dropwise over the surface of the cells.

Forty-eight hours after transfection, the proteins were harvested. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed *in situ* by adding 100 μ l 1 X Passive Lysis Buffer (Promega Corporation). The cell debris was scraped off the surface of the dish using a rubber policeman and collected in a microfuge tube. The lysates were centrifuged in a microfuge at 12,000 g for 2 minutes at 4°C to pellet the cell debris and the supernatant was transferred to a fresh microfuge tube. The lysate was stored at -80°C or assayed directly.

Transfections were performed at least three times in duplicate or triplicate for each luciferase plasmid tested. Various experiments were performed to establish the optimal conditions for transfecting cells. A number of controls were included with each experiment. Firefly luciferase plasmids were cotransfected with pRL-CMV (Promega Corporation) as an internal control for the efficiency of DNA uptake. Two sets of duplicate dishes were also included, one was transfected with pGL2-Basic vector (Promega Corporation) to control for background firefly luciferase activity. The other was transfected with pGL2-Control (Promega Corporation) to control for transfection efficiency between different experiments. At first, a set of dishes was left untreated to control for background *Renilla* luciferase activity, but this was found to be unnecessary as background *Renilla* activity was negligible.

2.2.5.2. Co-transfections of CV-1 cells

Co-transfections were performed, in which expression vectors coding for the transcription factors, upstream stimulatory factor 1 (USF1), upstream stimulatory factor2a (USF2a), peroxisome proliferator-activated receptor- α (PPAR α) and retinoid X receptor- α (RXR α) were transfected along with the various ACC β promoter reporter constructs. Cells were transfected with an equal amount of ACC β 5'-flanking region reporter construct and expression vector. Cells in each dish were transfected with an equal amount of DNA, pGL2-Basic was used to equalise amounts of DNA.

2.2.5.3. Transient Transfections of H9c2 Myoblasts and Myotubes

a) Myoblasts

One day before transfections were carried out, cells were plated at a density of 3.135×10^4 cells/well in 24-well cell culture plates (15.6mm, Costar). The cells were transfected using the same protocol as that used to transfect CV-1 cells with the following modification, 0.8 μ g of the promoter reporter construct was used to transfect cells.

b) Myotubes

Completely differentiated H9c2 myotubes were transfected employing the same protocols used for myoblasts.

2.2.6. Reporter Gene Assays

The activities of the firefly and *Renilla* luciferase were assayed using the Dual Luciferase Reporter Assay System (Promega Corporation) and a luminometer (Monolight 3010) or Bio-Orbit 1253 luminometry system. Firefly luciferase was measured by adding 20 μ l of cell lysate to 100 μ l of Luciferase Assay Reagent II (LARII) containing the firefly luciferase substrate, luciferin and measuring luminescence (light units) in the luminometer. Thereafter, 100 μ l of a second reagent, Stop & Glo, which quenches the firefly luciferase and contains the substrate for *Renilla* luciferase, coelenterazine, was added. Luminescence was measured to determine the activity of the internal *Renilla*, control vector.

The relative luciferase activity (RLA) value was obtained in order to account for variations in transfection efficiency. RLA was calculated as follows: Firefly luciferase activity (light units) for each sample was divided by the *Renilla* luciferase activity (light units). The normalised activity for each sample was expressed as a percentage of the positive control vector, pGL2-Control which was assigned the value of 100%; this value was obtained in order to compare the results of independent experiments.

2.3 Statistical Analyses

Data are presented as mean $\pm$ S.E.M. Analysis of variance (ANOVA) and the Bonferonni or Tukey-Kramer tests were used to determine significant differences between groups. A value of $p < 0.05$ was regarded as significant.

3.1. Characterisation of the Metabolic Gene Expression Profile in Response to Hypobaric Hypoxia

We initially attempted to characterise metabolic gene expression profiles in response to hypobaric hypoxia using northern blot hybridisation. The cDNA probes for metabolic genes other than ACC β were available in the laboratory and protocols for the use of these probes had been previously optimised. However, we were unable to set up a protocol for assessing ACC β mRNA levels using this method; we obtained very weak or no signals. However, we attempted to solve this problem using the following approaches: (i) amounts of RNA per sample used were increased to 25 μ g, (ii) amount of probe used in the hybridisation reaction was increased (2-fold), (iii) using various methods of removing unincorporated α -P³²-dCTP, (iv) stringency of hybridisation conditions were reduced (hybridisation temperature was gradually reduced from 65°C to 60°C) and (v) number and length of washes to remove unbound probe were reduced.

There was also a limitation on the number of animals available for research as the rat colonies at the University of Cape Town Animal Unit were culled at this time due to a *Cryobacterium pseudomonas* infection. The disease-free animals used in this study were imported at a considerable cost. Due to the relatively low RNA levels in the myocardium and the limited amounts of tissue available, the troubleshooting was hampered and not all avenues to optimise this technique could be explored. Consequently, alternative approaches were sought to reduce the amount of tissue needed relative to the amounts required for northern blot analysis. We found that reverse transcription and quantitative real-time PCR was an ideal approach. This method is a reliable and sensitive method for mRNA quantitation. The technology to carry out this work was not available locally and therefore tissue samples were sent to laboratories in the USA, which had optimised systems to detect transcript levels of our genes of interest.

3.1.1. Induction of Right Ventricular Hypertrophy with Exposure to Hypobaric Hypoxia

In order to establish the morphological changes induced by acute and chronic hypobaric hypoxia, rats were exposed to 11% ambient oxygen for 48 hours or one week. Thereafter, they were sacrificed along with age and sex-matched control animals, heart tissue was collected and analysed. The morphometric data are summarised in **Table 3.1**. We found that there were marked differences in body weight of the 48 hour group versus the other groups, this was reflected in the higher heart and LVS masses, these differences did not appear to be due to the hypoxic stress, but rather that these animals may have been on average slightly larger than the animals in the other groups at the start of the experiment. Therefore, in order to take these differences into account all statistical analyses were performed only on ratios of mass measurements to detect changes in the morphology of the myocardium. There was a statistically significant increase (~30%; $p < 0.001$ vs. normoxic controls) in the heart:body mass ratio after one week of hyperbaric treatment indicative of myocardial hypertrophy. Moreover, the RV:LVS mass ratio also increased significantly by 54% ($p < 0.001$ vs. normoxic controls) after 7 days of hyperbaric treatment, indicating the development of right ventricular hypertrophy after seven days.

Table 3.1. Morphometric and Haematological Characteristics of Control and Hypoxic rats.

Characteristic	Control	Hypobaric Hypoxia	
		48 hours	1 week
n	8	8	8
Body Mass (g)	156.6 ± 2.8	233.4 ± 5.2	172.8 ± 3.7
Total Heart Mass (mg)	652.5 ± 21.7	972.5 ± 38.5	926.3 ± 11.9
LVS Mass (mg)	422.5 ± 11.0	611.35 ± 12.1	471.3 ± 23.7
RV Mass (mg)	157.5 ± 8.4	196.3 ± 7.8	257.5 ± 21.9
RV Mass / LVS Mass (%)	37.3 ± 2.1	32.1 ± 1.3	57.10 ± 8.3*
Heart: body mass ratio (%)	0.42±0.01	0.42±0.02	0.54±0.01***

RV – right ventricle; LV- left ventricle; LVS – left ventricle + septum

*p < 0.05 vs. normoxic controls; ***p < 0.001 vs. normoxic controls

Values are mean ± Standard error of Mean (SEM)

To confirm the development of right ventricular hypertrophy, the expression of atrial natriuretic peptide (ANP) in the RV of these animals was measured. ANP was used as a marker of the foetal gene expression pattern, which is observed with the development of hypertrophy. ANP gene expression in the RV of hypoxic animals increased insignificantly after 48 hours. There was a significant 6.5-fold ($p < 0.001$ vs. normoxic controls) increase after one week confirming the induction of RV hypertrophy after exposure to hypobaric hypoxia for 7 days (**Figure 3.1**) inferred from morphometric data. In subsequent and more extensive studies using this model, it was found that there was no change in ANP mRNA levels in the LVS [202]. Consequently, these results show the development of RV hypertrophy in response to hypobaric hypoxia as previously observed in this model [179, 180].

3.1.3. Erythrocytotic Response to Hypobaric Hypoxia

We measured haematocrit levels of blood samples obtained by cardiac puncture to assess for erythrocytosis. Haematocrit levels were found to increase 18% in hypoxic rats after 48 hours of hypobaric hypoxia from $43.86 \pm 1.53\%$ of whole blood in controls to $51.75 \pm 1.68\%$ ($p < 0.01$ vs. normoxic controls). A further rise of approximately 40% ($61.3 \pm 1.8\%$; $p < 0.001$ vs. normoxic controls) above control levels was observed after one week.

Vascular endothelial growth factor (VEGF) is involved in angiogenesis and is induced by hypoxia. Although we did not directly assess angiogenesis in cardiac tissue, VEGF mRNA levels were measured to determine if there was any change in the regulation of VEGF transcript levels in response to hypobaric hypoxia (**Figure 3.2**). There were no significant alterations in VEGF mRNA levels in either ventricle.

3.1.2. Upregulation of Glucose Oxidation Rather than Glucose Uptake in Response to Hypobaric Hypoxia

In order to assess transcriptional regulation of cardiac glucose metabolism in response to hypobaria, we measured GLUT1, GLUT4 and PDK4 transcript levels. There was no significant alteration in GLUT1 transcript levels in either ventricle (**Figure 3.3**) with hypobaria. However, there was a modest transient increase ($23.7 \pm 9.1\%$; $p = \text{ns}$ vs. normoxic controls) in GLUT1 mRNA levels in the RV (**Figure 3.3a**) after 48 hrs hypobaric hypoxia compared to controls, these levels returned to baseline after 1 week. GLUT 4 transcript levels in the RV (**Figure 3.4a**) were lower ($29.2 \pm 12.8\%$; $p = \text{ns}$ vs. normoxic controls) compared to control levels after 48 hrs and this attenuation ($30.0 \pm 6.1\%$; $p = \text{ns}$ vs. normoxic controls) was maintained after 7 days of exposure to the stimulus. No changes in GLUT4 gene expression were detected in the LVS (**Figure 3.4b**). PDK4 gene expression was significantly downregulated in both ventricles after 7 days of exposure to hypoxia ($p < 0.001$ vs. normoxic controls). Under hypoxic conditions, PDK4 levels were diminished by $85 \pm 3.4\%$ in the RV and by $84 \pm 2.1\%$ in the LVS (**Figure 3.5**).

3.1.4. Downregulation of Fatty Acid Metabolism Regulators

The mRNA levels of several genes involved in FAO were measured to assess the transcriptional regulation of FAO in response to hypobaric hypoxia. Levels of PPAR α mRNA, a key regulator of FAO gene expression, were not significantly altered in either ventricle; although there was a downward trend ($30.6 \pm 10.0\%$; $p = \text{ns}$ vs. normoxic controls) in PPAR α expression in the LVS after one week (**Figure 3.6**).

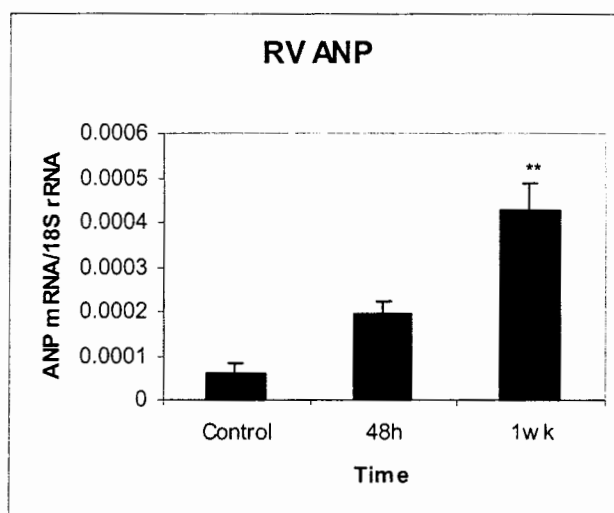
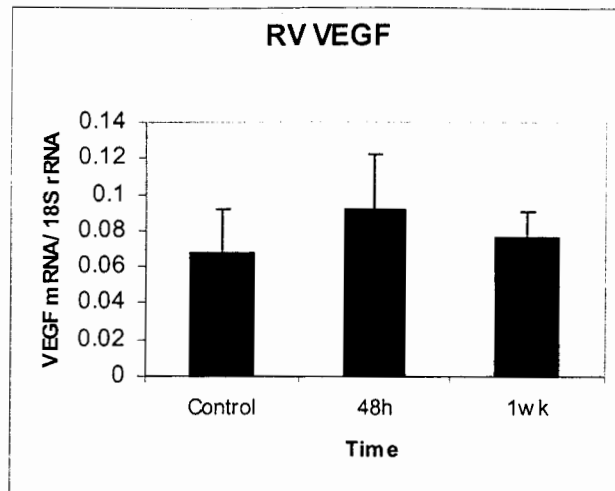
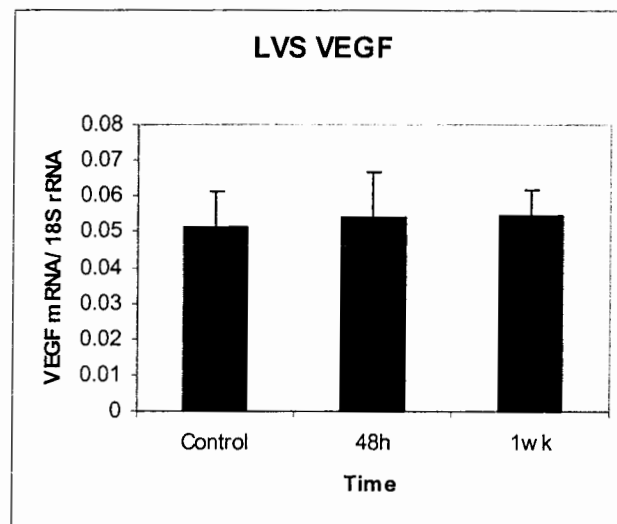


Figure 3.1. Effects of 48 hours and one week of exposure to hypobaric hypoxia on ANP expression in the right ventricle (RV).

Reverse transcription and quantitative real-time PCR analyses showed that there was upward trend in ANP expression which is significant after one week of hypobaria (** $p < 0.001$ vs. normoxic controls). The increase in ANP expression was indicative of a reversion to the foetal gene expression pattern ($n = 8$).



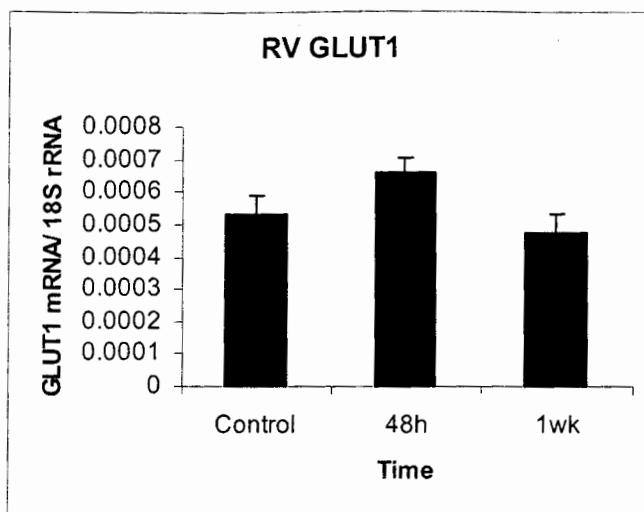
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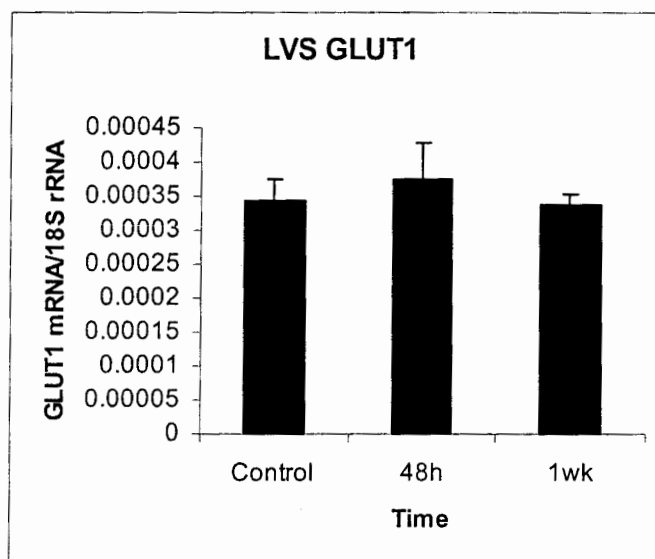
b)

Figure 3.2. Effects of 48 hours and one week hypobaric hypoxia on VEGF expression.

Results obtained using reverse transcription and quantitative real-time PCR analysis demonstrated that VEGF mRNA levels in the (a) right ventricle (RV) and (b) left ventricle and septum (LVS) were unaltered by acute and chronic hypobaric hypoxia ($n = 5$; $p = ns$).



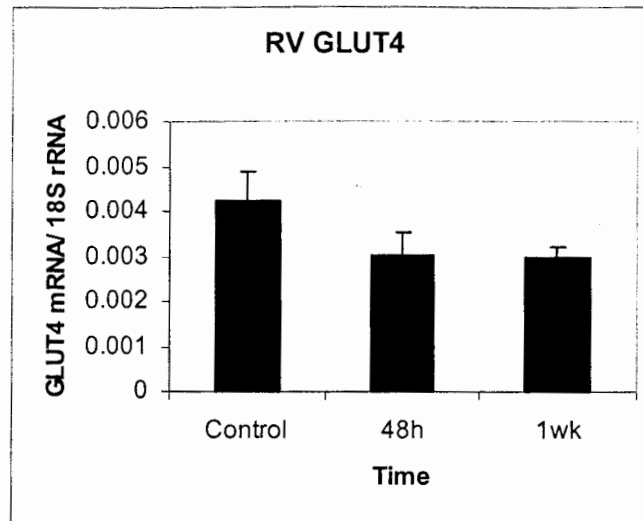
a)



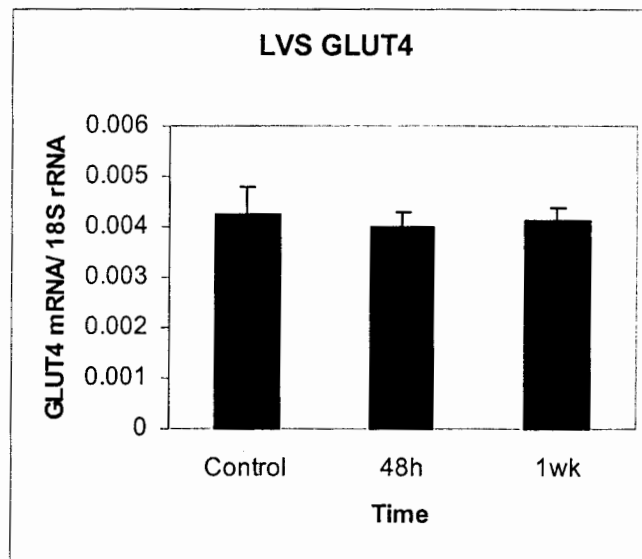
b)

Figure 3.3. GLUT1 gene expression levels in response to hypobaria.

No statistically significant changes in GLUT1 gene expression were detected in the (a) right ventricle (RV) or (b) the left ventricle and septum (LVS) in response to hypobaria using quantitative real-time PCR analyses. There was a slight increase in GLUT1 transcript levels in the RV after 48 hours but levels decreased to control levels after one week ($n = 5$; $p = ns$).



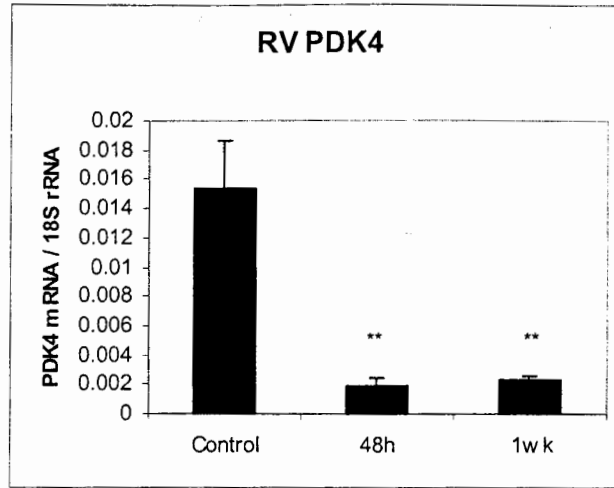
a)



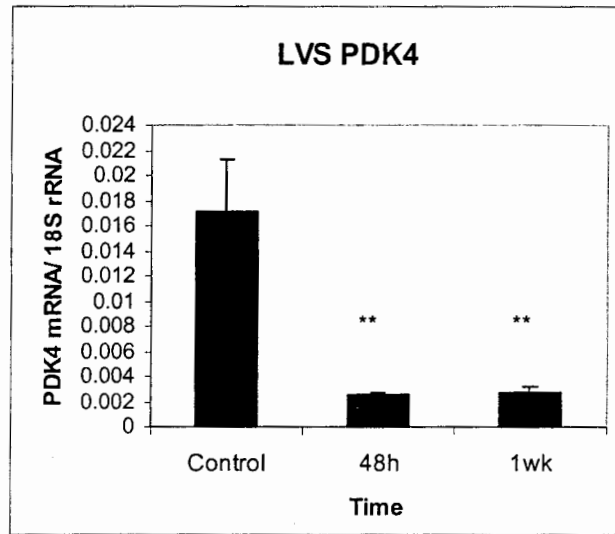
b)

Figure 3.4. GLUT4 gene expression levels in response to hypobaria.

The results of quantitative real-time PCR analyses showed that GLUT4 mRNA levels did not alter significantly in (a) the right ventricle (RV), but a downtrend was detected in the RV after 48 hours and maintained after 7 days. GLUT4 expression in (b) the left ventricle and septum (LVS) was unaltered by hypoxia ($n = 5$; $p = \text{ns}$).



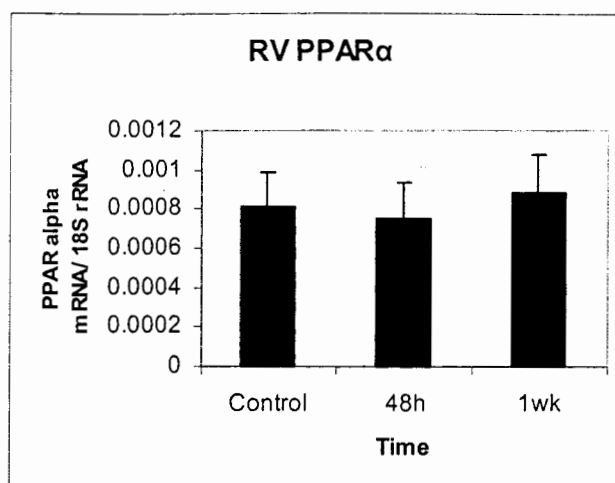
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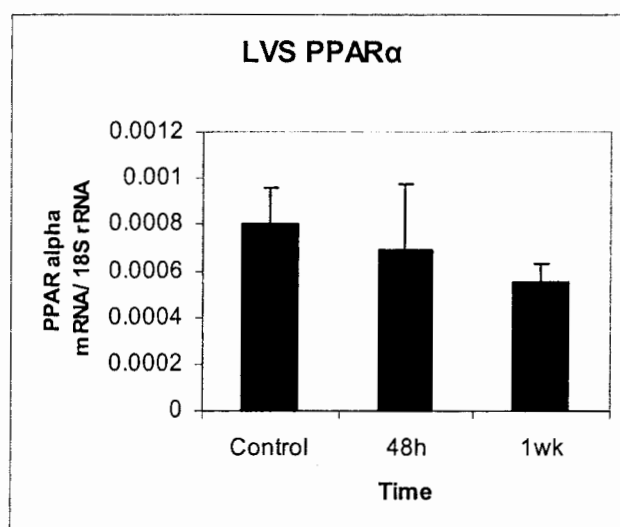
b)

Figure 3.5. PDK4 mRNA levels were significantly lower in response to hypobaric hypoxia.

PDK4 mRNA levels were significantly decreased in both (a) right ventricle (RV; ** $p < 0.001$ vs. normoxic controls) and (b) the left ventricle and septum (LVS; ** $p < 0.01$ vs. normoxic controls) after 48 hours and 7 days hypobaric hypoxia ($n = 5$).



a)



b)

Figure 3.6. PPAR α mRNA levels in response to hypobaric hypoxia.

PPAR α gene expression was not statistically significantly altered by acute or chronic hypoxia in (a) the right ventricle (RV) or (b) left ventricle and septum (LVS). There was a small decrease in PPAR α gene expression in the LVS after 7 days ($n = 5$; $p = \text{ns}$).

There was a co-ordinate downward trend in the expression of genes regulating FAO in the RV after seven days: α -MCD was diminished by $46.9 \pm 12.7\%$ ($p = \text{ns}$ vs. normoxic controls; **Figure 3.7a**) and MCAD gene expression was reduced by $18.8 \pm 8.1\%$ (**Figure 3.9a**). A similar trend was identified in the LVS: MCD mRNA levels were reduced by $34.2 \pm 9.9\%$ (**Figure 3.7b**), mCPT1 mRNA levels were reduced by $27.3 \pm 7.1\%$ (**Figure 3.8b**) and MCAD gene expression was decreased by $21 \pm 5\%$ (**Figure 3.9b**). Interestingly, UCP3 gene expression (**Figure 3.10**) was significantly decreased in both ventricles.

3.1.5. Analogous Acetyl-CoA Carboxylase Isoform Gene Expression in Response to Hypobaric Hypoxia

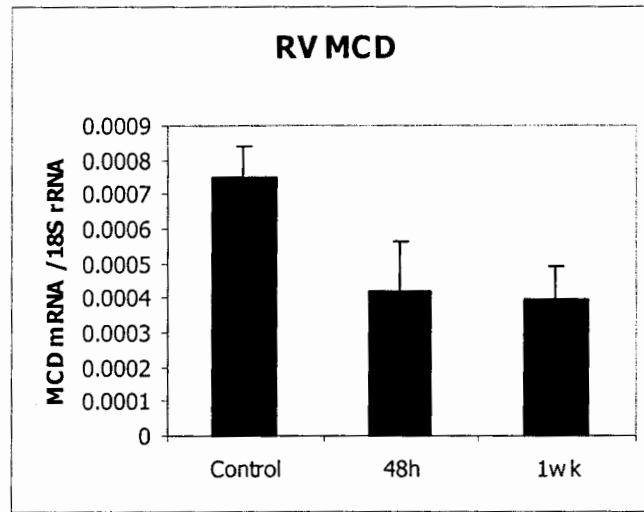
One of the aims of this study was to determine whether ACC α and ACC β were distinctly regulated, for this reason we have evaluated transcript levels of these isoforms in response to hypobaria. We found that ACC α mRNA levels were approximately four-fold lower than ACC β levels, in agreement with previous work showing that ACC β is the cardiac-enriched isoform [127]. Decreased expression of both isoforms was observed in response to hypertrophy (RV) and hypoxia (LVS).

There were no significant changes in ACC α mRNA levels in the RV in response to hypobaric hypoxia (**Figure 3.11a**). There was a downward trend in ACC α mRNA levels in this ventricle after 7 days ($28.41 \pm 5.4\%$; $p = \text{ns}$ vs. normoxic controls). In the LVS (**Figure 3.11b**), an insignificant decrease in ACC α mRNA levels ($23.4 \pm 8.6\%$; $p = \text{ns}$ vs. normoxic controls) was observed after 48 hrs but the decrease was very significant after 1 wk ($23.4 \pm 8.6\%$; $p < 0.01$ vs. normoxic controls).

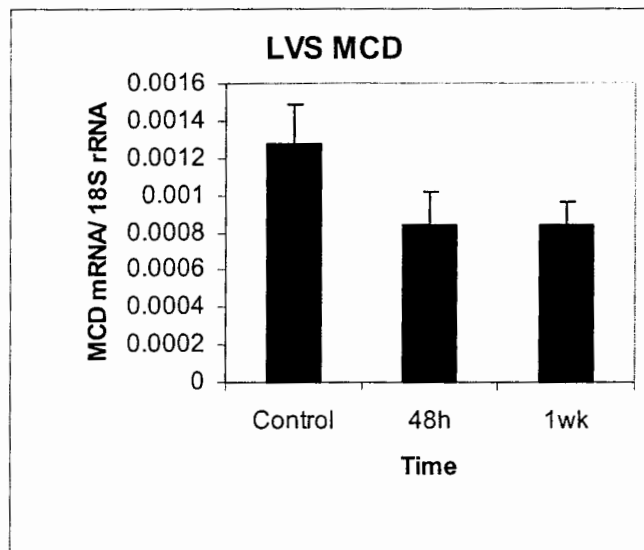
Similarly, ACC β mRNA levels were decreased by $37.0 \pm 15.0\%$ in the RV (**Figure 3.12a**) after 48 hours ($p < 0.05$ vs. normoxic controls) and the decline was maintained after 7 days ($37.22 \pm 0.7\%$; $p < 0.05$ vs. normoxic controls). ACC β transcription was also significantly altered in the LVS (**Figure 3.12b**) after 48 hours ($21.5 \pm 6.9\%$; $p < 0.05$ vs. normoxic controls) and the decline was more significant after 7 days ($27.1 \pm 0.3\%$; $p < 0.01$ vs. normoxic controls). These results indicate that ACC β transcriptional regulation is affected by hypoxia rather than hypertrophy.

3.2. A Novel Sequence in the 5'-Flanking Region of the Rat ACC β Gene Contains Several Promoter Elements

In order to delineate molecular mechanisms regulating ACC β gene expression in the rat myocardium, we used the Rat Genome Walker kit (Clontech) to isolate the 5'-flanking region of the rat ACC β gene. This kit allows for the isolation of genomic sequences upstream of a known sequence. Three of the four rat genomic libraries screened yielded a fragment upstream of the cDNA. The three libraries namely, DL-*PvuI*, DL-*DraII* and DL-*EcoRV* yielded fragments of approximately 1.8, 1.6 and 1.5 kb, respectively. The entire cloned fragments could not be sequenced; sequencing in the 3' to 5' direction was hindered by a GC-rich sequence. Sequencing in the 5' to 3' direction yielded a fragment approximately 1 kb in length, but a poly-A sequence of this fragment prevented further sequencing in this direction. A 947 bp consensus sequence upstream of the translation start site was obtained from these three fragments. Computer-based analyses using the nucleotide Basic Local Alignment Search Tool (BLASTN; [203]) demonstrated that this sequence aligned with the *Rattus norvegicus* chromosome 12 whole genome shotgun (WGS) supercontig (Genbank accession no. NW_047377) (**Figure 3.13**) facilitating the mapping of this region to chromosome 12 of the rat genome.



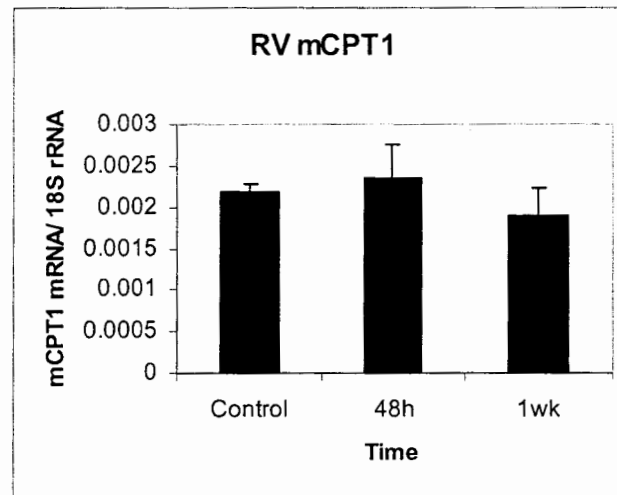
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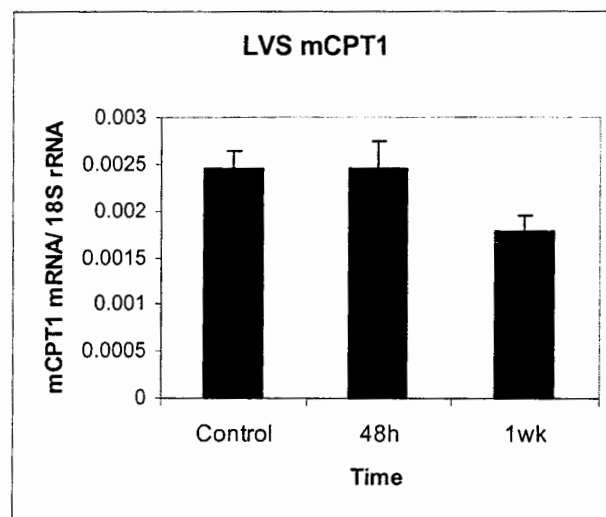
b)

Figure 3.7. MCD mRNA levels in response to hypobaric hypoxia.

There was a statistically insignificant decrease in the expression of MCD in (a) the right ventricle (RV) and (b) the left ventricle and septum (LVS) in response to hypobaria ($n = 5$; $p = ns$).



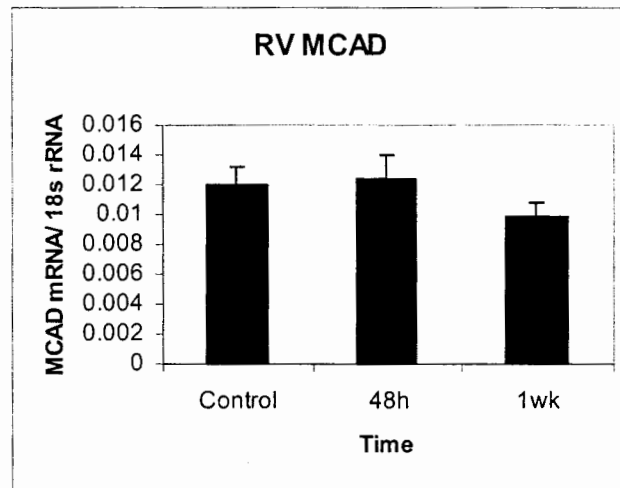
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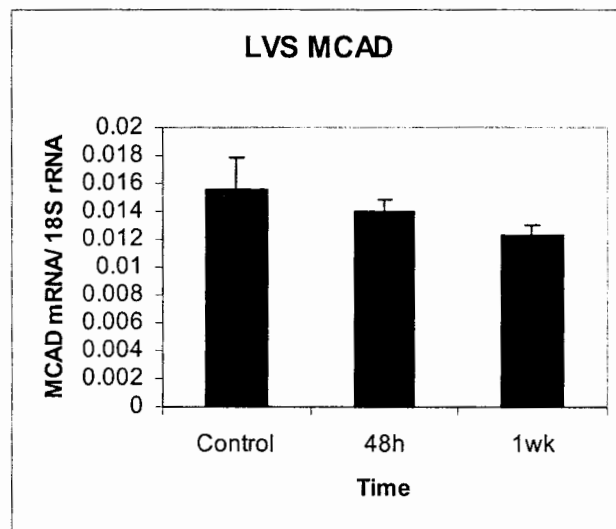
b)

Figure 3.8. mCPT 1 mRNA levels in response to hypobaric hypoxia.

Quantitative real-time PCR investigation showed that mRNA levels of mCPT1 were unchanged by hypoxia in (a) the right ventricle (RV) and levels were decreased in (b) the left ventricle and septum (LVS) after 7 days ($n = 5$; $p = ns$).



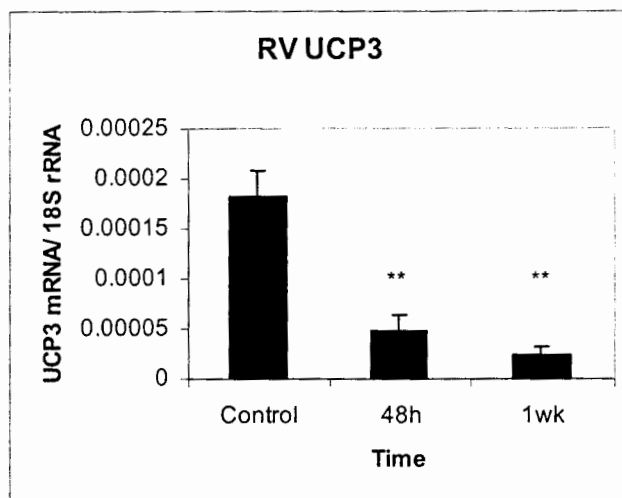
a)



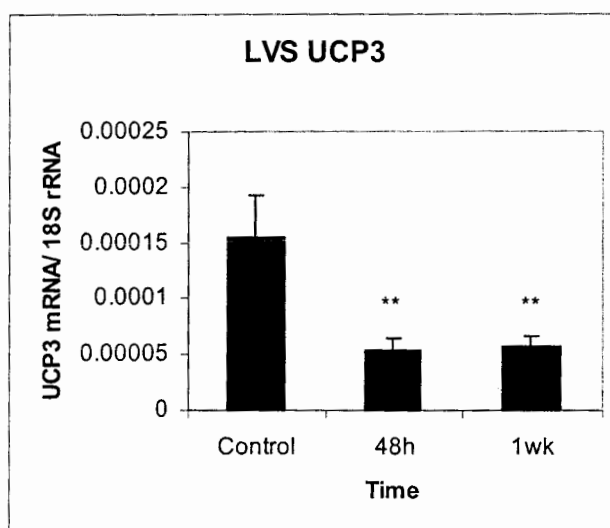
b)

Figure 3.9. MCAD mRNA levels in response to hypobaria.

MCAD gene expression was insignificantly reduced in the (a) right ventricle (RV) and the (b) left ventricle and septum (LVS) after 1 week of hypobaria (n = 5; p = ns).



a)



b)

Figure 3.10. UCP3 mRNA levels in response to hypobaric hypoxia.

UCP3 gene expression was significantly (** $p < 0.001$ vs. normoxic controls) diminished in both the (a) right ventricle (RV) and (b) left ventricle and septum (LVS) after 7 days of hypobaric hypoxia ($n = 5$).

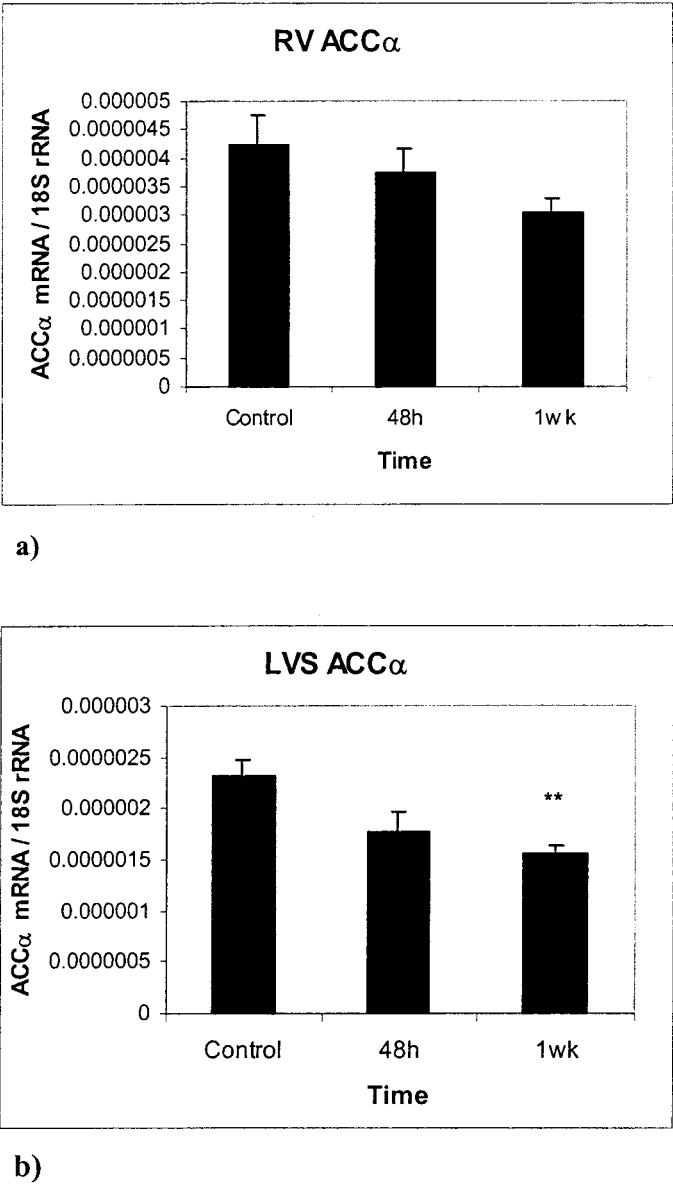
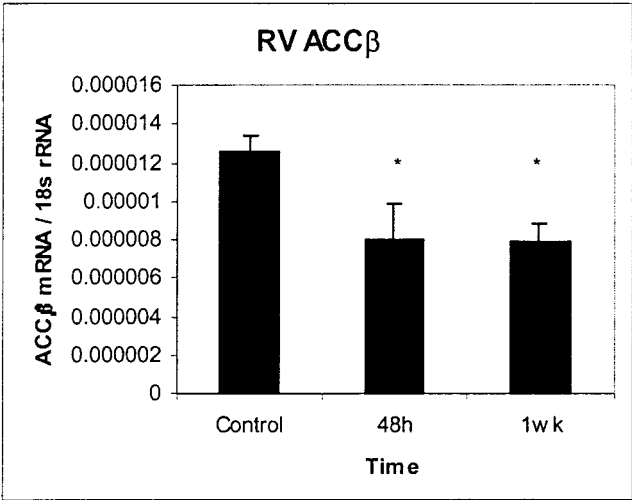
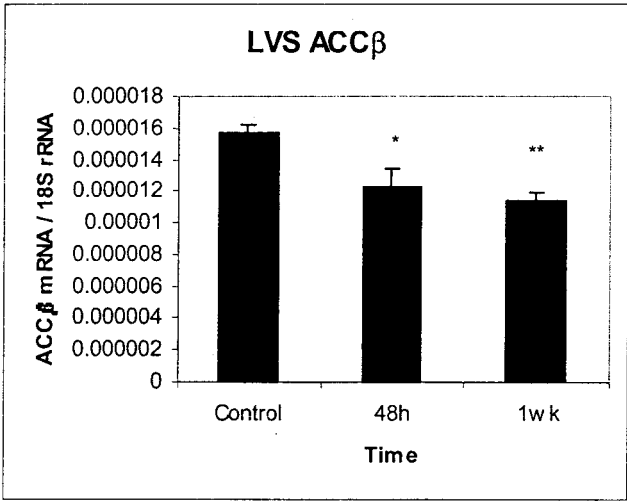


Figure 3.11. ACCα mRNA levels in response to hypobaric hypoxia. ACCα gene expression in the (a) right ventricle (RV) was not significantly altered in response to hypobaric hypoxia. There was a significant decrease in ACCα mRNA levels (**p < 0.01 vs. normoxic controls) in the (b) left ventricle and septum (LVS) after 7 days (n = 8).



a)



b)

Figure 3.12. ACCβ mRNA levels in response to hypobaria.

ACCβ mRNA levels were significantly downregulated in both ventricles in response to hypobaria. In the (a) right ventricle (RV), there was a significant decrease in ACCβ mRNA levels after 48 hours (*p < 0.05 vs. normoxic controls) which was maintained after 7 days (*p < 0.05 vs. normoxic controls). ACCβ mRNA levels in the (b) left ventricle and septum (LVS) were significantly decreased after 48 hours (*p < 0.05 vs. normoxic controls) and this reduction was more significant after 1 week (**p < 0.01 vs. normoxic controls).

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```

Figure 3.13. Results of BLASTN alignment of the 5'- Flanking region of the rat ACC β gene with a contig containing *Rattus norvegicus* chromosome 12.

The figure is taken directly from the BLASTN output and shows the 5'-flanking region (Query) aligned with the *Rattus norvegicus* chromosome supercontig (NW 047377.1; Sbjct). Numbering indicates nucleotide positions). The sequence is numbered relative to the translation start site which is designated as +1.

Inspection of the 947 bp revealed several promoter-linked elements required for the formation of a basal transcription complex. Two typical TATA boxes were present at positions -34 / -30 and -873 / -869 relative to the translation start site. Three CAAT boxes were found at positions -87/-83, -496 / -492 and -766 / -762. We used the online program TESS to search the response element database, TRANSFAC [40] and SIGNALSCAN [41] to screen for consensus sequences to which transcription factors bind. Multiple consensus sequences for transcription factor binding sites or response elements potentially regulating ACC β gene expression were identified in the 947 bp sequence. Putative response elements involved in metabolic gene regulation were identified (**Figure 3.14**). For example, six copies of the E-box (CANNTG), the binding site for basic/helix-loop-helix (bHLH) transcription factors were identified. Five binding sites for Sp1 transcription factors were found as well as one activator protein-1 (AP-1) response element (TGACTCA) was identified. Two nuclear receptor response elements (NRRE) with the consensus sequence, AGGTCA, were also identified. A comprehensive list of response elements is given in Appendix C. We also searched for short fragments which matched that of the human ACC β gene promoter, PII β [173], **Figure 3.15** shows the alignment of the two sequences. There was a 33.55% identity between the two sequences but no noteworthy fragments of alignment were detected, the longest continuous alignment was 8 bp. Similarly, the 947 bp sequence did not show any small significant regions of identity with the contig containing the human ACC α (Genbank accession no. NT_010795).

3.3. Determination of a Transcription Start Site of the Rat ACC β Gene and Isolation of the Predominant Promoter Region

We initially applied primer extension analysis to isolate the transcription start site of the rat ACC β gene, this approach was unsuccessful, the methodology is given in Appendix B. Therefore, 5'-rapid amplification of cDNA ends (5'-RACE) approach was used. We successfully used two 5'-RACE protocols. First, we used the GeneRacer™ Kit (Invitrogen) which required the use of RNA isolated in the laboratory. The second 5'-RACE kit used was the Marathon-Ready™ cDNA kit (Clontech) as this kit contained commercially prepared cDNA with an oligonucleotide ligated to the 5'-ends. We had previously found that RNA isolated in our laboratory was partially degraded, most likely caused the presence of RNases and shearing due to handling despite stringent precautions. The use of commercially prepared cDNA provided in the Marathon-Ready™ cDNA kit was used to increase the likelihood of isolating transcripts present at low levels in the rat myocardium.

Using these 5'-RACE methods, we were able to clone a fragment approximately 150 bp in size into a vector and ten clones were sequenced. A one hundred and thirty basepair consensus sequence upstream of the initiator codon was obtained and aligned with the novel genomic 947 bp region. The 5'-RACE product contained part of the incipient ACC β cDNA sequence and the first 9 bp of the 947 bp sequence upstream of the translation start site. The remaining 121 bp sequence upstream of the 9 bp segment did not align with the previously isolated 947 bp sequence upstream of the ACC β cDNA sequence.

Using BLAST [203], the 5'-RACE product has been found to be identical to a rat ACC β muscle-specific promoter sequence deposited in GenBank (GenBank Accession No. AY 217098) in January 2003, about one year after this sequence was isolated in this study (**Figure 3.16**). On 23 October 2003, the *Rattus norvegicus* WGS supercontig (Genbank accession no. NW 047377.1; GI 34872873) containing the a portion of the rat chromosome 12, was deposited into Genbank, the ACC β muscle-specific promoter and we were able to map it to a region 19,540 bp upstream of the translation start site of the ACC β gene. **Figure 3.17.** shows the genomic structure of the part of the rat chromosome 12 containing the ACC β gene.

Query 4 cacattcatgcacatatatgtacatatctatatgtaggggtatatatgcatatacacaca
 || ||| | || | || ||| | | ||
 Subject 45 caagatcacactgtccattggtggcagagtcctggagcgggtggctcacactgcccattggt

Query 64 tatattacaagtataatatatgtgtatatgtatgtatacatatccctaaatacattaagt
 ||| | | | | | | || || | || |
 Subject 105 gctctgagtgagatggtgaatgctgatggctcagatgtgtacagcccaactgcatccag

Query 124 acaaccactcagtctatctaattgttacttgtatgtgttttcaaggctgaccatttgttat
 ||||| || | | | | || || || || |
 Subject 165 cactgcactcagtncccaggggggatgaactcattgaactcttaacagcccagcaatgat

Query 184 tggataaccagttggcacgctcttccccaggaagattatttctcttgttcccagcatcct
 |||| || || || | | || ||
 Subject 225 gtaggtaccatccttatccccattttgcagatgaggagactgatgatcagggaggcatt

Query 244 tagctgctcgtagttc.....
 || || | ||
 Subject 285 taacttgcccaaggcttccccaccttgtaagtggcagaaccgacatgtgaaccagggtca

Query 260
 Subject 345 gtttccaggggaccaaacaagggtgcccggtgacagttgactttggccgccaatcatgcct

Query 260 tttgtgtcaggttgaggcct
 || || || | ||
 Subject 405 caatatagcgaagaactggcctggagccggggatcttggttggtcccagtccttggtc

Query 280 ggtgtctctcccctgatcggtttattttttattttattagt.atgcgtgtcttgcctgca
 ||| || | || | | || || | || ||
 Subject 465 tgtggctaattgcacatgtgacctctggccaccatttgctcatctacaacacaagcaca

Query 339 catatatatgtgtaccacatgtatgcccagtgcccttctgaggtcagaggagggcatgggc
 | | | || || || || || || || | | ||
 Subject 525 ctggactcgatgatccctgtgcttcccc...gcattcagctgtaggttgacagcaggtggc

Query 399 tcacctgaa..actcaaattagcgtgattgtgagctgccacgtaggcactgggaattgg
 | ||| |||| || || || || || || || || |
 Subject 582 ttttgagaaacactctgatctgtgacacccagatctacaggccgagtgacaggtgaaggt

Query 457 agccaggctcttctgcaagagcagtcagtgcgcttagctgctgagccatttctccagcctg
 ||| | | | | || || || ||
 Subject 642 gtccaccagtcagcctcttacgccccagcagccacacttggcctgtccccatcagtttaa

Query 517 atatattcctgattcctcattcctgatttgagctctgggatggccgaggttgaaaactttg
 |||| || || | | | || || | || ||
 Subject 702 ttatctctgaatttgcaaaaggacattgcgccacttgctgaagggccccctgaccaccccc

Query 577 tgttctgtaagaatcttctcgtgactcatcctgt...ggcggaggcagatcggcaaaaag
 || || || | | |||| || || | || |
 Subject 762 tctttgtcccgataactgttgcttctcttctcctgctgggctgacctgtgtgtccagctgg

Query 634 caagcggccctcttatgacagcagacccttcccctctgccatgttttctgcttctcc
 | | | | | | | || | | || |
 Subject 822 ccattcgtgccagtatagccaaacacaagttgtgctgagctctcaccctcccttctggac

Query 694 tggcaccgtttcattgaggaccacaaatgaactccgggagcatgcaatgcattcacacca
 ||| | ||| || | || | || || || ||
 Subject 882 aggcgtctgacagatgaaccccttggaaacatctgtcgatgc.....tgtttctctgtct

```

Query 754   cagcatataccacatctaggttagcaacatgccttgtggtccaggacagaagcgctgag
           ||      |      |      |      ||      ||||      ||||      |
Subject 936   acccagggctgttgtgattacaggactgtttaagcctgtaccagagatggagagttggg

Query 814   gtcagaggagaagcccatttctgcttattgtatccagtacagggaccccaataacccttgc
           ||      |      |      |      |      |      |      |      |
Subject 996   caagagggcaattgcatgagcagtgaagtagggctaagtacagaaatgtggcaagctgga

Query 874   cccacatgctgtgttaggagaaaattccggtgatcatagctataaaactctctctccctt
           |      ||      |      ||      |      |      ||      |      ||||      ||||
Subject 1056   gggaggtggtcctcaagcccaagccctgggagcacagatagcctcttaactaccta

Query 934   gacaggttgtctgaatg
           |      |      ||||      |
Subject 1116   agcttgaggtcaggagaaggctgag

```

Figure 3.15. Results of alignment of the 5'-flanking sequence of the rat ACC β gene and the human ACC β promoter sequence deposited into Genbank.

The figure shows the 5'-flanking region and first codon (Query) aligned with the human ACC β promoter (PII β) sequence (Subject; Genbank accession no. AF268378S2). Numbering indicates nucleotide positions. The sequence is numbered relative to the translation start site which is in bold. An identity of 33.55% (309 bp) was found between the two sequences.

```

Query: 1  acatttacagtcacagccgagccactggcagcttagatccgggcctcagccacagaggag 60
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 659 acatttacagtcacagccgagccactggcagcttagatccgggcctcagccacagaggag 718

Query: 61  cttgaaaaatgtcctggatacacagctgggaagccatggggaaagcaaaagtgtgaacgc 120
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 719 cttgaaaaatgtcctggatacacagctgggaagccatggggaaagcaaaagtgtgaacgc 778

Query: 121 agttgtctgaatg 133
          |
Sbjct:779 a 779

```

Figure 3.16. Results of BLASTN alignment of the 5'-RACE product with a rat ACC β promoter sequence deposited into Genbank.

The aligned sequence is taken directly from the BLASTN output and shows the 5'-RACE product (Query) aligned with the rat ACC β muscle-specific promoter sequence (Sbjct; Genbank Accession No. AY 217098)). The nucleotides in bold indicate those from the 5'-RACE product which did not align with the sequence deposited into Genbank, those underlined represent the first codon. Numbering indicates nucleotide positions.

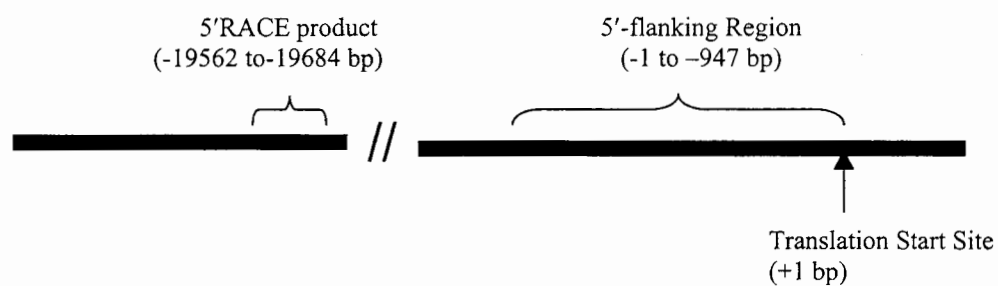


Figure 3.17. Diagram illustrating the genomic structure of the portion of rat chromosome 12 containing the ACC β gene and its regulatory sequences.

The figure shows the position of translation start site (+1 bp), the position of the 5'-flanking region isolated in this study and the position of the 5'RACE product.

3.4. Novel Rat ACC β 5'-flanking region shows Developmental Patterning of Transcriptional Regulation

Although the 5'-flanking region isolated by the Genome Walker approach was not found to contain the major promoter of ACC β in the rat myocardium as shown by analysis of the 5'-RACE product, the presence of numerous *cis*-elements indicated that this sequence was a potential regulatory element in transcription. To evaluate the capacity of this region to function as a promoter, we assessed its ability to regulate reporter gene transcription *in vitro*. No recognition sites for commonly used restriction enzymes were identified in the 947 bp sequence upstream of the translation start site of the rat ACC β gene therefore primers were designed to introduce restriction sites at the ends of the amplified PCR product.

Reporter constructs containing the entire 947 bp sequence could not be made due to the AT-rich nature (~72% AT-rich) of the 184 bp sequence at the 5'-end of the fragment, which prevented the design of workable primers from this sequence. In order to amplify this region, the primer required would have to be 40 bp long with a hybridization temperature of only 56°C, the GC-content of 29%. Hence, deletion constructs containing 764, 600 and 257 bp fragments of the 5'-flanking region linked to a luciferase reporter gene were generated and referred to as ACC764.luc, ACC600.luc and ACC257.luc, respectively (**Figure 3.18**). These constructs were designed to encompass as much of the 947 bp sequence as possible and also to identify the E-boxes and non-conventional PPREs which could regulation transcriptional changes in response to nutritional flux. ACC764.luc contained five E-boxes and two non-conventional PPREs, ACC600.luc contained four E-boxes and two non-conventional PPREs while ACC257.luc contained two E-boxes and and one non-conventional PPRE.

To determine whether the 5'-flanking region could drive transcription, protocols for transient transfection of cells were established. Initially, variations of the calcium phosphate-DNA transfection method were used, since it is relatively inexpensive. Although cells were successfully transfected using this method, there was a high level of cell death. Furthermore, cells changed morphology becoming more rounded in shape and lifting off the cell culture plates following transfection indicating that the precipitate damaged the cells (Appendix B). Consequently, a transient transfection protocol using the liposomal transfection reagent, FuGENE6 Transfection Reagent (Roche), was set up. Transient transfection experiments were conducted using the appropriate controls: an internal control was included to assess transfection efficiency and positive and negative luciferase control vectors were included to control for differences between experiments. The results presented represent normalised luciferase activity and are expressed as a percentage of the positive control vector.

Two cell lines: CV-1, a monkey kidney-derived cell line and, a rat heart-derived cell line, H9c2, immature myoblasts and differentiated myotubes were transfected with ACC764.luc (**Figure 3.19**). The different H9c2 cells were used to ascertain whether there were any differences in expression with development. The results of these experiments showed an induction of transcriptional activity in both cell lines used. Interestingly, the induction was approximately 3-fold higher in differentiated H9c2 myotubes compared to the induction observed in CV-1 cells and undifferentiated H9c2 myoblasts. These findings indicate that the 764 bp region immediately proximal to the ACC β initiator codon could drive transcription in a manner demonstrating developmental patterning.

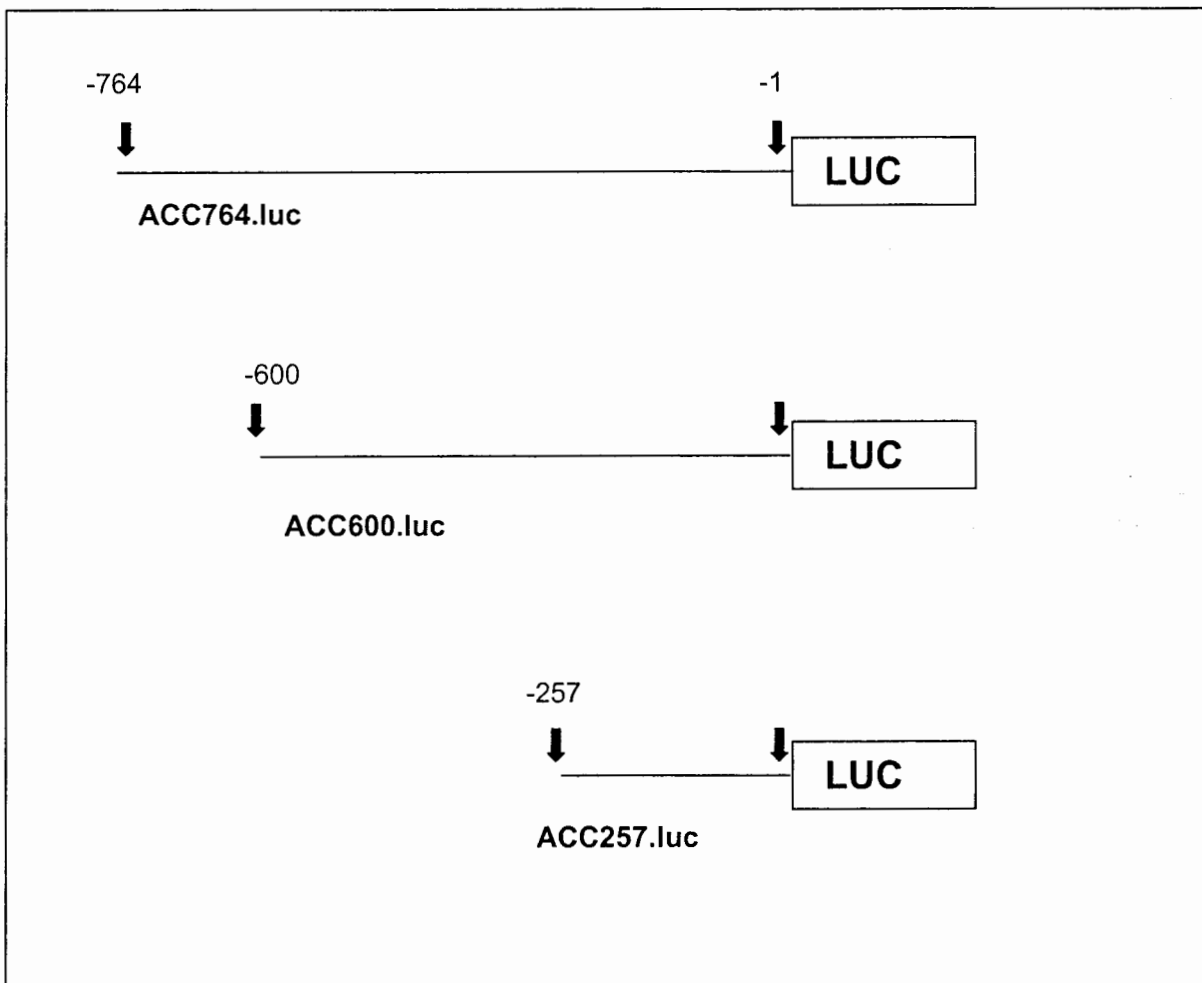


Figure 3.18. The Luciferase Reporter Constructs of 5'-flanking region of the Rat ACC β cDNA used for transient transfection.

Deletion mutants of the rat ACC β 5'-flanking region. The translation start site was considered as +1, the resultant reporter constructs were named ACC764.luc (-764 / -1), ACC600.luc (-600 / -1) and ACC257 (-257 / -1).

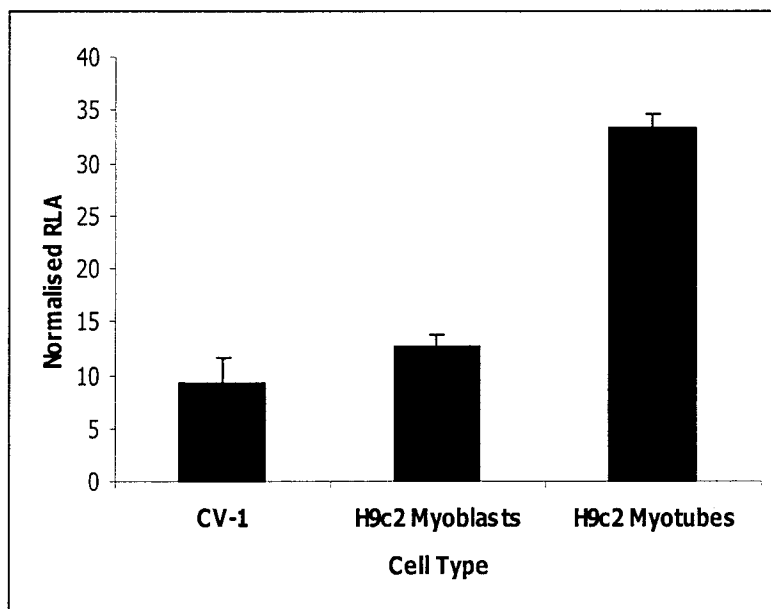


Figure 3.19. Activities of ACC764.luc in different cell types.

Results are normalised with respect to *Renilla* luciferase activity in the same sample and expressed as a percentage ($\pm$ SEM) of firefly luciferase activity obtained with the positive control vector, pGL2-Control. The figure represents the results of at least three experiments ($n= 6-12$).

Further transient transfections were carried out using three deletion constructs containing regions of the ACC β 5'-flanking region (**Figure 3.20** and **Figure 3.21**) to isolate regions controlling transcriptional activity. A similar pattern of expression was observed in all three cell types tested. The activity ($\pm$ SEM) obtained with construct ACC764.luc was highest in all cells transfected: $9.32 \pm 2.3\%$ in CV-1 cells; $12.71 \pm 1.06\%$ in H9c2 myoblasts and $33.25 \pm 1.33\%$ in H9c2 myotubes. The transcriptional activity ($\pm$ SEM) induced by ACC600.luc was the lowest of the three deletion constructs: $3.18 \pm 0.86\%$ in CV-1 cells, $4.20 \pm 0.3\%$ in H9c2 myoblasts and $11.13 \pm 1.5\%$ in H9c2 myotubes; approximately one third of the activity obtained with ACC764.luc. ACC257.luc induced intermediate transcriptional activity of: $6.38 \pm 1.92\%$ in CV-1 cells, $6.41 \pm 0.28\%$ in H9c2 myoblasts and $14.88 \pm 2.9\%$ in H9c2 myotubes. These results indicated that there was a silencer in the -600 / -258 region.

3.5. Transcription Factor Co-transfection Upregulates Transcription Driven by the ACC β 5'-flanking region

The presence of six E-boxes in the 5'-flanking region of the rat ACC β gene prompted an investigation to test the possibility that the transcription factors, USF1 and USF2a could activate the expression of a reporter gene driven by the 5'-flanking region. Co-transfection of the deletion constructs (ACC764.luc, ACC600.luc and ACC257.luc) along with USF1 and USF2a expression vectors, pUSF1 and pUSF2a, showed an induction of transcription (**Figure 3.22**). ACC600.luc and ACC257.luc were induced $4.43 (\pm 0.34)$ and $5.52 (\pm 0.34)$ -fold ($\pm$ SEM), respectively when co-transfected with the USF1 expression vector. However, the USF1-induced upregulation detected with ACC764.luc was $2.47 (\pm 0.26)$ -fold, almost half of that of the other two constructs. Similarly, co-transfection with a USF2a expression vector showed that the induction of transcription was lower with ACC764.luc than with the other two reporter constructs.

Co-transfection with the USF2a expression vector showed a greater induction of ACC β gene expression than that obtained with the USF1 expression vector; the reporter constructs, ACC764.luc, ACC600.luc and ACC.luc257 were induced $9.53 (\pm 1.92)$, $15.69 (\pm 1.94)$ and $17.26 (\pm 0.64)$ -fold ($\pm$ SEM), respectively. Co-transfecting cells with equal amounts of USF1 and USF2a expression vectors also increased gene expression. ACC764.luc, ACC600.luc and ACC.luc257 were induced $7.49 (\pm 0.86)$, $13.53 (\pm 1.51)$ and $12.84 (\pm 2.02)$ -fold ($\pm$ SEM) respectively (**Figure 3.19**). This induction was greater than co-transfection with USF1 alone, but less than the induction by USF2a alone. These results indicate that the two E-boxes at positions -231 / -226 and -71 / -66 are likely to be responsible for the increased gene expression generated by USF1 and USF2a. There also appears to be a repressor of transcription in the -764 to -601 region which suppresses the activating effect of the E-boxes in the 257 bp fragment. An unsuccessful effort was made to identify the *cis*-element/s to which these transcription factors bind using site-directed mutagenesis (**Appendix B**).

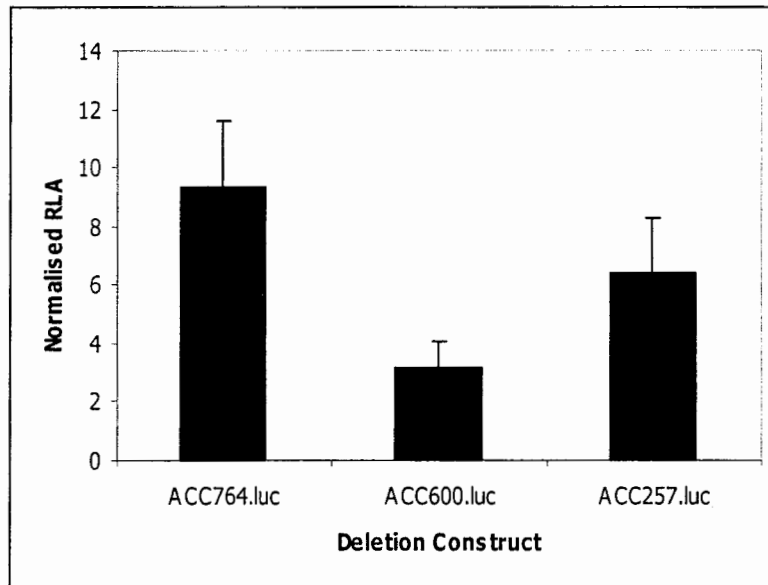
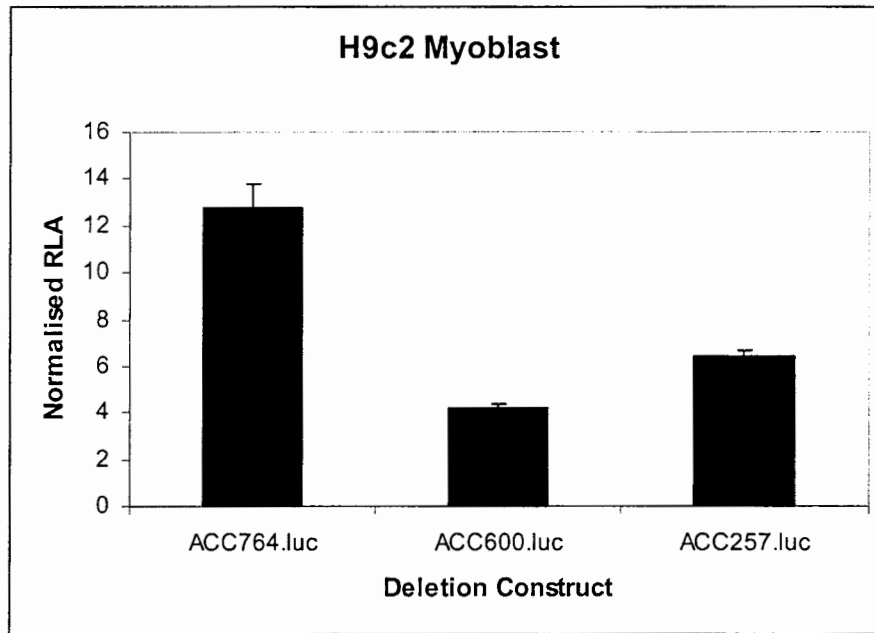
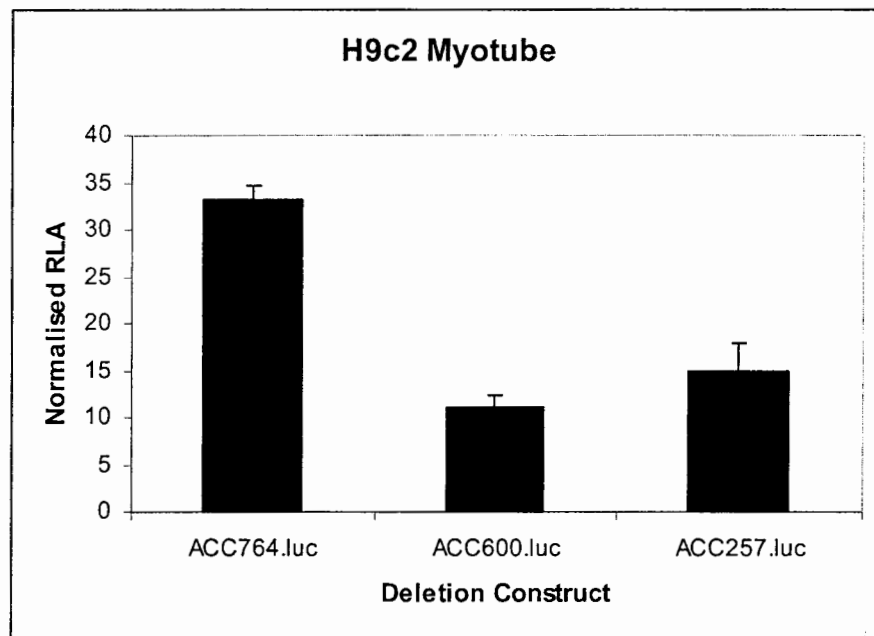


Figure 3.20. Activities of the Rat ACC β 5'-flanking region deletion constructs in CV-1 cells.

Results are normalised with respect to *Renilla* luciferase activity in the same sample and expressed as a percentage ($\pm$ SEM) of firefly luciferase activity obtained with the positive control vector, pGL2-Control ($n = 8$).



a)



b)

Figure 3.21. Activities of the Rat ACC β 5'-flanking region deletion constructs in (a) H9c2 Myoblasts (n= 12) and (b) H9c2 Myotubes (n= 8-12).

Results are normalised with respect to *Renilla* luciferase activity in the same sample and expressed as a percentage ($\pm$ SEM) of firefly luciferase activity obtained with the positive control vector, pGL2-Control.

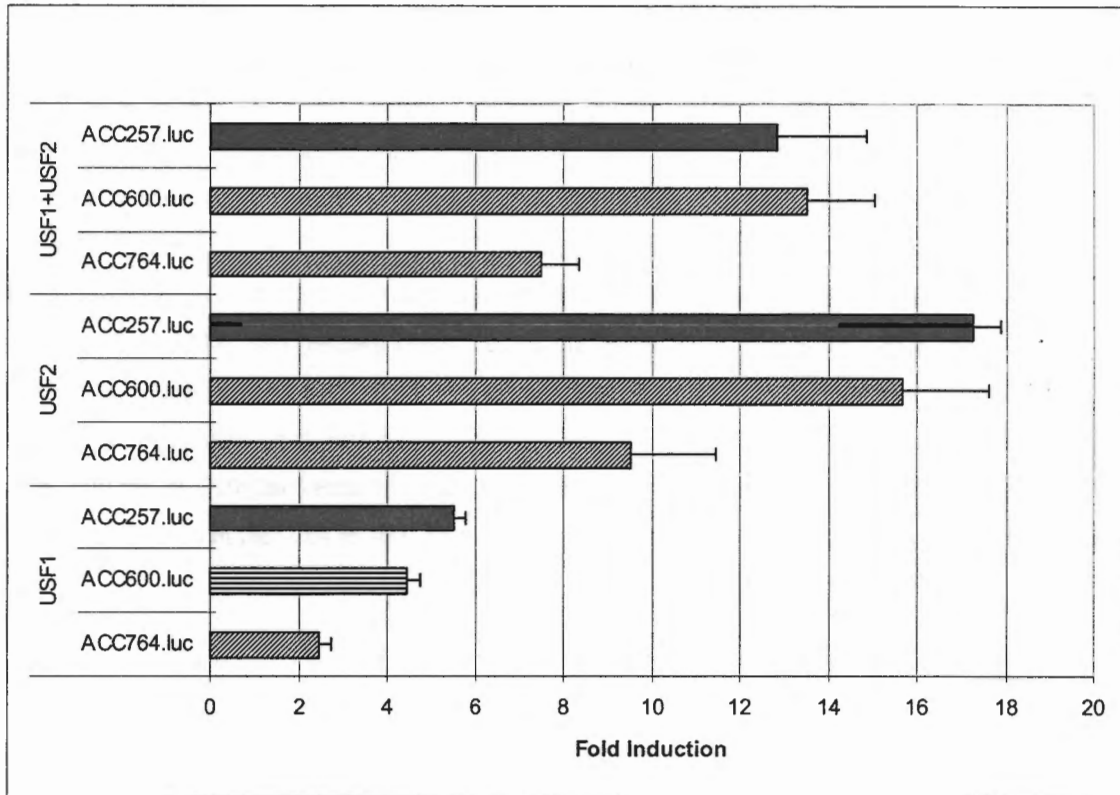


Figure 3.22. USF1 and USF2a upregulate transcription from the rat ACC β 5'-flanking region.

CV-1 cells were transfected with ACC β deletion constructs and relative luciferase activity normalised to the activity of a positive control vector, pGL2-Control. The figure shows the average fold induction ($\pm$ SEM) induced by USF1 and USF2a co-transfected with the ACC β deletion constructs, ACC764.luc, ACC600.luc and ACC257.luc. The data represent the average of three experiments (n 6).

The nucleotide sequence of the two nuclear receptor response elements (NRRE) in the ACC β 5'-flanking region indicated that these sites were putative binding sites for PPAR α and RXR α . Although these sites did not conform to the classical direct repeat of six nucleotides separated by one nucleotide, both NRREs were flanked by identical sequences on both the 3'-end (CTG) and the 5'-end (GAGGAG). Co-transfection of the deletion constructs (ACC764.luc, ACC600.luc and ACC257.luc) along with PPAR α and RXR α expression vectors, pPPAR α and pRXR α , showed an induction of transcription (Figure 3.22). The reporter constructs were induced 2.14 ($\pm$ 0.27), 5.21 ($\pm$ 1.04) and 2.63 ($\pm$ 0.22)-fold ($\pm$ SEM), respectively when co-transfected with the PPAR α expression vector (pPPAR α). Co-transfection with the RXR α expression vector (pRXR α) showed an induction of transcription with three reporter constructs ACC764.luc, ACC600.luc and ACC.luc257 of 1.91 ($\pm$ 0.19), 3.37 ($\pm$ 0.87) and 2.60 ($\pm$ 0.16)-fold ($\pm$ SEM), respectively. Cells co-transfected with equal amounts of pPPAR α and pRXR α also yielded increased gene expression. ACC764.luc, ACC600.luc and ACC.luc257 were induced 2.66 ($\pm$ 0.47), 6.46 ($\pm$ 0.47) and 3.05 ($\pm$ 0.29)-fold respectively (**Figure 3.23**). There was no indication of an additive effect when both transcription factors were used. The results indicate that PPAR α could induce transcription from the ACC600.luc vector to a greater extent than from the two other constructs. There also appears to be a repressor of transcription in the -764 to -601 region which suppresses the activating effect of PPAR α and RXR.

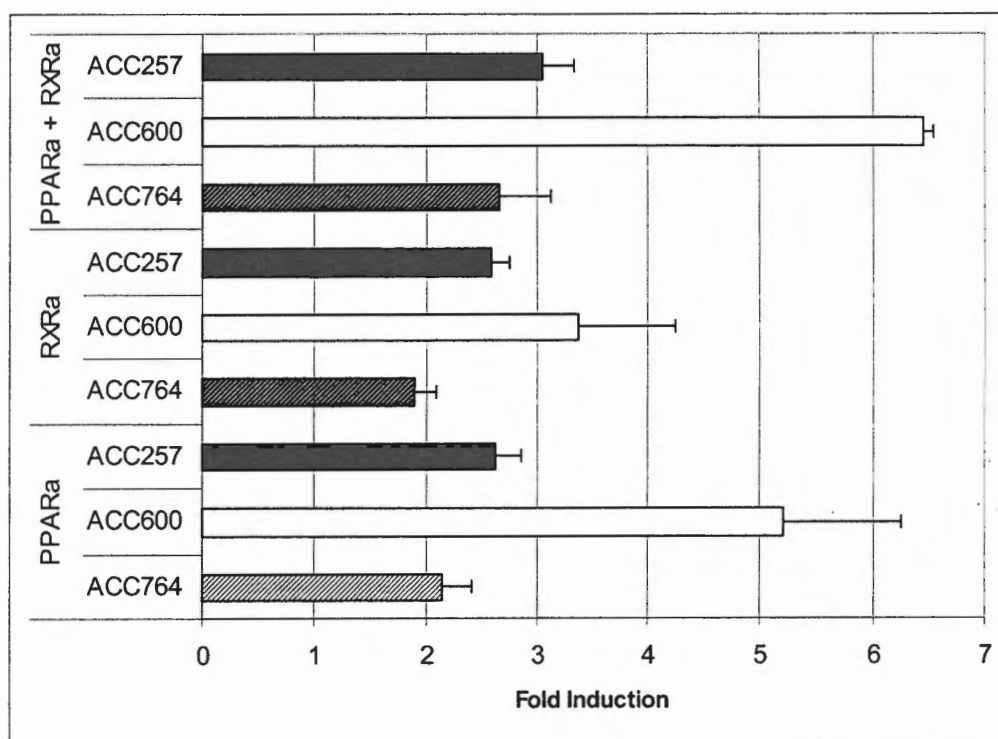


Figure 3.23. PPAR α and RXR α upregulate transcription from the rat ACC β 5'-flanking region.

CV-1 cells were transfected with ACC β deletion constructs and relative luciferase activity normalised to the activity of a positive control vector, pGL2-Control. The figure shows the average fold induction ($\pm$ SEM) induced by PPAR α and RXR α co-transfected with the ACC β deletion constructs, ACC764.luc, ACC600.luc and ACC257.luc. The data represent the average of at least three experiments (n 6).

The aim of this study was to further elucidate the regulation of ACC β gene expression in the rodent myocardium. With this intention, we have:

- 1) delineated the metabolic gene expression profile in cardiac tissue isolated from rats exposed to hypobaric hypoxia,
- 2) assessed the mRNA levels of both ACC isoforms, ACC α and ACC β , under hypobaric hypoxic conditions,
- 3) isolated and characterised a novel sequence in the 5'-flanking region of the rat ACC β gene, and
- 4) assessed the transcriptional regulatory activity of this novel sequence.

The major findings of this study are as follows:

- 1) ACC α and ACC β gene expression are similarly regulated by hypoxia and with the development of hypertrophy,
- 2) ACC gene expression is regulated in a manner analogous to the regulation of genes involved in FAO and
- 3) A novel sequence in the 5'-flanking region of the rat ACC β gene controls transcription in a developmentally regulated manner and may direct alterations in gene expression in response to changes in substrate availability.

Aberrations in cardiac metabolism have been observed in various forms of heart disease. Consequently, there has been increased interest in metabolic interventions, metabolic modulation, to improve contractile function in the diseased myocardium. An attractive prospective application of metabolic modulation is in the treatment of chronic conditions by altering the transcription of metabolic genes. To date, much of the work using metabolic modulation has revolved around reducing damage in the ischaemic and reperfused ischaemic heart [9]. These studies have revealed that inhibition of FAO during and after ischaemia reduces myocardial damage and promotes cardiac functional recovery.

Understanding molecular mechanisms inhibiting myocardial FAO are therefore of critical importance. A central step in the regulation FAO in the myocardium is catalysed by CPT 1, which controls the mitochondrial importation of fatty acids. The myocardial-enriched CPT 1 (mCPT 1) is allosterically inhibited by malonyl-CoA. The synthesis of malonyl-CoA is regulated by ACC, there are two isoforms of ACC in mammals (ACC α and ACC β) and both isoforms are expressed in the myocardium. ACC β gene expression is elevated in tissues such as cardiac and skeletal muscle, that have high energy requirements and primarily oxidise fatty acids [127] suggesting that ACC β plays a role in FAO. ACC activity in the heart has been studied under various pathophysiological and physiological conditions such as aerobic reperfusion of ischaemic hearts [170, 172], hypoxia [157] and development [167]. However, the transcriptional regulation of ACC β in the myocardium is not well understood. This study was therefore carried out in order to elucidate the transcriptional regulation of ACC β .

4.1. Characterisation of Cardiac Metabolic Gene Expression Profile in Response to Hypobaric Hypoxia

A cardiac metabolic gene expression profile in response to hypobaric hypoxia in a rodent model was constructed in order to contextualise the regulation of ACC β gene expression in terms of cardiac metabolism. Adaptation to hypobaric hypoxia imitates the acclimatisation to high altitude conditions and exposure to chronic hypoxia provides a useful animal model of the observed phenotype in patients with chronic obstructive or restrictive lung diseases and/or pulmonary hypertension. Northern blot analyses were initially unsuccessfully used to assess ACC β transcript levels, which was a significant goal of this project. The inconsistent results obtained, using this technique, were due to technical difficulties arising from the low

transcript levels in cardiac tissue. A further obstacle was the large amount of cardiac tissue required for the use of this technique when the number of research animals became a limiting factor. In light of these experimental limitations, this study was carried out in collaboration with Prof. Heinrich Taegtmeier and Dr Martin Young at the University of Texas-Houston Medical School (Houston, USA) where RT-PCR and quantitative real-time PCR were performed. Subsequent studies in this laboratory has extended this collaboration and used the results presented in this thesis as a primer for more extensive investigations into the transcriptional regulation of metabolic genes in response to hypobaria [202]. Part of this new data has been incorporated into the following discussion.

4.1.1. Development of Right Ventricular Hypertrophy and the Reactivation of the Foetal Gene Regulatory Programme

An initial response to hypobaric hypoxia is hypoxic pulmonary vasoconstriction and muscularisation of pulmonary vessels. This leads to increased afterload on the RV of the heart, pulmonary hypertension and the consequent development of right ventricular hypertrophy in order to sustain cardiac output [204]. Morphological data obtained in this study, demonstrated the development of right ventricular hypertrophy after one week of exposure hypobaria. The data concur with earlier studies using this model [179, 180].

The development of right ventricular hypertrophy in this study was confirmed by the increase in ANP gene expression in this ventricle. ANP is a cardiac hormone, which is normally expressed primarily in the atria of the adult heart while the foetal ventricle expresses higher levels of ANP mRNA and protein. This hormone is thought to regulate several processes in the embryonic myocardium including cell growth and proliferation [205]. ANP expression has been found to increase in the ventricles of the adult myocardium with the development of several forms of heart disease including the development of cardiac hypertrophy [44] and heart failure [206-208]. Moreover, ANP protein levels are elevated before the onset of morphological hypertrophy and it is therefore used as an early indicator of the development of hypertrophy [209].

ANP has come to be regarded as a marker of the foetal gene regulatory programme. This gene programme is typified by the reactivation of embryonic sarcomeric and metabolic genes observed in several forms of cardiac disease. The reactivation of foetal genes is considered to be an adaptive mechanism enabling the myocardium to adjust to changes in environment. Embryonic genes regulating cardiac fuel metabolism are also switched on with the initiation of the foetal gene programme during which there is a co-ordinate downregulation of genes regulating FAO [208]. This results in an increased dependence on glucose as the primary fuel source, a pattern of fuel selection detected in the embryonic heart. Hence the morphological data and increased ANP gene expression found in the present investigation demonstrate the development of RV hypertrophy and indicated a switch to the foetal gene expression programme in the RV of hearts subjected to hypobaric hypoxia.

4.12. Erythrocytotic Response to Hypoxia

An adaptive response to hypobaric hypoxia is the activation of the oxygen sensor, hypoxia inducible factor 1 (HIF1) [210]. HIF1 is known to upregulate more than 30 genes in response to hypoxia and has recently been implicated in post-translational modifications proteins involved in adaptation to decreased oxygen availability [211]. The production of erythropoietin is promoted by HIF1, increasing the red blood cell mass thus improving the oxygen-carrying capacity of

blood [212]. The elevated haematocrit levels detected in hypoxic animals in the present study demonstrate an appropriate erythrocytotic response to hypoxia, with the resultant increase in red blood cell mass. This occurrence increases the haemoglobin content in blood improving blood oxygen-carrying capacity and thus tissue oxygenation. This form of altitude-induced modulation of oxygen delivery to overcome hypoxia as has been previously observed in human with exposed to high altitudes [213].

The elevated haematocrit levels may serve to gradually normalise oxygen delivery and to moderate the extent of transcriptional regulation of metabolic genes in response to the limitation in oxygen availability. A more acute restriction of oxygen accessibility may bring about the activation of other mechanisms to overcome or mitigate the effects of lower oxygen delivery, such as increasing oxygen delivery by increasing blood flow, the upregulation of the anaerobic contribution to metabolism or alteration of cardiac oxidative substrate usage to generate ATP using oxygen-sparing means.

For example, a mechanism induced by HIF1 in response to hypoxia, is angiogenesis, in order to amplify blood flow and thus oxygen delivery [214]. This process is mediated by vascular endothelial growth factor (VEGF), which is a mitogen directing new blood vessel formation [215]. We did not directly inspect for the development of angiogenesis. We did, however, assess VEGF mRNA levels to examine for the presence of a regulatory mechanism controlling VEGF mRNA levels and therefore the transcriptional regulation of new vessel formation. The data obtained here suggest that if angiogenesis were detected in the myocardia of rats exposed to hypobaria, this development may not be controlled by regulatory mechanisms inducing VEGF mRNA levels as they were not activated in response to hypobaria in this model. The VEGF mRNA data support the notion that the elevated haematocrit levels normalise oxygen delivery and diminish the necessity for other adaptive responses to hypoxia such as angiogenesis at least at the transcriptional level of regulation.

4.1.3. Regulators of Metabolism

Changes in cardiac metabolism have been detected in defence against hypobaric hypoxia. Human populations such as the Himalayan Sherpas, which have resided at high altitudes for generations, have exhibited an increased preference for carbohydrates as a cardiac oxidative substrate. This preference is beneficial under these circumstances since energy synthesis using these substrates is “oxygen-sparing” relative to fatty acids [213].

In the present study, we assessed the transcript levels of two glucose transporters in the myocardium, namely GLUT1 which is considered to be the foetal isoform and GLUT4, the adult transporter. We did not detect significant changes in the transcript levels of these transporters in response to hypobaria. There was, however, a modest upregulation of GLUT1 transcript levels and a similar downregulation in GLUT4 mRNA levels in the RV after 48 hours.

These changes are modest compared to those obtained in a separate study [216] where GLUT mRNA levels were found to change significantly in response to two and 14 days of hypobaric hypoxia. Sivitz et al.(1992) found that GLUT1 mRNA levels increased 2-3 fold in both ventricles after 2 days and this increase was attenuated by 50% after 14 days. GLUT4 mRNA levels remained unchanged in the LV but decreased 74% in the RV after two days, levels returned to baseline after 14 days. The results obtained by Sivitz et al. [216] also indicated that there was a reversion to the foetal phenotype in the RV as an acute response to hypobaric hypoxia (2 days). However, after this initial development of compensatory hypertrophy in the RV, there is a return to the adult phenotype. We did not measure transcript levels at 14 days in the present study but this was done in a subsequent study carried out in this laboratory [202] which found a similar weak

transient reversion to the foetal gene expression pattern after 48 hrs followed by a return to the adult expression pattern after 7 days. A further “strengthening” of the adult gene expression pattern was detected after 14 days with an additional decrease in GLUT1 and increase in GLUT4 transcript levels. The discrepancy between the results of the present study and those of Sivitz et.al.[216] may be due to differences between the strains of rats used. Sprague–Dawley rats were used in the other investigation whereas Wistar rats were used in this investigation; other studies have also found similar regulatory patterns along with quantitative differences in the induced changes, between the two strains [217, 218].

The compensatory adaptive mechanism in response to hypobaric hypoxia also extended to protein levels as indicated by studies of GLUT proteins. Dill et.al.[219] found that cardiac GLUT1 and GLUT4 protein levels increased significantly after seven days of hypobaric hypoxia. These workers [219] found that after 4 weeks of hypobaric hypoxia, GLUT1 protein levels returned to baseline while GLUT4 protein levels remained significantly upregulated, most likely as an adaptation to chronic hypobaric hypoxia. Furthermore, there appears to be a reversion to the adult phenotype after a period of compensatory adaptation to chronic hypoxia. These results may reflect differences in GLUT regulation at the transcriptional and translational levels in response to hypobaria.

In vitro models of hypertrophy induced by pharmacological agonists demonstrated that the induction of hypertrophy was associated with increased GLUT1 gene expression [220]. Moreover, a recent report by Morissette et al. [221] proposed that an upregulation of GLUT1 was essential for the development of hypertrophy. The mechanism indicated for the development of hypertrophy was that GLUT1 also has a signalling function, independent of glucose transport, interacting with other proteins allowing for signal transduction in response to various hypertrophic stimuli [221]. Hence the moderate induction of GLUT1 observed in the RV after 48 hrs of hypobaria in this study may have been sufficient to induce hypertrophy. The reversion to the foetal gene programme associated with the development of hypertrophy is also indicated by the modest downregulation of the adult glucose transporter, GLUT4 mRNA levels in the RV of rats subjected to 48 hrs of hypobaria. These data reinforce the evidence from morphological data and increased ANP mRNA levels suggesting the reactivation of the foetal gene regulatory programme in the RV with hypobaria.

In order to assess the effects of hypobaria on the transcriptional regulation of glucose oxidation, we measured the mRNA levels of PDK4, an inhibitor of this process. Cardiac PDK4 is involved in regulating the PDH activity to changing environmental conditions, by inactivating it through phosphorylation. Furthermore, PDK4 has been associated with circumstances where FAO is chronically upregulated in muscle [187-189]. We found that PDK4 mRNA levels decreased significantly in both ventricles implying that this downregulation is brought about by a hypoxic rather than a hypertrophic stimulus due to increased load. The results of this study differ slightly from those of Sharma et.al. [202], given that they found PDK4 mRNA levels normalised after 7 days. These differences may be due to the slightly different rate of onset of the compensatory mechanism induced by hypoxia. The decreased PDK4 transcript levels concur with previous work showing a downregulation in PDK4 mRNA levels with hypoxia [222] and those of Lydell et. al. [223], who found no decrease in PDH expression or activity with hypertrophy.

The decreased cardiac PDK4 transcript levels in hypobaric hypoxia imply an increased flux through PDH allowing more pyruvate generated by glycolysis to enter the TCA cycle and the optimisation of glucose usage. These results indicate augmented carbohydrate utilisation as an oxidative substrate in the hypoxic myocardium, which is advantageous under hypoxic conditions as the yield of ATP per mole of oxygen is higher compared to the ATP derived from the FAO pathway. This effort to increase glucose oxidation may be part of an adaptive response to the early, speedy reduction in oxygen

supply. The regulatory mechanism is probably directed by PPAR α -mediated downregulation of PDK4 transcription [222] and thus reflects increased PPAR α activity.

The mRNA levels of several genes regulating FAO were also measured in order to assess the transcriptional and post-transcriptional mechanisms controlling FAO under hypobaric hypoxic conditions. The transcription factor, PPAR α and several of its target genes, namely MCD, MCAD, PDK4 and UCP3 were included in this study. No significant changes in PPAR α gene expression in response to hypobaria were detected. Subsequent studies in this laboratory expanding on this work have found evidence of regulation of PPAR α at the transcript level. An immediate downregulation of PPAR α was detected in both ventricles within 12 hours of hypoxia, but these levels returned to control levels inside of 24 hours (Essop, unpublished data). Longer exposure to hypobaria (14 days) resulted in an additional decrease in PPAR α mRNA levels in the RV but not in the LVS. Hence PPAR α gene expression appeared to be controlled by a hypoxic stimulus after very brief exposure (less than 12 hrs) to hypobaria but with prolonged exposure to this stimulus, it was the hypertrophic rather than the hypoxic stimulus, which regulated PPAR α transcript levels.

The role of PPAR α in response to hypoxia is currently unresolved. Razeghi et al. [222] found decreased PPAR α levels in response to hypoxia. However, Huss et al. [102] found that hypoxia deactivates PPAR α by reducing the availability of its obligate dimerisation partner, RXR. The results obtained using the hypobaric hypoxia model indicate that PPAR α is regulated by hypoxia.

Interestingly, UCP3 transcript levels declined significantly in both ventricles in response to hypobaria after 48 hours and the reduction was maintained after 1 week, indicating that the expression of this gene is regulated by hypoxia rather than hypertrophy. Increased UCP3 expression has been linked to chronic elevations in plasma free fatty acid levels [194], however studies in the laboratory found no significant differences in plasma glucose or non-esterified fatty acids (NEFA) with hypobaria after 48 hours and 1 week (Ngumbela, unpublished data). Hence these results suggest alternate regulation of UCP3 in the hypobaric environment. The characterisation of the minimal human UCP3 gene promoter has exposed the presence of a PPRE, which has been found to regulate UCP3 expression [224, 225]. UCP3 is considered to be a PPAR α -target gene in the myocardium [195] and these data support the downregulation of PPAR α activity in response to hypoxia despite the insignificant alteration in PPAR α transcript levels.

These results support the proposal that UCP3 is involved in FAO as a fatty acid transporter rather than in uncoupling of oxidative phosphorylation through proton translocation [191]. In light of the downregulation of UCP3 gene expression, further investigation was carried out in this laboratory to assess cardiac mitochondrial function in response to one week of hypobaric hypoxia and the role of UCP3 [226]. They found a decrease in oxygen consumption and ATP synthesis, but no change in respiratory uncoupling in reaction to hypobaria, these findings further corroborate the proposal for the involvement of UCP3 in the regulation of FAO rather than in uncoupling. If UCP3 is indeed a fatty acid anion transporter, the significant downregulation in its gene expression under hypoxic conditions would favour carbohydrate utilisation over FA.

In summary, there was a co-ordinated downward trend in transcript levels of other PPAR-target genes namely, MCD, MCAD and significant downregulation in PDK4 and UCP3, in both ventricles in response to hypoxia for one week. These proteins are all regulators of FAO and therefore the decreased expression was indicative of diminished FAO in response to hypoxia as observed in other studies [222]. Therefore, we suggest that these results reflect an attempt by the heart to

increase glucose oxidation and decrease FAO as part of an adaptive response to hypobaria as glucose is a more oxygen-efficient fuel.

4.1.4. Summary of Adaptive Responses to Hypobaric Hypoxia

Substrate utilisation by the heart is dynamic and changes along with developmental status, substrate supply and pathophysiologic states [123]. The changes in fuel substrate selection underline the adaptable nature of the mammalian heart. This trait enables it to select specific substrates at different stages of development and also gives emphasis to the complex, multiple regulatory steps required to rapidly and efficiently switch from one fuel substrate to another.

Although the results of the present study were not conclusive, the subsequent study in this laboratory [202], confirmed that the trends in metabolic gene regulation were indeed significant. The failure to obtain significant changes in gene expression in this study may be attributed to the low number of animals used in RT and quantitative PCR experiments by our collaborators. Therefore results of this work in conjunction with further work in this laboratory [202] indicate that this model of hypobaric hypoxia activates an adaptive form of hypertrophy [227]. However, this study only measured expression up until day 7 where the results suggested a switch to the foetal gene expression pattern in the RV. These results together with those of Sharma et. al. [202], indicate that after an initial adaptive period at day 2, followed by a return to the adult gene expression pattern with continued exposure to hypobaria at day 14. These adaptations resemble the adaptive mechanisms described in populations that have resided at high altitudes for generations and most likely used by the myocardium during the early stages of disorders such as chronic lung disease and pulmonary hypertension to adjust to the limited oxygen availability [176, 177].

The results indicate that three compensatory mechanisms were activated to enable the rat myocardium to adjust to chronic hypobaria:

- 1) hypertrophic response in the RV to adapt to increased afterload,
- 2) erythrocytotic response to increase oxygen delivery to the myocardium and
- 3) a switch to oxygen-sparing fuel substrates

To summarise the characterisation of cardiac metabolic gene expression pattern in response to hypobaric hypoxia, we have:

- 1) **Established that RV hypertrophy is accompanied by a reversion to the foetal pattern of gene expression after one week of exposure to hypobaric hypoxia.**

This switch in gene expression pattern has been observed in other models of hypertrophy [208].

- 2) **Delineated a cardiac metabolic gene expression profile in response to hypobaric hypoxia.**

There is a downward trend in the expression of FAO genes in both ventricles indicative of decreased FAO. This would suggest increased cardiac glucose utilisation, an adaptive mechanism against hypobaric hypoxia [176].

4.1.5. Analogous Regulation of ACC Isoform Gene Expression

One of the principal aims of characterising the cardiac metabolic gene expression profile of the rat myocardium in response to hypobaric hypoxia was to contextualise the expression pattern of ACC β in the framework of cardiac metabolism, in order to further investigate its function/s. Contrary to our hypothesis, ACC α and ACC β gene expression were downregulated in both ventricles under hypobaric hypoxic and hypertrophic conditions, indicating that the isoforms are alike in their regulation of FAO under these circumstances. These results were also unexpected as ACC is thought to downregulate cardiac FAO and therefore under conditions associated with decreased FAO, such as hypoxia, elevated ACC mRNA levels were anticipated.

Although we did not measure ACC protein and activity levels in this study, we speculated that the diminished ACC mRNA levels may point to an alternate role for ACC in the myocardium other than regulating mCPT 1 activity, especially ACC β , which is the predominant isoform in the myocardium. *In vitro* studies by Lee and Kim [228] in the rat cardiac-derived H9c2 cell line support an alternate role for ACC β . Their findings indicate that ACC β is not directly involved in the regulation of FAO by means of regulating mCPT 1 via the synthesis of malonyl-CoA. Rather, they proposed that it plays a role in myogenesis, myoblast differentiation and maturation, processes involving a switch in fuel substrate preference by the myocardium. Hence the function of ACC β appears to be in the regulation of energy homeostasis or the maintenance of the adult muscle phenotype [76, 174].

Interestingly, cardiac ACC β and UCP3 mRNA are significantly diminished in response to hypoxia. Investigation of ACC β -null mice indicated that cardiac UCP3 transcript levels were normal under normoxic and standard dietary conditions [154]. The results of this study suggest that the hypoxia-induced stress activates a common mechanism, which brings about a reduction in the transcript levels of both genes.

The precise function/s and mechanism of ACC β action therefore remain unclear, however data from various sources have lead to conjecture that this protein may have a signalling function. Studies in this laboratory have shown that cardiac ACC β gene expression is upregulated in response to a carbohydrate-enriched diet [229] and ACC β transcript and protein levels were also elevated in the murine myocardium in response to fasting [230] but no corresponding increase in ACC activity was detected. Furthermore, ACC β has been implicated in direct interaction with other proteins [231] and its localisation to the outer mitochondrial membrane [133] suggesting that ACC β acts as a signalling protein transmitting signals between the mitochondrion and cytosol in response to changes in substrate availability [174].

GLUT1, another transmembranal protein involved in metabolism, has recently been suggested to have a signalling function during the development of hypertrophy [221]. This proposal was made when it was found that *in vitro* induction of hypertrophy required an upregulation of GLUT1 transcript and protein levels, however no elevation in GLUT1 activity could be detected. GLUT1 has also been implicated in protein-protein interactions [232, 233]. The similarities between ACC β regulation in the adaptation to fasting [230] and GLUT1 regulation during the development of hypertrophy [220] support the emerging concept of the evolution of signalling roles for metabolic proteins functioning at membrane interfaces separating cellular and sub-cellular compartments.

Thus, our results question our hypothesis proposing the distinct regulation of ACC isoform gene expression and indicate that these proteins perform similar functions under hypoxic conditions. Surprisingly, transcriptional regulation of both

ACC isoforms is analogous to that of genes involved in FAO. We speculate that ACC β has a signalling role functioning as a conduit for signals at the interface between the mitochondrion and cytosol. Future studies using techniques such as the yeast-two hybrid system to detect protein-protein interactions should lead to the verification of this suggestion through the identification of proteins with which ACC β may interact.

4.2. The 5'-Flanking Region of the Rat ACC β Gene Contains Several Promoter Elements and Induces Transcription

The counterintuitive regulation of the rat ACC, especially ACC β , mRNA levels observed in response to hypobaric hypoxia prompted further investigation of the transcriptional regulation of this gene. In order to gain insight into the transcriptional regulation of ACC β , we attempted to isolate the ACC β gene promoter in the myocardium. We isolated a 947 bp fragment in the 5'-flanking region of the rat ACC β gene and were recently able to align this sequence with part of chromosome 12 of the rat genome.

A search for *cis*-acting DNA elements within the sequence identified putative elements implicated in basal gene expression. Two TATA boxes and three CAAT boxes were identified, these elements are generally part of the basal transcriptional machinery. The presence of a TATA box at position -34 / -30 and a CAAT box at position -87 / -83 relative to the adenine of the initiator ATG codon suggested that this region was a potential core promoter sequence. Sequence analysis also revealed multiple putative transcription factor binding sites associated with metabolic gene regulation. Six E-boxes were found in this sequence, these are binding sites for basic helix-loop-helix (bHLH) transcription factors. Five Sp1 elements were also found, these are binding sites for members of the Sp family of transcription of these response elements indicated that the 947 bp sequence contained a putative promoter region involved in the transcriptional regulation of a gene encoding a protein involved in metabolism. There was a low sequence identity between the 947 bp sequence and the functional human ACC β promoter sequence (Genbank accession No. AF268378S2), but certain response elements such as multiple E-boxes were present in both sequences. E-boxes had been implicated in the regulation of the ACC β promoter [173].

The results of 5'-RACE analyses demonstrated that the flanking region immediately proximal to the initiator codon of the rat ACC β protein does not contain the predominant promoter of this gene in the myocardium. In retrospect, the method used in the attempt to obtain the rat ACC β promoter through the isolation of the sequence immediately upstream of the initiator codon was not the best approach we could have employed. A better way would have been to use 5'-RACE to isolate the gene promoter rather than employing Genome Walker technology. We isolated the predominant ACC β promoter in the rat myocardium using 5'-RACE analyses, this sequence was subsequently been deposited into Genbank as the rat ACC β muscle-specific gene promoter (Genbank accession No. AY 21798) by another investigative group in January 2003. To date this group has not published data regarding the transcriptional activity of this putative promoter or the putative rat liver-specific ACC β promoter sequence (Genbank accession No. AY 21799).

Recent acceleration in the quantity of data released by the Rat Genome Sequencing Project Consortium (http://rgd.mcw.edu/sequences/rgp_info.html) led to the entry of the *Rattus norvegicus* chromosome 12 WGS supercontig (Genbank accession no. NW_047377) into Genbank on 23 October 2003. Consequently, we were able to map the ACC β gene and its two putative promoters to chromosome 12. BLASTN analyses indicated that the putative rat muscle-specific

promoter situated 19,540 bp upstream of the first codon. A similar result was recently obtained with the characterisation of the human ACC α gene; in that case the gene promoters were also located about 19.5 kb upstream of the coding [152].

The large 5'-flanking region between the transcription and translation start sites is thought to be indicative of elaborate regulation of gene expression and post-transcriptional processes. These findings highlight the importance of regulating FA levels in the cell via ACC β as has been observed with the complex control of ACC α at all levels of regulation. This complexity is necessitated by the multiple roles, which FAs play in the cell. In addition to providing an important oxidative fuel substrate FAs have other functions such as structural roles, primarily as the hydrophobic constituent of membranes and signaling regulatory functions. For example, FAs are known to regulate gene expression, particularly that of proteins involved in FA transport and metabolism [235-237]. The tight indirect regulation of FA levels via the regulation of malonyl-CoA by means of ACC-isoforms and MCD, as well as the regulation of these enzymes, is therefore of critical importance especially in highly oxidative tissues such as skeletal and cardiac muscle.

4.2.1. The Rat ACC β 5'-flanking Region Induces Transcription *in vitro* and Demonstrates Developmental Patterning

Although 5'-RACE analysis demonstrated that the 5'-flanking region isolated in this study was not the predominant promoter of the rat ACC β gene in muscle, the sequence contained numerous promoter elements involved in the regulation of cardiac genes suggesting that it could regulate transcription. Furthermore, the possibility that this region was involved in the regulation of ACC β transcription could not be excluded as data obtained to date have indicated that the regulation of ACC isoforms is complex.

Most of the existing information regarding the regulation of ACC relates to ACC α . Multiple promoters of the ACC α gene have been identified in the rat [144], bovine [151] and human [152]. Characterisation of the rat and human ACC α transcripts has revealed several mRNA variants resulting from multiple gene promoters and differential splicing [145, 152]. The rat ACC α promoters have been best characterised, one of these promoters is constitutively active in all tissues and not affected by changing environmental conditions. The second promoter exhibited variations in tissue expression and was activated by external stimuli demonstrating rigorous regulation of the expression of this gene with changing environmental conditions. Splicing of the transcripts derived from these promoters also varied suggesting dissimilar post-transcriptional regulation.

Recent data regarding ACC β gene expression also indicates an equally complex system regulating gene expression of this isoform. Two ACC β gene promoters have been discovered in the human, one has been identified and characterised in muscle and the liver [76, 173]. Therefore, in order to determine whether the 5'-flanking region of the rat ACC β had the capacity to drive transcription, reporter gene constructs containing fragments of the 5'-flanking region upstream of a reporter luciferase gene were generated and used to transiently transfect cells.

We could not construct reporter plasmids spanning the entire 947 bp fragment upstream of the rat ACC β initiation codon due to the AT-rich (~72%) nature of the 183 bp at the 5'-end of the fragment. This feature had also hampered the sequencing of the entire 5'-flanking region obtained using the Genome Walker approach and in this instance prevented the design of workable primers for PCR-amplification of this region. In addition, the lack of common restriction enzyme recognition sites in the 183 bp sequence did not permit the isolation of a fragment containing this sequence by digestion

for further use in the assembly of a reporter construct. Furthermore, AT-rich sequences have been associated with strong base-unpairing properties, a feature linked to strong promoter activity [234]. This was a major setback in this study as the fragment contained a TATA box, a CAAT box as well as an Sp1 binding site, which could all form a core promoter. Consequently, the transcriptional activity regulated by the entire 947 bp sequence isolated in this study could not be delineated.

Two cell lines (CV-1 and H9c2) were transfected to determine whether there was tissue-specific transcriptional regulation. Furthermore, undifferentiated H9c2 myoblasts and differentiated myotubes were transfected to identify variation in transcriptional activity with development. H9c2 cells have been found to exhibit both skeletal and cardiac muscle features (i.e. expresses both skeletal and cardiac isoforms of some proteins) [238] and therefore the transcriptional upregulation in H9c2 myotubes indicates that this sequence may regulate gene expression in mature myocytes, but it cannot be categorically stated that this regulation is specific to cardiomyocytes.

The results obtained demonstrated that the 764 bp region immediately upstream of the rat ACC β initiation codon was able to drive transcription of a reporter gene *in vitro* and was therefore by definition a core promoter [31]. Furthermore, the sequence was able to induce levels of reporter activity in H9c2 myotubes, which were three-fold higher than the levels obtained in myoblasts. These results showed that this 5'-flanking region was preferentially active in mature myocytes and pointed to the presence of endogenous factors in mature myocytes, which were responsible for driving transcription from the 5'-flanking region. This indicated that this region was involved in developmental patterning.

The pattern of expression was similar to the endogenous ACC β expression pattern obtained by Kim and co-workers [231] in H9c2 myoblast and myotubes indicative of ACC β involvement in myogenesis. This process is accompanied by a switch in gene expression from the foetal to adult expression programme and a resultant change in substrate preference from glucose to fatty acids. The switch in substrate utilisation is also considered to be part of an adaptive mechanism in response to changing oxygen availability during the postnatal period, when there is a shift from hypoxic foetal conditions to normoxic postnatal conditions [167]. Taken together the higher levels of transcription induced by ACC764.luc in H9c2 myotubes suggest that the 764 bp fragment is a gene regulatory sequence involved in controlling gene expression during myogenesis.

Analysis of gene expression using the three deletion constructs, ACC764.luc, ACC600.luc and ACC257.luc, revealed a similar pattern of expression for the three constructs in the three cell types tested. The ACC257.luc containing 257 bp directly upstream of the initiator codon was able to drive transcription *in vitro* and the core promoter is contained in this region. The ACC600.luc construct consistently showed the lowest capacity to drive transcription in the three cell types tested suggesting that there was a silencer in the -600 / -258 region. Two putative response elements, AP-1 site and E-box were identified in this region. Both these elements have been implicated in the repression of gene expression and further work is required to identify the repressor *cis*-element in this region.

4.2.2. Transcription Factors Involved in the Regulation of Metabolic Gene Expression Enhance Transcriptional Activity from the 5'-flanking Region

In the present study, we found that transcription factors involved in the regulation of cardiac metabolic genes could induce transcription from the ACC β 5'-flanking region. The presence of six E-boxes in the 947 bp region upstream of the rat

ACC β translated region implied that there was a regulatory role for these elements in the transcription induced by this sequence. In order to determine the involvement of USFs in the regulation of transcription induced from the 5'-flanking region of the rat ACC β gene, CV-1 cells were co-transfected with luciferase constructs containing segments of the 5'-flanking region along with expression vectors overexpression USF1 and USF2a. USF1 and USF2a were found to induce transcription from the 5'-flanking region. The rank order of regulation of ACC β by USF proteins, from the highest to the lowest level, was USF2a, USF1/USF2a and USF1. Since USFs bind DNA as homo- or heterodimers of USF1 and USF2a, the results demonstrated that USF2a homodimers upregulate transcription to a greater extent than USF1/USF2a heterodimers and USF1 homodimers. USF2 has been found to physically interact with PGC-1 and in this way regulates the PPAR α -mediated transcription of mCTP 1 [62]. USF2 is thought to play a more important role in cardiac metabolic gene expression than USF1 [62].

The co-transfection experiments revealed that the induction of gene expression detected in cells co-transfected with USF expression vectors and the luciferase construct containing 764 bp of the rat ACC β 5'-flanking region was only about 50% of the induction obtained in cells co-transfected with reporter constructs containing 600 and 257 bp regions of the 5'-flanking region. This result points to the presence of a repressor of USF induction in the -764/-601 bp region relative to the translation start site. Moreover, the region involved in the USF-induced gene expression was narrowed down to the 257 bp region upstream of the translation start site, there are two E-boxes and a degenerate E-box in this region.

In order to fully delineate the regulation of transcriptional activity from a promoter region, both the *cis*- and *trans*-acting elements involved must be identified and the interaction of these factors must be established. Additional work using techniques such as site-directed mutagenesis and electrophoretic mobility shift assays is required to identify the region involved in USF binding and transcriptional regulation as well as the co-activators required for USF-induction of gene expression. We attempted to carry out site-directed mutagenesis in order to determine which *cis*-acting factors in the ACC β 5'-flanking region were involved in USF-induced gene expression but technical difficulties and time constraints did not allow for the optimisation of experimental conditions (Appendix B). Consequently, the induction of transcriptional activity by USFs in this study was not definitively established.

Both nuclear receptors, PPAR α and RXR α , increased transcription driven by the rat ACC β 5'-flanking region indicating that the 764 bp region contained in these vectors may be involved in the regulation of metabolic gene expression. Co-transfection of both nuclear receptors simultaneously did not demonstrate an additive effect on the induction of transcription signifying that these factors mediate transcription via the same *cis*-element. Future co-transfection experiments using ligands for specific for PPAR α and RXR α such as WY14643 and phytanic acid, respectively, will show if either or both of these NRs are able to mediate transcription from this sequence.

PPAR α was able to induce transcription from the ACC600.luc vector to a greater extent than from ACC764.luc and ACC257.luc, which contain two and one NRREs, respectively. However, the large SEM values obtained, even though the results represent least three independent experiments, in which each construct was tested in duplicate, were large. Therefore, although a definitive deduction cannot be made from these results, they do suggest that transcription factor binding site involved in the induction of transcription by PPAR α and RXR α is present in the 257 bp region contained in ACC257.luc. Furthermore these results suggest that elements in the -258 /- 600 bp region allows for greater upregulation of transcription by PPAR α and that *trans*-elements in the -764 to -601 bp region inhibited this upregulation. We did not carry out techniques such as site directed mutagenesis or mobility shift assays to determine *cis*- and *trans*-element

interactions, we can therefore only speculate at the mechanism of USF, PPAR α and RXR α mediation of transcription in this case. The NR may be able to act directly with the irregular PPREs contained in the reporter constructs. Conversely, the absence of a conventional PPRE in the 5'-flanking region also suggests that the NRs may act indirectly in transcriptional regulation. For example, Kim et. al. [174] found that transcription from human ACC β gene promoter in myocytes was mediated by MyoD and MRF4 involved an upstream mechanism directed by the NRs, RXR α and RAR α .

The results of co-transfection experiments showed that ACC600.luc was the reporter construct most responsive to the overexpression of transcription factors. This could indicate a common transcriptional mechanism, which is activated by USFs, as well as by PPAR α and RXR α . Such a mechanism would entail the presence of an enhancer or silencer in the -600 to -158 bp region, which is activated or repressed, by endogenous factors, resulting in increased activation from this region.

The presence of numerous consensus sequences for the binding of transcription factors in the rat ACC β 5'-flanking region, as well as the finding suggesting that this region interacts with transcription factors involved in metabolism suggest that this region plays a regulatory role in metabolic gene expression in reaction to nutritional flux. Another putative regulatory role for this region is at the post-transcriptional level of regulation. Evidence is emerging that transcription factors play multiple roles acting at different levels of regulation controlling the activity of gene expression. Transcription factors have been found to bind RNA transcripts and in this way regulate the export of these transcripts from the nucleus into the cytoplasm [239]. The involvement of transcription factors in the control of translation has also been established [240]. Consequently regulation of metabolic genes by transcription factors at the post-transcriptional level may add a further level of complexity to what is already known about the control of cardiac metabolism. This is an aspect of cardiac metabolic regulation, which as yet has not received attention and future investigations into this area should prove interesting.

The findings of this investigation into the transcriptional regulation of the rat ACC β gene can be summarised as follows:-

- 1) We have isolated and characterised the 947 bp sequence in the 5'-flanking region of the rat ACC β gene, immediately proximal to the initiator codon,
- 2) Established that this sequence is not the predominant ACC β gene promoter in the myocardium but was nonetheless the 764 bp sequence immediately proximal to the translation initiation site is able to drive gene expression in a manner demonstrating developmental patterning. The highest gene expression levels were detected in mature myocytes suggesting that factors driving myogenesis may regulate transcriptional activity from this sequence,
- 3) Ascertained that the 257 bp region upstream of the initiator codon contains a putative core promoter, and
- 4) Determined the ability of transcription factors known to be involved in the regulation of metabolic gene expression, namely, USFs and the nuclear receptors (PPAR α and RXR α), to induce transcriptional activity from the 5'-flanking region suggesting that this sequence may be control a transcriptional mechanism regulated by changes nutritional flux.

4.3. Study Limitations

There were several limitations encountered during this study. It was assumed that both ventricles of the heart were exposed to equal degrees of hypoxia and that effects of reduced oxygen delivery to the ventricles were the same, in the hypobaric model used. This may not have been the case and consequently some of the differences in gene expression

patterns observed in the two ventricles may reflect the level of hypoxia to which each ventricle was exposed and the resultant extent of adaptation.

Furthermore, we did not assess ventricular function and have used morphological and gene expression data to deduce that the right ventricular hypertrophy was not maladaptive. This is an area of ongoing research in this laboratory. Initial Langendorff perfusion experiments on the hearts of rats exposed to hypobaric hypoxia have found enhanced right ventricular function, proposed to result from increased RV mass (Ngumbela, unpublished data).

We did not assess protein levels and enzyme activities in studies using the rodent hypobaric model. As a result, we have not established whether the changes in transcript levels are translated into corresponding changes in protein levels and enzyme activity. Other studies using this model [216, 219] have shown that there is a co-ordinate regulation of cardiac metabolic gene expression and protein levels in response to hypobaric conditions. Future work in this area, with the construction of metabolic profiles should ascertain whether a correlation between metabolic gene transcript levels, protein levels and enzyme activity exists.

Although this study did not examine for the effects of neuroendocrinal stimulation, previous studies have shown that the metabolically active cardiac tissue is very sensitive to acute incidents of hypoxia. [241]. One such effect is elevated adrenergic stimulation and increased heart rate [242]. The method used to euthanise animals, by CO₂ asphyxiation, may have also affected gene expression may as CO₂ has been found to depress systolic blood pressure and may also elicit a neuroendocrinal response which could affect gene expression [243].

We could not construct reporter plasmids containing the entire 947 bp fragment upstream of the rat ACC β initiation codon as a result of technical problems, and were therefore unable to ascertain whether the entire 947 bp sequence in the 5'-flanking region of the rat ACC β gene is involved in regulating the expression of this gene. We cannot exclude the possibility that this sequence does not regulate ACC β gene expression at all. Emerging data from various genome projects show the complexity of the arrangement of regulatory and structural components of genes. An example is the recent report providing the first confirmation of a promoter of one gene positioned in the intron of another [244]. Also, although the presence of elements enhancing and repressing transcription driven by the 5'-flanking region has been established, the *cis*-elements and *trans*-elements involved in these processes remain to be identified and verified.

In this study, we hypothesised that **the cardiac enriched isoform of acetyl-CoA carboxylase (ACCB) is distinct in its regulatory control of cardiac fatty acid oxidation (FAO) compared to the acetyl-CoA carboxylase α -isoform (ACCA) and that evaluating the control of ACCB would provide further insights into its regulatory role in cardiac metabolic remodelling.** The results of this work have questioned this hypothesis, we found that the gene expression of ACC α and ACC β are similarly regulated in response to hypobaric hypoxia suggesting that the isoforms are analogous in their regulation of FAO. We speculate that ACC β may play a signalling role conducting messages between the cytosol and mitochondrion. The exact role/s of ACC β in cardiac FAO remains elusive and is the focus of ongoing research.

In order to further explore the regulation of ACC β gene expression and to obtain further knowledge into its regulatory role in cardiac metabolism, we isolated a 947 bp sequence in the 5'-flanking region of the rat ACC β gene, immediately proximal to the translation start site. Investigation of the 5'-flanking region revealed a core promoter sequence in the 257 bp region immediately upstream of the initiator codon. This sequence, however, was not the predominant promoter of the rat ACC β gene but is nevertheless able to induce reporter gene expression in a pattern analogous to that of ACC β in myocytes with development, suggesting that this sequence may play a role in regulating ACC β expression during myogenesis, further investigation is required to confirm or refute this proposal and to delineate the putative underlying transcriptional mechanisms. Another possible role for this region may be in the post-transcriptional regulation of ACC β , as there is evidence of transcription factors interacting with gene transcripts to regulate post-transcriptional processes.

This study has focused on the regulation of cardiac metabolism at the transcriptional level since it is at this stage that critical changes involved in the development of chronic cardiovascular diseases occur. Consequently, the manipulation of cardiac metabolic gene expression presents an innovative target in the implementation of metabolic modulation, i.e. preventing metabolic remodelling. The results of this study indicate that the use of metabolic modulation at the transcriptional level may be more complex than previously envisaged. This is mainly as a result of poor understanding of the function/s of metabolic enzymes as has recently been demonstrated by evidence supporting a signalling role for GLUT1 and the unexpected transcriptional regulation of ACC β observed during this investigation. Hence much work remains to determine the exact function/s of metabolic molecules so as to better define targets for therapeutic intervention.

REFERENCES

1. Gilbert, F.S. and S.R. Singer, *Developmental Biology*. 2003, Sunderland: Sinauer Associates Inc.
2. Taegtmeyer, H., *Metabolism--the lost child of cardiology*. J Am Coll Cardiol, 2000. **36**(4): p. 1386-8.
3. Langendorff, O., *Untersuchungen am uberlebenden Säugethierherzen*. Arch Ges Physiol Menschen Tiere, 1895. **61**: p. 291-332.
4. Opie, L.H., *Metabolism of the heart in health and disease. I*. Am Heart J, 1968. **76**(5): p. 685-98.
5. Shipp, J.C., L.H. Opie, and D. Challoner, *Fatty acid and glucose metabolism in the perfused heart*. Nature, 1961. **189**: p. 1018-9.
6. Randle, P.J., et al., *The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus*. Lancet, 1963. **1**: p. 785-9.
7. Neely, J.R. and H.E. Morgan, *Relationship between carbohydrate and lipid metabolism and the energy balance of heart muscle*. Ann Rev Physiol, 1974. **36**: p. 413-459.
8. Watkins, H., *Genetic clues to disease pathways in hypertrophic and dilated cardiomyopathies*. Circulation, 2003. **107**(10): p. 1344-6.
9. Stanley, W.C., et al., *Regulation of myocardial carbohydrate metabolism under normal and ischaemic conditions. Potential for pharmacological interventions*. Cardiovasc Res, 1997. **33**(2): p. 243-57.
10. Opie, L.H., *The Glucose Hypothesis: Relation to acute myocardial ischaemia*. J Mol Cell Cardiol, 1970. **1**: p. 107-115.
11. De Leiris, J., L.H. Opie, and W.F. Lubbe, *Effects of free fatty acid and enzyme release in experimental glucose on myocardial infarction*. Nature, 1975. **253**(5494): p. 746-7.
12. Oliver, M.F., V.A. Kurien, and T.W. Greenwood, *Relation between serum-free-fatty acids and arrhythmias and death after acute myocardial infarction*. Lancet, 1968. **1**(7545): p. 710-4.
13. Bricknell, O.L. and L.H. Opie, *Effects of substrates on tissue metabolic changes in the isolated rat heart during underperfusion and on release of lactate dehydrogenase and arrhythmias during reperfusion*. Circ Res, 1978. **43**(1): p. 102-15.
14. Bricknell, O.L., P.S. Daries, and L.H. Opie, *A relationship between adenosine triphosphate, glycolysis and ischaemic contracture in the isolated rat heart*. J Mol Cell Cardiol, 1981. **13**(10): p. 941-5.
15. Lopaschuk, G.D., R.B. Wambolt, and R.L. Barr, *An imbalance between glycolysis and glucose oxidation is a possible explanation for the detrimental effects of high levels of fatty acids during aerobic reperfusion of ischemic hearts*. J Pharmacol Exp Ther, 1993. **264**(1): p. 135-44.
16. Katz, E.B., et al., *Cardiac and adipose tissue abnormalities but not diabetes in mice deficient in GLUT4*. Nature, 1995. **377**(6545): p. 151-5.
17. Liao, R., et al., *Cardiac-specific overexpression of GLUT1 prevents the development of heart failure attributable to pressure overload in mice*. Circulation, 2002. **106**(16): p. 2125-31.
18. Abel, E.D., et al., *Cardiac hypertrophy with preserved contractile function after selective deletion of GLUT4 from the heart*. J Clin Invest, 1999. **104**(12): p. 1703-14.
19. Finck, B.N. and D.P. Kelly, *Peroxisome proliferator-activated receptor alpha (PPARalpha) signaling in the gene regulatory control of energy metabolism in the normal and diseased heart*. J Mol Cell Cardiol, 2002. **34**(10): p. 1249-57.
20. Blair, E., et al., *Mutations in the gamma(2) subunit of AMP-activated protein kinase cause familial hypertrophic cardiomyopathy: evidence for the central role of energy compromise in disease pathogenesis*. Hum Mol Genet, 2001. **10**(11): p. 1215-20.
21. Hasenfuss, G., et al., *Influence of pyruvate on contractile performance and Ca(2+) cycling in isolated failing human myocardium*. Circulation, 2002. **105**(2): p. 194-9.
22. Bristow, M., *Etomoxir: a new approach to treatment of chronic heart failure*. Lancet, 2000. **356**(9242): p. 1621-2.
23. Frey, N. and E.N. Olson, *Modulating cardiac hypertrophy by manipulating myocardial lipid metabolism?* Circulation, 2002. **105**(10): p. 1152-4.
24. Taegtmeyer, H., *Switching metabolic genes to build a better heart*. Circulation, 2002. **106**(16): p. 2043-5.
25. Hampsey, M., *Molecular genetics of the RNA polymerase II general transcriptional machinery*. Microbiol Mol Biol Rev, 1998. **62**(2): p. 465-503.
26. Ogbourne, S. and T.M. Antal, *Transcriptional control and the role of silencers in transcriptional regulation in eukaryotes*. Biochem J, 1998. **331** (Pt 1): p. 1-14.
27. Shilatifard, A., *Transcriptional elongation control by RNA polymerase II: a new frontier*. Biochim Biophys Acta, 2004. **1677**(1-3): p. 79-86.
28. Svejstrup, J.Q., *The RNA polymerase II transcription cycle: cycling through chromatin*. Biochim Biophys Acta, 2004. **1677**(1-3): p. 64-73.
29. Parvin, J.D., H.T. Timmers, and P.A. Sharp, *Promoter specificity of basal transcription factors*. Cell, 1992. **68**(6): p. 1135-44.
30. Holstege, F.C., et al., *Dissecting the regulatory circuitry of a eukaryotic genome*. Cell, 1998. **95**(5): p. 717-28.
31. Gill, G., *Regulation of the initiation of eukaryotic transcription*, in *Essays in Biochemistry. Regulation of gene expression*, K.E. Chapman and S.J. Higgins, Editors. 2001, Portland Press Ltd.: London.
32. Solovyev, V.V. and I.A. Shakhmuradov, *PromH: Promoters identification using orthologous genomic sequences*. Nucleic Acids Res, 2003. **31**(13): p. 3540-5.
33. Bell, B. and L. Tora, *Regulation of gene expression by multiple forms of TFIID and other novel TAFII-containing complexes*. Exp Cell Res, 1999. **246**(1): p. 11-9.
34. Albright, S.R. and R. Tjian, *TAFs revisited: more data reveal new twists and confirm old ideas*. Gene, 2000. **242**(1-2): p. 1-13.
35. Emami, K.H., A. Jain, and S.T. Smale, *Mechanism of synergy between TATA and initiator: synergistic binding of TFIID following a putative TFIIA-induced isomerization*. Genes Dev, 1997. **11**(22): p. 3007-19.
36. Werner, T., *Models for prediction and recognition of eukaryotic promoters*. Mamm Genome, 1999. **10**(2): p. 168-75.
37. Bucher, P., *Weight matrix descriptions of four eukaryotic RNA polymerase II promoter elements derived from 502 unrelated promoter sequences*. J Mol Biol, 1990. **212**(4): p. 563-78.
38. Thanos, D. and T. Maniatis, *Virus induction of human IFN beta gene expression requires the assembly of an enhanceosome*. Cell, 1995. **83**(7): p. 1091-100.

39. Bondarenko, V.A., et al., *Communication over a large distance: enhancers and insulators*. Biochem Cell Biol, 2003. **81**(3): p. 241-51.
40. Heinemeyer, T., et al., *Databases on transcriptional regulation: TRANSFAC, TRRD and COMPEL*. Nucleic Acids Res, 1998. **26**(1): p. 362-7.
41. Higo, K., et al., *Plant cis-acting regulatory DNA elements (PLACE) database: 1999*. Nucleic Acids Res, 1999. **27**(1): p. 297-300.
42. Daniel, S. and K.H. Kim, *Sp1 mediates glucose activation of the acetyl-CoA carboxylase promoter*. J Biol Chem, 1996. **271**(3): p. 1385-92.
43. Discher, D.J., et al., *Hypoxia regulates beta-enolase and pyruvate kinase-M promoters by modulating Sp1/Sp3 binding to a conserved GC element*. J Biol Chem, 1998. **273**(40): p. 26087-93.
44. Sack, M.N., et al., *A role for Sp and nuclear receptor transcription factors in a cardiac hypertrophic growth program*. Proc Natl Acad Sci U S A, 1997. **94**(12): p. 6438-43.
45. Santalucia, T., et al., *Hypertrophic agonists induce the binding of c-Fos to an AP-1 site in cardiac myocytes: implications for the expression of GLUT1*. Cardiovasc Res, 2003. **59**(3): p. 639-48.
46. Claessens, F. and D.T. Gwirth, *DNA recognition by nuclear receptors*. Essays Biochem, 2004. **40**: p. 59-72.
47. Jones, N., *Transcriptional regulation by dimerization: two sides to an incestuous relationship*. Cell, 1990. **61**(1): p. 9-11.
48. Ephrussi, A., et al., *B lineage-specific interactions of an immunoglobulin enhancer with cellular factors in vivo*. Science, 1985. **227**(4683): p. 134-40.
49. Fisher, F. and C.R. Goding, *Single amino acid substitutions alter helix-loop-helix protein specificity for bases flanking the core CANN TG motif*. Embo J, 1992. **11**(11): p. 4103-9.
50. Murre, C., et al., *Structure and function of helix-loop-helix proteins*. Biochim Biophys Acta, 1994. **1218**(2): p. 129-35.
51. Fisher, D.E., L.A. Parent, and P.A. Sharp, *High affinity DNA-binding Myc analogs: recognition by an alpha helix*. Cell, 1993. **72**(3): p. 467-76.
52. Sirtito, M., et al., *Ubiquitous expression of the 43- and 44-kDa forms of transcription factor USF in mammalian cells*. Nucleic Acids Res, 1994. **22**(3): p. 427-33.
53. Ferre-D'Amare, A.R., et al., *Structure and function of the b/HLH/Z domain of USF*. Embo J, 1994. **13**(1): p. 180-9.
54. Tsui, H.W., et al., *Molecular mechanisms underlying SHP-1 gene expression*. Eur J Biochem, 2002. **269**(12): p. 3057-64.
55. Rippe, R.A., et al., *Binding of upstream stimulatory factor to an E-box in the 3'-flanking region stimulates alpha1(I) collagen gene transcription*. J Biol Chem, 1997. **272**(3): p. 1753-60.
56. Navankasattusas, S., et al., *The basic helix-loop-helix protein upstream stimulating factor regulates the cardiac ventricular myosin light-chain 2 gene via independent cis regulatory elements*. Mol Cell Biol, 1994. **14**(11): p. 7331-9.
57. Xiao, Q. and K. Ojamaa, *Regulation of cardiac alpha-myosin heavy chain gene transcription by a contractile-responsive E-box binding protein*. J Mol Cell Cardiol, 1998. **30**(1): p. 87-95.
58. Vallet, V.S., et al., *Glucose-dependent liver gene expression in upstream stimulatory factor 2 -/- mice*. J Biol Chem, 1997. **272**(35): p. 21944-9.
59. Bengtsson, S.H., et al., *Transcriptional regulation of the human carboxyl ester lipase gene in THP-1 monocytes: an E-box required for activation binds upstream stimulatory factors 1 and 2*. Biochem J, 2002. **365**(Pt 2): p. 481-8.
60. Lefrancois-Martinez, A.M., et al., *Upstream stimulatory factor proteins are major components of the glucose response complex of the L-type pyruvate kinase gene promoter*. J Biol Chem, 1995. **270**(6): p. 2640-3.
61. Kennedy, H.J., et al., *Upstream stimulatory factor-2 (USF2) activity is required for glucose stimulation of L-pyruvate kinase promoter activity in single living islet beta-cells*. J Biol Chem, 1997. **272**(33): p. 20636-40.
62. Moore, M.L., E.A. Park, and J.B. McMillin, *Upstream stimulatory factor represses the induction of carnitine palmitoyltransferase-Ibeta expression by PGC-1*. J Biol Chem, 2003. **278**(19): p. 17263-8.
63. Roy, A.L., et al., *Cloning of an inr- and E-box-binding protein, TFII-I, that interacts physically and functionally with USF1*. Embo J, 1997. **16**(23): p. 7091-104.
64. Kivinen, A., et al., *USF2 is connected to GAAAATATGATA element and associates with androgen receptor-dependent transcriptional regulation in prostate*. Prostate, 2004. **59**(2): p. 190-202.
65. Qyang, Y., et al., *Cell-type-dependent activity of the ubiquitous transcription factor USF in cellular proliferation and transcriptional activation*. Mol Cell Biol, 1999. **19**(2): p. 1508-17.
66. Viollet, B., et al., *Immunohistochemical characterization and transacting properties of upstream stimulatory factor isoforms*. J Biol Chem, 1996. **271**(3): p. 1405-15.
67. Weintraub, H., et al., *Muscle-specific transcriptional activation by MyoD*. Genes Dev, 1991. **5**(8): p. 1377-86.
68. Arnold, H.H. and B. Winter, *Muscle differentiation: more complexity to the network of myogenic regulators*. Curr Opin Genet Dev, 1998. **8**(5): p. 539-44.
69. French, B.A., et al., *Heterodimers of myogenic helix-loop-helix regulatory factors and E12 bind a complex element governing myogenic induction of the avian cardiac alpha-actin promoter*. Mol Cell Biol, 1991. **11**(5): p. 2439-50.
70. Lassar, A.B., et al., *Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo*. Cell, 1991. **66**(2): p. 305-15.
71. Benezra, R., et al., *The protein Id: a negative regulator of helix-loop-helix DNA binding proteins*. Cell, 1990. **61**(1): p. 49-59.
72. Jen, Y., H. Weintraub, and R. Benezra, *Overexpression of Id protein inhibits the muscle differentiation program: in vivo association of Id with E2A proteins*. Genes Dev, 1992. **6**(8): p. 1466-79.
73. Gerber, A.N., et al., *Two domains of MyoD mediate transcriptional activation of genes in repressive chromatin: a mechanism for lineage determination in myogenesis*. Genes Dev, 1997. **11**(4): p. 436-50.
74. Vallett, S.M., et al., *A direct role for sterol regulatory element binding protein in activation of 3-hydroxy-3-methylglutaryl coenzyme A reductase gene*. J Biol Chem, 1996. **271**(21): p. 12247-53.
75. Salles, J., et al., *Acetyl-CoA carboxylase and SREBP expression during peripheral nervous system myelination*. Biochim Biophys Acta, 2003. **1631**(3): p. 229-38.
76. Oh, S.Y., et al., *Acetyl-CoA carboxylase beta gene is regulated by sterol regulatory element-binding protein-1 in liver*. J Biol Chem, 2003. **278**(31): p. 28410-7.
77. McEwan, I.J., *Sex, drugs and gene expression: signalling by members of the nuclear receptor superfamily*. Essays Biochem, 2004. **40**: p. 1-10.
78. Kumar, R., B.H. Johnson, and E.B. Thompson, *Overview of the structural basis for transcription regulation by nuclear hormone receptors*. Essays Biochem, 2004. **40**: p. 27-39.

79. Soutoglou, E. and I. Talianidis, *Coordination of PIC assembly and chromatin remodeling during differentiation-induced gene activation*. Science, 2002. **295**(5561): p. 1901-4.
80. Lee, T.I. and R.A. Young, *Transcription of eukaryotic protein-coding genes*. Annu Rev Genet, 2000. **34**: p. 77-137.
81. Strahl, B.D. and C.D. Allis, *The language of covalent histone modifications*. Nature, 2000. **403**(6765): p. 41-5.
82. Acevedo, M.L. and W.L. Kraus, *Transcriptional activation by nuclear receptors*. Essays Biochem, 2004. **40**: p. 73-88.
83. Tanese, N., et al., *Molecular cloning and analysis of two subunits of the human TFIID complex: hTAFII130 and hTAFII100*. Proc Natl Acad Sci U S A, 1996. **93**(24): p. 13611-6.
84. Barberis, A. and L. Gaudreau, *Recruitment of the RNA polymerase II holoenzyme and its implications in gene regulation*. Biol Chem, 1998. **379**(12): p. 1397-405.
85. Gabellini, D., R. Tupler, and M.R. Green, *Transcriptional derepression as a cause of genetic diseases*. Curr Opin Genet Dev, 2003. **13**(3): p. 239-45.
86. Gaston, K. and P.S. Jayaraman, *Transcriptional repression in eukaryotes: repressors and repression mechanisms*. Cell Mol Life Sci, 2003. **60**(4): p. 721-41.
87. McKinsey, T.A., C.L. Zhang, and E.N. Olson, *Control of muscle development by dueling HATs and HDACs*. Curr Opin Genet Dev, 2001. **11**(5): p. 497-504.
88. Miyata, K.S., et al., *Chicken ovalbumin upstream promoter transcription factor (COUP-TF) binds to a peroxisome proliferator-responsive element and antagonizes peroxisome proliferator-mediated signaling*. J Biol Chem, 1993. **268**(26): p. 19169-72.
89. Taegtmeyer, H., *Genetics of energetics: transcriptional responses in cardiac metabolism*. Ann Biomed Eng, 2000. **28**(8): p. 871-6.
90. van Bilsen, M., G.J. van der Vusse, and R.S. Reneman, *Transcriptional regulation of metabolic processes: implications for cardiac metabolism*. Pflugers Arch, 1998. **437**(1): p. 2-14.
91. Lopaschuk, G.D. and M.A. Spafford, *Energy substrate utilization by isolated working hearts from newborn rabbits*. Am J Physiol, 1990. **258**(5 Pt 2): p. H1274-80.
92. Lopaschuk, G.D., M.A. Spafford, and D.R. Marsh, *Glycolysis is predominant source of myocardial ATP production immediately after birth*. Am J Physiol, 1991. **261**(6 Pt 2): p. H1698-705.
93. Barger, P.M. and D.P. Kelly, *PPAR signaling in the control of cardiac energy metabolism*. Trends Cardiovasc Med, 2000. **10**(6): p. 238-45.
94. Barger, P.M., et al., *Deactivation of peroxisome proliferator-activated receptor-alpha during cardiac hypertrophic growth*. J Clin Invest, 2000. **105**(12): p. 1723-30.
95. Gulick, T., et al., *The peroxisome proliferator-activated receptor regulates mitochondrial fatty acid oxidative enzyme gene expression*. Proc Natl Acad Sci U S A, 1994. **91**(23): p. 11012-6.
96. Issemann, I. and S. Green, *Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators*. Nature, 1990. **347**(6294): p. 645-50.
97. Gilde, A.J. and M. Van Bilsen, *Peroxisome proliferator-activated receptors (PPARs): regulators of gene expression in heart and skeletal muscle*. Acta Physiol Scand, 2003. **178**(4): p. 425-34.
98. Gottlicher, M., et al., *Fatty acids activate a chimera of the clofibrate acid-activated receptor and the glucocorticoid receptor*. Proc Natl Acad Sci U S A, 1992. **89**(10): p. 4653-7.
99. Elholm, M., et al., *Acyl-CoA esters antagonize the effects of ligands on peroxisome proliferator-activated receptor alpha conformation, DNA binding, and interaction with Co-factors*. J Biol Chem, 2001. **276**(24): p. 21410-6.
100. Murakami, K., et al., *Fatty-acyl-CoA thioesters inhibit recruitment of steroid receptor co-activator 1 to alpha and gamma isoforms of peroxisome-proliferator-activated receptors by competing with agonists*. Biochem J, 2001. **353**(Pt 2): p. 231-8.
101. Kliewer, S.A., et al., *Convergence of 9-cis retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors*. Nature, 1992. **358**(6389): p. 771-4.
102. Huss, J.M., F.H. Levy, and D.P. Kelly, *Hypoxia inhibits the peroxisome proliferator-activated receptor alpha/retinoid X receptor gene regulatory pathway in cardiac myocytes: a mechanism for O2-dependent modulation of mitochondrial fatty acid oxidation*. J Biol Chem, 2001. **276**(29): p. 27605-12.
103. Vega, R.B., J.M. Huss, and D.P. Kelly, *The Coactivator PGC-1 Cooperates with Peroxisome Proliferator-Activated Receptor alpha in Transcriptional Control of Nuclear Genes Encoding Mitochondrial Fatty Acid Oxidation Enzymes*. Mol Cell Biol, 2000. **20**(5): p. 1868-76.
104. Lehman, J.J. and D.P. Kelly, *Transcriptional activation of energy metabolic switches in the developing and hypertrophied heart*. Clin Exp Pharmacol Physiol, 2002. **29**(4): p. 339-45.
105. Juge-Aubry, C.E., et al., *Regulation of the transcriptional activity of the peroxisome proliferator-activated receptor alpha by phosphorylation of a ligand-independent trans-activating domain*. J Biol Chem, 1999. **274**(15): p. 10505-10.
106. Barger, P.M., et al., *p38 mitogen-activated protein kinase activates peroxisome proliferator-activated receptor alpha: a potential role in the cardiac metabolic stress response*. J Biol Chem, 2001. **276**(48): p. 44495-501.
107. Leone, T.C., C.J. Weinheimer, and D.P. Kelly, *A critical role for the peroxisome proliferator-activated receptor alpha (PPARalpha) in the cellular fasting response: the PPARalpha-null mouse as a model of fatty acid oxidation disorders*. Proc Natl Acad Sci U S A, 1999. **96**(13): p. 7473-8.
108. Campbell, F.M., et al., *A role for peroxisome proliferator-activated receptor alpha (PPARalpha) in the control of cardiac malonyl-CoA levels: reduced fatty acid oxidation rates and increased glucose oxidation rates in the hearts of mice lacking PPARalpha are associated with higher concentrations of malonyl-CoA and reduced expression of malonyl-CoA decarboxylase*. J Biol Chem, 2002. **277**(6): p. 4098-103.
109. Finck, B.N., et al., *The cardiac phenotype induced by PPARalpha overexpression mimics that caused by diabetes mellitus*. J Clin Invest, 2002. **109**(1): p. 121-30.
110. Hopkins, T.A., et al., *Control of cardiac pyruvate dehydrogenase activity in peroxisome proliferator-activated receptor-alpha transgenic mice*. Am J Physiol Heart Circ Physiol, 2003. **285**(1): p. H270-6.
111. Yusuf, S., et al., *Global burden of cardiovascular diseases: part I: general considerations, the epidemiologic transition, risk factors, and impact of urbanization*. Circulation, 2001. **104**(22): p. 2746-53.
112. Yusuf, S., et al., *Global burden of cardiovascular diseases: Part II: variations in cardiovascular disease by specific ethnic groups and geographic regions and prevention strategies*. Circulation, 2001. **104**(23): p. 2855-64.
113. McGarry, J.D. and N.F. Brown, *The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis*. Eur J Biochem, 1997. **244**(1): p. 1-14.

114. Weiss, B.C., et al., *Rat heart expresses two isoforms of Mitochondrial Carnitine Palmitoyltransferase I*. J Biol Chem, 1994. **269**(29): p. 18712-18715.
115. Weiss, B.C., et al., *Use of a selective inhibitor of liver carnitine palmitoyltransferase I (CPT I) allows quantification of its contribution to total CPT I activity in the rat heart*. J Biol Chem, 1994. **269**(42): p. 26443-48.
116. Edwards, M.R., M.I. Bird, and E.D. Saggerson, *Effects of DL-2-bromopalmitoyl-CoA and bromoacetyl-CoA in rat liver and heart mitochondria. Inhibition of carnitine palmitoyltransferase and displacement of [¹⁴C]malonyl-CoA from mitochondrial binding sites*. Biochem J, 1985. **230**(1): p. 169-79.
117. McGarry, J.D., et al., *Observations on the affinity for carnitine, and malonyl-CoA sensitivity, of carnitine palmitoyltransferase I in animal and human tissues. Demonstration of the presence of malonyl-CoA in non-hepatic tissues of the rat*. Biochem J, 1983. **214**(1): p. 21-8.
118. Saddik, M., et al., *Acetyl-CoA carboxylase regulation of fatty acid oxidation in the heart*. J Biol Chem, 1993. **268**(34): p. 25836-45.
119. Bird, M.I. and E.D. Saggerson, *Binding of malonyl-CoA to isolated mitochondria. Evidence for high- and low-affinity sites in liver and heart and relationship to inhibition of carnitine palmitoyltransferase activity*. Biochem J, 1984. **222**(3): p. 639-47.
120. Awan, M.M. and E.D. Saggerson, *Malonyl-CoA metabolism in cardiac myocytes and its relevance to the control of fatty acid oxidation*. Biochem J, 1993. **295** (Pt 1): p. 61-6.
121. Cook, G.A. and M.D. Lappi, *Carnitine palmitoyltransferase in the heart is controlled by a different mechanism than the hepatic enzyme*. Mol Cell Biochem, 1992. **116**(1-2): p. 39-45.
122. Lopaschuk, G.D., et al., *Regulation of fatty acid oxidation in the mammalian heart in health and disease*. Biochim Biophys Acta, 1994. **1213**(3): p. 263-76.
123. Taegtmeyer, H., R. Hems, and H.A. Krebs, *Utilization of energy-providing substrates in the isolated working rat heart*. Biochem J, 1980. **186**(3): p. 701-11.
124. Bianchi, A., et al., *Identification of an isozymic form of acetyl-CoA carboxylase*. J Biol Chem, 1990. **265**(3): p. 1502-9.
125. Abu-Elheiga, L., et al., *Human acetyl-CoA carboxylase 2. Molecular cloning, characterization, chromosomal mapping, and evidence for two isoforms*. J Biol Chem, 1997. **272**(16): p. 10669-77.
126. Ullrich, C.K., et al., *Assignment of acetyl-CoA carboxylase-beta (ACACB) to human chromosome band 12q24.1 by in situ hybridization*. Cytogenet Cell Genet, 1997. **77**(3-4): p. 176-7.
127. Abe, K., Y. Shinohara, and H. Terada, *Isolation and characterization of cDNA encoding rat heart type acetyl-CoA carboxylase*. Biochim Biophys Acta, 1998. **1398**(3): p. 347-52.
128. Lopez-Casillas, F., et al., *Structure of the coding sequence and primary amino acid sequence of acetyl-coenzyme A carboxylase*. Proc Natl Acad Sci U S A, 1988. **85**(16): p. 5784-8.
129. Widmer, J., et al., *Identification of a second human acetyl-CoA carboxylase gene*. Biochem J, 1996. **316** (Pt 3): p. 915-22.
130. Ha, J., et al., *Cloning of human acetyl-CoA carboxylase cDNA*. Eur J Biochem, 1994. **219**(1-2): p. 297-306.
131. Ha, J., et al., *Cloning of human acetyl-CoA carboxylase-beta and its unique features*. Proc Natl Acad Sci U S A, 1996. **93**(21): p. 11466-70.
132. Abu-Elheiga, L., et al., *Human acetyl-CoA carboxylase: characterization, molecular cloning, and evidence for two isoforms*. Proc Natl Acad Sci U S A, 1995. **92**(9): p. 4011-5.
133. Abu-Elheiga, L., et al., *The subcellular localization of acetyl-CoA carboxylase 2*. Proc Natl Acad Sci U S A, 2000. **97**(4): p. 1444-9.
134. Kantor, P.F., G.D. Lopaschuk, and L.H. Opie, *Myocardial Energy Metabolism*, in *Heart Physiology and Pathophysiology*, N. Sperelakis, et al., Editors. 2001, Academic Press: San Diego. p. 544-595.
135. Thampy, K.G., *Formation of malonyl coenzyme A in rat heart. Identification and purification of an isozyme of A carboxylase from rat heart*. J Biol Chem, 1989. **264**(30): p. 17631-4.
136. Thampy, K.G. and S.J. Wakil, *Regulation of acetyl-coenzyme A carboxylase. II. Effect of fasting and refeeding on the activity, phosphate content, and aggregation state of the enzyme*. J Biol Chem, 1988. **263**(13): p. 6454-8.
137. Mabrouk, G.M., et al., *Acute hormonal control of acetyl-CoA carboxylase. The roles of insulin, glucagon, and epinephrine*. J Biol Chem, 1990. **265**(11): p. 6330-8.
138. Davies, S.P., A.T. Sim, and D.G. Hardie, *Location and function of three sites phosphorylated on rat acetyl-CoA carboxylase by the AMP-activated protein kinase*. Eur J Biochem, 1990. **187**(1): p. 183-90.
139. Kim, K.H., et al., *Role of reversible phosphorylation of acetyl-CoA carboxylase in long-chain fatty acid synthesis*. Faseb J, 1989. **3**(11): p. 2250-6.
140. Hardie, D.G., *Minireview: the AMP-activated protein kinase cascade: the key sensor of cellular energy status*. Endocrinology, 2003. **144**(12): p. 5179-83.
141. Munday, M.R., et al., *Identification by amino acid sequencing of three major regulatory phosphorylation sites on rat acetyl-CoA carboxylase*. Eur J Biochem, 1988. **175**(2): p. 331-8.
142. Kim, K.H., *Regulation of mammalian acetyl-coenzyme A carboxylase*. Annu Rev Nutr, 1997. **17**: p. 77-99.
143. Thampy, K.G. and S.J. Wakil, *Regulation of acetyl-coenzyme A carboxylase. I. Purification and properties of two forms of acetyl-coenzyme A carboxylase from rat liver*. J Biol Chem, 1988. **263**(13): p. 6447-53.
144. Luo, X.C., et al., *Structural features of the acetyl-CoA carboxylase gene: mechanisms for the generation of mRNAs with 5' end heterogeneity*. Proc Natl Acad Sci U S A, 1989. **86**(11): p. 4042-6.
145. Kim, K.H. and H.J. Tae, *Pattern and regulation of acetyl-CoA carboxylase gene expression*. J Nutr, 1994. **124**(8 Suppl): p. 1273S-1283S.
146. Tae, H.J., X. Luo, and K.H. Kim, *Roles of CCAAT/enhancer-binding protein and its binding site on repression and derepression of acetyl-CoA carboxylase gene*. J Biol Chem, 1994. **269**(14): p. 10475-84.
147. Huang, C. and H.C. Freake, *Thyroid hormone regulates the acetyl-CoA carboxylase P1 promoter*. Biochem Biophys Res Commun, 1998. **249**(3): p. 704-8.
148. Daniel, S., et al., *Dephosphorylation of Sp1 by protein phosphatase 1 is involved in the glucose-mediated activation of the acetyl-CoA carboxylase gene*. J Biol Chem, 1996. **271**(25): p. 14692-7.
149. Lopez-Casillas, F. and K.H. Kim, *The 5' untranslated regions of acetyl-coenzyme A carboxylase mRNA provide specific translational control in vitro*. Eur J Biochem, 1991. **201**(1): p. 119-27.
150. El Khadir-Mounier, C., et al., *Cloning and characterization of the 5' end and promoter region of the chicken acetyl-CoA carboxylase gene*. Biochem J, 1996. **314** (Pt 2): p. 613-9.

151. Mao, J., et al., *Genomic distribution of three promoters of the bovine gene encoding acetyl-CoA carboxylase alpha and evidence that the nutritionally regulated promoter I contains a repressive element different from that in rat*. *Biochem J*, 2001. **358**(Pt 1): p. 127-35.
152. Mao, J., S.S. Chirala, and S.J. Wakil, *Human acetyl-CoA carboxylase 1 gene: presence of three promoters and heterogeneity at the 5'-untranslated mRNA region*. *Proc Natl Acad Sci U S A*, 2003. **100**(13): p. 7515-20.
153. Abu-Elheiga, L., et al., *Continuous fatty acid oxidation and reduced fat storage in mice lacking acetyl-CoA carboxylase 2*. *Science*, 2001. **291**(5513): p. 2613-6.
154. Abu-Elheiga, L., et al., *Acetyl-CoA carboxylase 2 mutant mice are protected against obesity and diabetes induced by high-fat/high-carbohydrate diets*. *Proc Natl Acad Sci U S A*, 2003. **100**(18): p. 10207-12.
155. Harwood, H.J., Jr., *Acetyl-CoA carboxylase inhibition for the treatment of metabolic syndrome*. *Curr Opin Investig Drugs*, 2004. **5**(3): p. 283-9.
156. Debard, C., et al., *Expression of key genes of fatty acid oxidation, including adiponectin receptors, in skeletal muscle of Type 2 diabetic patients*. *Diabetologia*, 2004. **47**(5): p. 917-25.
157. Wang, D., L.M. Buja, and J.B. McMillin, *Acetyl coenzyme A carboxylase activity in neonatal rat cardiac myocytes in culture: citrate dependence and effects of hypoxia*. *Arch Biochem Biophys*, 1996. **325**(2): p. 249-55.
158. Seng, T.W., et al., *Cyclohexanedione herbicides are inhibitors of rat heart acetyl-CoA carboxylase*. *Bioorg Med Chem Lett*, 2003. **13**(19): p. 3237-42.
159. Dyck, J.R., et al., *Phosphorylation control of cardiac acetyl-CoA carboxylase by cAMP-dependent protein kinase and 5'-AMP activated protein kinase*. *Eur J Biochem*, 1999. **262**(1): p. 184-90.
160. Winder, W.W. and D.G. Hardie, *Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise*. *Am J Physiol*, 1996. **270**(2 Pt 1): p. E299-304.
161. Vavvas, D., et al., *Contraction-induced changes in acetyl-CoA carboxylase and 5'-AMP-activated kinase in skeletal muscle*. *J Biol Chem*, 1997. **272**(20): p. 13255-61.
162. Witters, L.A. and B.E. Kemp, *Insulin activation of acetyl-CoA carboxylase accompanied by inhibition of the 5'-AMP-activated protein kinase*. *J Biol Chem*, 1992. **267**(5): p. 2864-7.
163. Clark, H., D. Carling, and D. Saggerson, *Covalent activation of heart AMP-activated protein kinase in response to physiological concentrations of long-chain fatty acids*. *Eur J Biochem*, 2004. **271**(11): p. 2215-24.
164. Stephens, T.J., et al., *Progressive increase in human skeletal muscle AMPKalpha2 activity and ACC phosphorylation during exercise*. *Am J Physiol Endocrinol Metab*, 2002. **282**(3): p. E688-94.
165. Coven, D.L., et al., *Physiological role of AMP-activated protein kinase in the heart: graded activation during exercise*. *Am J Physiol Endocrinol Metab*, 2003. **285**(3): p. E629-36.
166. Lopaschuk, G.D., et al., *Acetyl-CoA carboxylase involvement in the rapid maturation of fatty acid oxidation in the newborn rabbit heart*. *J Biol Chem*, 1994. **269**(41): p. 25871-8.
167. Makinde, A.O., J. Gamble, and G.D. Lopaschuk, *Upregulation of 5'-AMP-activated protein kinase is responsible for the increase in myocardial fatty acid oxidation rates following birth in the newborn rabbit*. *Circ Res*, 1997. **80**(4): p. 482-9.
168. Woods, A., et al., *Identification of phosphorylation sites in AMP-activated protein kinase (AMPK) for upstream AMPK kinases and study of their roles by site-directed mutagenesis*. *J Biol Chem*, 2003. **278**(31): p. 28434-42.
169. Shaw, R.J., et al., *The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress*. *Proc Natl Acad Sci U S A*, 2004. **101**(10): p. 3329-35.
170. Kudo, N., et al., *Characterization of 5'-AMP-activated protein kinase activity in the heart and its role in inhibiting acetyl-CoA carboxylase during reperfusion following ischemia*. *Biochim Biophys Acta*, 1996. **1301**(1-2): p. 67-75.
171. Kovacic, S., et al., *Akt activity negatively regulates phosphorylation of AMP-activated protein kinase in the heart*. *J Biol Chem*, 2003. **278**(41): p. 39422-7.
172. Kudo, N., et al., *High rates of fatty acid oxidation during reperfusion of ischemic hearts are associated with a decrease in malonyl-CoA levels due to an increase in 5'-AMP-activated protein kinase inhibition of acetyl-CoA carboxylase*. *J Biol Chem*, 1995. **270**(29): p. 17513-20.
173. Lee, J.J., et al., *Cloning of human acetyl-CoA carboxylase beta promoter and its regulation by muscle regulatory factors*. *J Biol Chem*, 2001. **276**(4): p. 2576-85.
174. Kim, J.Y., J.J. Lee, and K.S. Kim, *Acetyl-CoA carboxylase beta expression mediated by MyoD and muscle regulatory factor 4 is differentially affected by retinoic acid receptor and retinoid X receptor*. *Exp Mol Med*, 2003. **35**(1): p. 23-9.
175. Smith, R.M., J. McCarthy, and M.N. Sack, *TNF alpha is required for hypoxia-mediated right ventricular hypertrophy*. *Mol Cell Biochem*, 2001. **219**(1-2): p. 139-43.
176. Holden, J.E., et al., *Enhanced cardiac metabolism of plasma glucose in high-altitude natives: adaptation against chronic hypoxia*. *J Appl Physiol*, 1995. **79**(1): p. 222-8.
177. Hochachka, P.W., et al., *³¹P magnetic resonance spectroscopy of the Sherpa heart: a phosphocreatine/adenosine triphosphate signature of metabolic defense against hypobaric hypoxia*. *Proc Natl Acad Sci U S A*, 1996. **93**(3): p. 1215-20.
178. Conway, M.A., et al., *Detection of low phosphocreatine to ATP ratio in failing hypertrophied human myocardium by ³¹P magnetic resonance spectroscopy*. *Lancet*, 1991. **338**(8773): p. 973-6.
179. Hunter, C., et al., *Growth of the heart and lungs in hypoxic rodents: a model of human hypoxic disease*. *Clin Sci Mol Med*, 1974. **46**(3): p. 375-91.
180. Leach, E., P. Howard, and G.R. Barer, *Resolution of hypoxic changes in the heart and pulmonary arterioles of rats during intermittent correction of hypoxia*. *Clin Sci Mol Med*, 1977. **52**(2): p. 153-62.
181. Depre, C., J.L. Vanoverschelde, and H. Taegtmeyer, *Glucose for the heart*. *Circulation*, 1999. **99**(4): p. 578-88.
182. Fink, R.I., et al., *Evidence that glucose transport is rate-limiting for in vivo glucose uptake*. *Metabolism*, 1992. **41**(8): p. 897-902.
183. Flier, J.S., et al., *Distribution of glucose transporter messenger RNA transcripts in tissues of rat and man*. *J Clin Invest*, 1987. **79**(2): p. 657-61.
184. Laybutt, D.R., et al., *Selective chronic regulation of GLUT1 and GLUT4 content by insulin, glucose, and lipid in rat cardiac muscle in vivo*. *Am J Physiol*, 1997. **273**(3 Pt 2): p. H1309-16.
185. Shepherd, P.R. and B.B. Kahn, *Glucose transporters and insulin action--implications for insulin resistance and diabetes mellitus*. *N Engl J Med*, 1999. **341**(4): p. 248-57.
186. Bowker-Kinley, M.M., et al., *Evidence for existence of tissue-specific regulation of the mammalian pyruvate dehydrogenase complex*. *Biochem J*, 1998. **329** (Pt 1): p. 191-6.

187. Wu, P., et al., *Mechanism responsible for inactivation of skeletal muscle pyruvate dehydrogenase complex in starvation and diabetes*. Diabetes, 1999. **48**(8): p. 1593-9.
188. Wu, P., et al., *Starvation and diabetes increase the amount of pyruvate dehydrogenase kinase isoenzyme 4 in rat heart*. Biochem J, 1998. **329** (Pt 1): p. 197-201.
189. Sugden, M.C., et al., *Expression and regulation of pyruvate dehydrogenase kinase isoforms in the developing rat heart and in adulthood: role of thyroid hormone status and lipid supply*. Biochem J, 2000. **352** Pt 3: p. 731-8.
190. Stavinoha, M.A., et al., *Diurnal Variations in the Responsiveness of Cardiac and Skeletal Muscle to Fatty Acids*. Am J Physiol Endocrinol Metab, 2004.
191. Himms-Hagen, J. and M.E. Harper, *Physiological role of UCP3 may be export of fatty acids from mitochondria when fatty acid oxidation predominates: an hypothesis*. Exp Biol Med (Maywood), 2001. **226**(2): p. 78-84.
192. Hoeks, J., et al., *Differential response of UCP3 to medium versus long chain triacylglycerols; manifestation of a functional adaptation*. FEBS Lett, 2003. **555**(3): p. 631-7.
193. Boss, O., et al., *Uncoupling protein-3: a new member of the mitochondrial carrier family with tissue-specific expression*. FEBS Lett, 1997. **408**(1): p. 39-42.
194. Schrauwen, P., et al., *Effect of acute exercise on uncoupling protein 3 is a fat metabolism-mediated effect*. Am J Physiol Endocrinol Metab, 2002. **282**(1): p. E11-7.
195. Young, M.E., et al., *Uncoupling protein 3 transcription is regulated by peroxisome proliferator-activated receptor (alpha) in the adult rodent heart*. Faseb J, 2001. **15**(3): p. 833-45.
196. Thompson, M.P. and D. Kim, *Links between fatty acids and expression of UCP2 and UCP3 mRNAs*. FEBS Lett, 2004. **568**(1-3): p. 4-9.
197. Sambrook, J., E.F. Fritsch, and T. Maniatis, *Molecular Cloning. A Laboratory Manual*. 1989, New York: Cold spring Harbor Laboratory Press.
198. Depre, C., et al., *Unloaded heart in vivo replicates fetal gene expression of cardiac hypertrophy*. Nat Med, 1998. **4**(11): p. 1269-75.
199. Takahashi, Y., et al., *Inhibition of the transcription of CYP1A1 gene by the upstream stimulatory factor 1 in rabbits. Competitive binding of USF1 with AhR.Arnt complex*. J Biol Chem, 1997. **272**(48): p. 30025-31.
200. Meier, J.L., et al., *The cellular transcription factor USF cooperates with varicella-zoster virus immediate-early protein 62 to symmetrically activate a bidirectional viral promoter*. Mol Cell Biol, 1994. **14**(10): p. 6896-906.
201. Robinson, C.E., et al., *DNA bending is induced by binding of the peroxisome proliferator-activated receptor gamma 2 heterodimer to its response element in the murine lipoprotein lipase promoter*. Biochem Biophys Res Commun, 1998. **244**(3): p. 671-7.
202. Sharma, S., et al., *Dynamic changes of gene expression in hypoxia-induced right ventricular hypertrophy*. Am J Physiol Heart Circ Physiol, 2004. **286**(3): p. H1185-92.
203. Altschul, S.F., et al., *Gapped BLAST and PSI-BLAST: a new generation of protein database search programs*. Nucleic Acids Res, 1997. **25**(17): p. 3389-402.
204. Emery, C.J., D. Bee, and G.R. Barer, *Mechanical properties and reactivity of vessels in isolated perfused lungs of chronically hypoxic rats*. Clin Sci (Lond), 1981. **61**(5): p. 569-80.
205. Cameron, V.A. and L.J. Ellmers, *Minireview: natriuretic peptides during development of the fetal heart and circulation*. Endocrinology, 2003. **144**(6): p. 2191-4.
206. Burnett, J.C., Jr., et al., *Atrial natriuretic peptide elevation in congestive heart failure in the human*. Science, 1986. **231**(4742): p. 1145-7.
207. Adachi, S., et al., *Distribution of mRNAs for natriuretic peptides in RV hypertrophy after pulmonary arterial banding*. Am J Physiol, 1995. **268**(1 Pt 2): p. H162-9.
208. Sack, M.N., et al., *Fatty acid oxidation enzyme gene expression is downregulated in the failing heart*. Circulation, 1996. **94**(11): p. 2837-42.
209. Bar, H., et al., *Upregulation of embryonic transcription factors in right ventricular hypertrophy*. Basic Res Cardiol, 2003. **98**(5): p. 285-94.
210. Huang, L.E. and H.F. Bunn, *Hypoxia-inducible factor and its biomedical relevance*. J Biol Chem, 2003. **278**(22): p. 19575-8.
211. Hopfl, G., O. Ogunshola, and M. Gassmann, *Hypoxia and high altitude. The molecular response*. Adv Exp Med Biol, 2003. **543**: p. 89-115.
212. Maxwell, P.H., C.W. Pugh, and P.J. Ratcliffe, *Inducible operation of the erythropoietin 3' enhancer in multiple cell lines: evidence for a widespread oxygen-sensing mechanism*. Proc Natl Acad Sci U S A, 1993. **90**(6): p. 2423-7.
213. Hochachka, P.W. and C. Monge, *Evolution of human hypoxia tolerance physiology*. Adv Exp Med Biol, 2000. **475**: p. 25-43.
214. Semenza, G.L., *Regulation of hypoxia-induced angiogenesis: a chaperone escorts VEGF to the dance*. J Clin Invest, 2001. **108**(1): p. 39-40.
215. Levy, A.P., N.S. Levy, and M.A. Goldberg, *Post-transcriptional regulation of vascular endothelial growth factor by hypoxia*. J Biol Chem, 1996. **271**(5): p. 2746-53.
216. Sivitz, W.I., et al., *Pretranslational regulation of two cardiac glucose transporters in rats exposed to hypobaric hypoxia*. Am J Physiol, 1992. **263**(3 Pt 1): p. E562-9.
217. Galan, X., M. Llobera, and I. Ramirez, *Lipoprotein lipase in developing rat tissues: differences between Wistar and Sprague-Dawley rats*. Biol Neonate, 1993. **64**(5): p. 295-303.
218. Bunag, R.D. and T.L. Teravainen, *Tail-cuff detection of systolic hypertension in different strains of ageing rats*. Mech Ageing Dev, 1991. **59**(1-2): p. 197-213.
219. Dill, R.P., et al., *Ageing and glucose transporter plasticity in response to hypobaric hypoxia*. Mech Ageing Dev, 2001. **122**(6): p. 533-45.
220. Montessuit, C. and A. Thorburn, *Transcriptional activation of the glucose transporter GLUT1 in ventricular cardiac myocytes by hypertrophic agonists*. J Biol Chem, 1999. **274**(13): p. 9006-12.
221. Morissette, M.R., et al., *Upregulation of GLUT1 expression is necessary for hypertrophy and survival of neonatal rat cardiomyocytes*. J Mol Cell Cardiol, 2003. **35**(10): p. 1217-27.
222. Razeghi, P., et al., *Hypoxia in vivo decreases peroxisome proliferator-activated receptor alpha-regulated gene expression in rat heart*. Biochem Biophys Res Commun, 2001. **287**(1): p. 5-10.
223. Lydell, C.P., et al., *Pyruvate dehydrogenase and the regulation of glucose oxidation in hypertrophied rat hearts*. Cardiovasc Res, 2002. **53**(4): p. 841-51.

224. Solanes, G., et al., *The human uncoupling protein-3 gene promoter requires MyoD and is induced by retinoic acid in muscle cells*. *Faseb J*, 2000. **14**(14): p. 2141-3.
225. Riquet, F.B., et al., *In vivo characterisation of the human UCP3 gene minimal promoter in mice tibialis anterior muscles*. *Biochem Biophys Res Commun*, 2003. **311**(3): p. 583-91.
226. Essop, M.F., et al., *Hypoxia-induced decrease of UCP3 gene expression in rat heart parallels metabolic gene switching but fails to affect mitochondrial respiratory coupling*. *Biochem Biophys Res Commun*, 2004. **314**(2): p. 561-4.
227. Meerson, F.Z., *The failing heart: adaptation and deadaptation*. 1983, New York: Raven Press.
228. Lee, J.K. and K.H. Kim, *Roles of acetyl-CoA carboxylase beta in muscle cell differentiation and in mitochondrial fatty acid oxidation*. *Biochem Biophys Res Commun*, 1999. **254**(3): p. 657-60.
229. Manga, N., et al., *USF1 Trans-Activates the rat cardiac acetyl-CoA carboxylase gene - a potential regulatory factor in response to nutritional flux*. *Circulation*, 2002. **106**(19): p. 985.
230. Makaula, S., et al., *PPAR-alpha induction of cardiac acetyl-CoA carboxylase expression is nullified by post-translational regulation in response to nutritional flux*. *Circulation*, 2002. **106**(19): p. 984.
231. Kim, J.M., et al., *Evidence that acetyl-CoA carboxylase isoforms play different biological roles in H9c2 cardiomyocyte*. *Biochem Biophys Res Commun*, 1998. **248**(3): p. 490-6.
232. Giorgino, F., et al., *The sentrin-conjugating enzyme mUbc9 interacts with GLUT4 and GLUT1 glucose transporters and regulates transporter levels in skeletal muscle cells*. *Proc Natl Acad Sci U S A*, 2000. **97**(3): p. 1125-30.
233. Bandyopadhyay, G., et al., *Glucose activates mitogen-activated protein kinase (extracellular signal-regulated kinase) through proline-rich tyrosine kinase-2 and the Glut1 glucose transporter*. *J Biol Chem*, 2000. **275**(52): p. 40817-26.
234. Bode, J., et al., *Biological significance of unwinding capability of nuclear matrix-associating DNAs*. *Science*, 1992. **255**(5041): p. 195-7.
235. Duplus, E., M. Glorian, and C. Forest, *Fatty acid regulation of gene transcription*. *J Biol Chem*, 2000. **275**(40): p. 30749-52.
236. Jump, D.B. and S.D. Clarke, *Regulation of gene expression by dietary fat*. *Annu Rev Nutr*, 1999. **19**: p. 63-90.
237. van der Lee, K.A., et al., *Long-chain fatty acid-induced changes in gene expression in neonatal cardiac myocytes*. *J Lipid Res*, 2000. **41**(1): p. 41-7.
238. Kimes, B.W. and B.L. Brandt, *Properties of a clonal muscle cell line from rat heart*. *Exp Cell Res*, 1976. **98**(2): p. 367-81.
239. Rivera-Pomar, R., et al., *RNA binding and translational suppression by bicoid*. *Nature*, 1996. **379**(6567): p. 746-9.
240. Graves, L.E., S. Segal, and E.B. Goodwin, *TRA-1 regulates the cellular distribution of the tra-2 mRNA in C. elegans*. *Nature*, 1999. **399**(6738): p. 802-5.
241. Rumsey, W.L., et al., *Adaptation to hypoxia alters energy metabolism in rat heart*. *Am J Physiol*, 1999. **276**(1 Pt 2): p. H71-80.
242. Bryan, R.M., Jr., *Cerebral blood flow and energy metabolism during stress*. *Am J Physiol*, 1990. **259**(2 Pt 2): p. H269-80.
243. Amagasa, S., et al., *Effects of halothane and isoflurane anesthesia on sympathetic adrenal nerve responses to carbon dioxide challenge in rats*. *J Anesth*, 1993. **7**(4): p. 454-61.
244. Xu, M., et al., *Last intron of the chemokine-like factor gene contains a putative promoter for the downstream CKLF super family member 1 gene*. *Biochem Biophys Res Commun*, 2004. **313**(1): p. 135-41.

APPENDIX A

The following stock solutions were used. Distilled, deionised water was used in all cases. Unless otherwise indicated the solutions were autoclaved and stored at room temperature.

1) **10X MOPS**

41.2 g 3-(N-morpholino)propanesulphonic acid (MOPS)

6.56 g sodium acetate

10 ml 0.5 M EDTA

Make to 800 ml with water, adjust pH to 7.0 using NaOH. Make to 1L. Store in dark at 4°C.

2) **20X SSC**

175.3 g NaCl

88.2 g Sodium citrate

Make to 800 ml with water, adjust pH to 7.0 using NaOH. Make to 1L.

3) **0.5 M EDTA**

18.62 g ethylenediaminetetra-acetic acid (EDTA)

Make to 80ml with water, adjust pH to 8.0 using NaOH pellets. Make to 100ml.

4) **Luria Broth**

950 ml water

10 g bacto-tryptone

5 g bacto-yeast extract

10 g NaCl

Adjust pH to 7.0 using 5N NaOH. Make to 1L.

5) **10X PBS**

8 g NaCl

0.2 g KCl

1.44 g Na₂HPO₄

0.24 g KH₂PO₄

Make to 800 ml with water, adjust pH to 7.4 using HCl. Make to 1L.

6) **50 X TAE**

242 g Tris

57.1 ml Glacial acetic acid

100 ml 0.5 M EDTA

Make to 1L.

7) **Plasmid Isolation Solutions****Solution 1: 50 mM glucose/ 10 mM EDTA/ 25 mM Tris-HCl**

20 ml 0.5M EDTA pH8

25 ml 1M Tris

9 g Glucose

Make to 1L with water and sterile filter.

Solution 2: 200 mM NaOH/ 1% SDS

8 ml 10 M NaOH

372 ml H₂O

20 ml 20% SDS

Prepare fresh and do not autoclave.

Solution 3: 3 M potassium acetate; 11.5% glacial acetic acid

294.4 g Potassium acetate

115 ml glacial acetic acid

Make to 1L with H₂O.

APPENDIX B

TECHNICAL DIFFICULTIES ENCOUNTERED AND ADDITIONAL PROTOCOLS USED

1) Primer Extension Analysis

We initially attempted to isolate the transcription start site of the rat ACC β gene using the primer extension analysis. Attempts were made to optimise this method by increasing the mRNA template through the isolation of mRNA from cardiac tissue using the Oligotex™ mRNA Mini Kit (Promega Corporation). Various experimental parameters were also varied. Measures were also taken to reduce RNA degradation such as using DEPC-treated water and tubes as well as sterile RNase free pipette tips. However, efforts to optimise this method proved to be unsuccessful. This was most likely due to:

- i) the low levels of ACC β mRNA and
- ii) the instability of the mRNA molecules which are easily degraded in spite of stringent measures taken to prevent RNA degradation.

The protocol used for primer extension analysis is as follows:

Primer extension was carried out using variations of protocols adapted from Haas and Strauss [Haas, 1990 #68] and Lay et al [Lay, 2000 #67]. Very briefly, 13 μ g of total RNA or 2 μ g mRNA was annealed to 0.4 pmol of the ACC inner primer at 80°C for 5 minutes, thereafter the temperature was decreased at a rate of 5°C / minute. The reaction was carried out in an annealing buffer (2 mM Tris pH 8.0, 30 mM KCl, 0.2 mM EDTA) in a final volume of 15 μ l. This was followed by an extension step, 15 μ l of extension buffer consisting of: 30 mM Tris pH 8.0, 15 mM MgCl₂, 8 mM DTT, 0.2 mM dNTP including α^{32} P-dCTP, 40U Superscript™II RNAaseH⁻ Reverse transcriptase (Life Technologies) was added to the annealing mixture and allowed to incubate at 42°C for 1 hour. Reactions were carried out in a GeneAmp PCR System 2400 (Applied Biosystems) taking the proper safety precautions. Five microlitres of the reaction mix was run in a 6% denaturing polyacrylamide gel at 150V for 4 hours. The gel was transferred to Whattman 3MM paper after the run, dried in a gel dryer and autoradiographed at -80°C using X-ray film (Amersham). This protocol did not yield results therefore, a 5'- rapidly amplified cDNA ends (5'-RACE) protocol was used.

2) Calcium Phosphate Transfection Method

To start we attempted to transiently transfect CV-1 cells using variations of calcium phosphate method. In order to establish a protocol for transfection using this method, CV-1 cells were transfected with the pGL2-Control vector (Promega Corporation). Two variations of the calcium phosphate protocols, Graham et al [Graham, 1991 #66] and the Profection® Mammalian Transfection Systems (Promega Corporation) were used to find the most efficient protocol.

Numerous transfection experiments were performed in order to optimise conditions including the amount of time cells were exposed to the precipitate and the amount of vector used. However, although cells were transfected, there was a high level of cell death and cells changed morphology becoming more rounded in shape and lifting off the cell culture plates following transfection indicating that the precipitate damaged the cells. We found that this problem could be avoided

using a liposomal transfection reagent, FuGENE 6 Transfection Reagent (Roche) and established the following protocol which was used in this study.

3) Site-directed Mutagenesis

The QuikChange® Site-Directed Mutagenesis Kit (Stratagene) was unsuccessfully used to introduce a point mutation into the E-box at position -71/-66 of the 5'-UTR of the rat ACC β gene in the construct, ACC257.luc. The following complementary primers were synthesised according to the manufacturer's instructions:

Forward: SDMfwd

5'-CCA ATA CCC TTG CCC CAC ACG CTG TGT TAG AGA AAA TTC-3'

Reverse: SDMrev

5'-GAA TTT TCT CTA ACA CAG CGT GTG GGG CAA GGG TAT TGG-3'

The base change introduced is indicated in bold.

In brief, the PCR-based method used is as follows:

ACC257.luc plasmid which is methylated was isolated as described above and used as a template in a PCR reaction using the primers SDMfwd and SDMrev. *PfuTurbo*® DNA polymerase which replicates both strands of the plasmid with high fidelity without removing the mutant oligonucleotide primers was included in the reaction to synthesise a mutated plasmid. The PCR product was treated with the restriction enzyme *Dpn I* to digest the methylated parent DNA strand. The *Dpn I* treated reaction was transformed into supercompetent XL1-Blue cells. This protocol did not yield the desired mutated plasmid.

Analysis of the initial PCR product showed that the PCR did not yield product, several endeavours were made to optimise PCR conditions by:

- i) changing cycling parameters
- ii) using different amounts of template.
- iii) confirming template purity,
- iv) verifying primer integrity.

REFERENCES

1. Haas, R.C. and A.W. Strauss, Separate nuclear genes encode sarcomere-specific and ubiquitous human mitochondrial creatine kinase isoenzymes. *J Biol Chem*, 1990. 265(12): p. 6921-7.
2. Lay, J.M., et al., Structure and developmental expression of the mouse CCK-B receptor gene. *Biochem Biophys Res Commun*, 2000. 272(3): p. 837-42.
3. Graham, F.L. and L. Prevec, Chapter 11, Manipulation of Adenovirus vectors, in *Methods in Molecular Biology*, Vol 7: Gene Transfer and Expression Protocols, E.J. Murray, Editor. 1991, The Humana Press Inc.: Clifton, NJ.

APPENDIX C

RESULTS OF SCAN FOR TRANSCRIPTION FACTORS

Factor or Site Name	Loc.(Str.)	Signal Sequence	SITE #
ABREA2HVA1	site 434 (-)	CCTACGTGGC	<u>S000140</u>
ABREATCONSENSUS	site 434 (-)	YACGTGGC	<u>S000406</u>
ABRELATERD1	site 436 (-)	ACGTG	<u>S000414</u>
ACGTABREMOTIFA2OSEM	site 434 (-)	ACGTGKC	<u>S000394</u>
ACGTATERD1	site 437 (+)	ACGT	<u>S000415</u>
ACGTATERD1	site 437 (-)	ACGT	<u>S000415</u>
AMMORESIIUDCRN1A1	site 658 (-)	GGWAGGGT	<u>S000374</u>
AMYBOX1	site 176 (-)	TAACARA	<u>S000020</u>
ARFAT	site 282 (+)	TGTCTC	<u>S000270</u>
BOXIIPCCHS	site 434 (-)	ACGTGGC	<u>S000229</u>
C8GCARGAT	site 339 (+)	CWWWWWWWWG	<u>S000410</u>
C8GCARGAT	site 339 (-)	CWWWWWWWWG	<u>S000410</u>
CAATBOX1	site 738 (+)	CAAT	<u>S000028</u>
CAATBOX1	site 862 (+)	CAAT	<u>S000028</u>
CAATBOX1	site 182 (-)	CAAT	<u>S000028</u>
CAATBOX1	site 424 (-)	CAAT	<u>S000028</u>
CAATBOX1	site 452 (-)	CAAT	<u>S000028</u>
CAATBOX1	site 706 (-)	CAAT	<u>S000028</u>
CAATBOX1	site 840 (-)	CAAT	<u>S000028</u>
CANBNNAPA	site 884 (-)	CNAACAC	<u>S000148</u>
CARGCW8GAT	site 339 (+)	CWWWWWWWWG	<u>S000431</u>
CARGCW8GAT	site 339 (-)	CWWWWWWWWG	<u>S000431</u>
CCAATBOX1	site 861 (+)	CCAAT	<u>S000030</u>
CCAATBOX1	site 182 (-)	CCAAT	<u>S000030</u>
CCAATBOX1	site 452 (-)	CCAAT	<u>S000030</u>
CEREGLUBOX2PSLEGA	site 566 (+)	TGAAAACT	<u>S000033</u>
DOFCOREZM	site 630 (+)	AAAG	<u>S000265</u>
DOFCOREZM	site 259 (-)	AAAG	<u>S000265</u>
DOFCOREZM	site 572 (-)	AAAG	<u>S000265</u>
DOFCOREZM	site 962 (-)	AAAG	<u>S000265</u>
DPBFCOREDCCDC3	site 278 (-)	ACACNNG	<u>S000292</u>
EBOXBNNAPA	site 174 (+)	CANNTG	<u>S000144</u>
EBOXBNNAPA	site 192 (+)	CANNTG	<u>S000144</u>
EBOXBNNAPA	site 354 (+)	CANNTG	<u>S000144</u>
EBOXBNNAPA	site 400 (+)	CANNTG	<u>S000144</u>
EBOXBNNAPA	site 717 (+)	CANNTG	<u>S000144</u>
EBOXBNNAPA	site 876 (+)	CANNTG	<u>S000144</u>
EBOXBNNAPA	site 174 (-)	CANNTG	<u>S000144</u>
EBOXBNNAPA	site 192 (-)	CANNTG	<u>S000144</u>
EBOXBNNAPA	site 354 (-)	CANNTG	<u>S000144</u>
EBOXBNNAPA	site 400 (-)	CANNTG	<u>S000144</u>
EBOXBNNAPA	site 717 (-)	CANNTG	<u>S000144</u>
EBOXBNNAPA	site 876 (-)	CANNTG	<u>S000144</u>
GATABOX	site 186 (+)	GATA	<u>S000039</u>
GATABOX	site 516 (+)	GATA	<u>S000039</u>
GATABOX	site 28 (-)	GATA	<u>S000039</u>
GATABOX	site 104 (-)	GATA	<u>S000039</u>
GATABOX	site 138 (-)	GATA	<u>S000039</u>
GATABOX	site 844 (-)	GATA	<u>S000039</u>
GCN4OSGLUB1	site 598 (-)	TGAGTCA	<u>S000277</u>
GT1CONSENSUS	site 893 (+)	GRWAAW	<u>S000198</u>
GT1CONSENSUS	site 997 (-)	GRWAAW	<u>S000198</u>
GT1CONSENSUS	site 677 (-)	GRWAAW	<u>S000198</u>
GTGANTG10	site 427 (+)	GTGA	<u>S000378</u>
GTGANTG10	site 597 (+)	GTGA	<u>S000378</u>
GTGANTG10	site 399 (-)	GTGA	<u>S000378</u>
GTGANTG10	site 746 (-)	GTGA	<u>S000378</u>
IBOXCORE	site 186 (+)	GATAA	<u>S000199</u>
INRNTPSADB	site 131 (+)	YTCANTYY	<u>S000395</u>
INRNTPSADB	site 532 (+)	YTCANTYY	<u>S000395</u>
INTRONLOWER	site 333 (-)	TGCAGG	<u>S000086</u>
LRENPCABE	site 433 (-)	ACGTGGCA	<u>S000231</u>
MARTBOX	site 308 (+)	TTWTWTTTWT	<u>S000067</u>
MYB1AT	site 188 (+)	WAACCA	<u>S000408</u>
MYB2CONSENSUSAT	site 192 (-)	YAACKG	<u>S000409</u>
MYB2CONSENSUSAT	site 900 (-)	YAACKG	<u>S000409</u>

MYBCORE	site	192 (+)	CNGTTR	<u>S000176</u>
MYBCORE	site	900 (+)	CNGTTR	<u>S000176</u>
MYBGAHV	site	176 (-)	TAACAAA	<u>S000181</u>
MYBST1	site	185 (+)	GGATA	<u>S000180</u>
MYBST1	site	104 (-)	GGATA	<u>S000180</u>
MYBST1	site	844 (-)	GGATA	<u>S000180</u>
MYCATERD1	site	354 (-)	CATGTG	<u>S000413</u>
MYCATERD1	site	876 (-)	CATGTG	<u>S000413</u>
MYCATRD22	site	354 (+)	CACATG	<u>S000174</u>
MYCATRD22	site	876 (+)	CACATG	<u>S000174</u>
MYCCONSUSAT	site	174 (+)	CANNTG	<u>S000407</u>
MYCCONSUSAT	site	192 (+)	CANNTG	<u>S000407</u>
MYCCONSUSAT	site	354 (+)	CANNTG	<u>S000407</u>
MYCCONSUSAT	site	400 (+)	CANNTG	<u>S000407</u>
MYCCONSUSAT	site	717 (+)	CANNTG	<u>S000407</u>
MYCCONSUSAT	site	876 (+)	CANNTG	<u>S000407</u>
MYCCONSUSAT	site	174 (-)	CANNTG	<u>S000407</u>
MYCCONSUSAT	site	192 (-)	CANNTG	<u>S000407</u>
MYCCONSUSAT	site	354 (-)	CANNTG	<u>S000407</u>
MYCCONSUSAT	site	400 (-)	CANNTG	<u>S000407</u>
MYCCONSUSAT	site	717 (-)	CANNTG	<u>S000407</u>
MYCCONSUSAT	site	876 (-)	CANNTG	<u>S000407</u>
NAPINMOTIFBN	site	2 (+)	TACACAT	<u>S000070</u>
NAPINMOTIFBN	site	83 (-)	TACACAT	<u>S000070</u>
NAPINMOTIFBN	site	346 (-)	TACACAT	<u>S000070</u>
POLASIG1	site	300 (-)	AATAAA	<u>S000080</u>
POLASIG1	site	307 (-)	AATAAA	<u>S000080</u>
POLASIG1	site	312 (-)	AATAAA	<u>S000080</u>
POLASIG3	site	218 (-)	AATAAT	<u>S000088</u>
POLLEN1LELAT52	site	892 (+)	AGAAA	<u>S000245</u>
POLLEN1LELAT52	site	222 (-)	AGAAA	<u>S000245</u>
POLLEN1LELAT52	site	504 (-)	AGAAA	<u>S000245</u>
POLLEN1LELAT52	site	831 (-)	AGAAA	<u>S000245</u>
POLLEN1LELAT52	site	963 (-)	AGAAA	<u>S000245</u>
POLLEN1LELAT52	site	980 (-)	AGAAA	<u>S000245</u>
QELEMENTZMZM13	site	378 (+)	AGGTCA	<u>S000254</u>
QELEMENTZMZM13	site	812 (+)	AGGTCA	<u>S000254</u>
RAV1AAT	site	779 (+)	CAACA	<u>S000314</u>
RAV1BAT	site	400 (+)	CACCTG	<u>S000315</u>
ROOTMOTIFTAPOX1	site	65 (+)	ATATT	<u>S000098</u>
ROOTMOTIFTAPOX1	site	519 (+)	ATATT	<u>S000098</u>
ROOTMOTIFTAPOX1	site	78 (-)	ATATT	<u>S000098</u>
RYREPEATBNNAPA	site	10 (+)	CATGCA	<u>S000264</u>
RYREPEATBNNAPA	site	734 (+)	CATGCA	<u>S000264</u>
RYREPEATLEGUMINBOX	site	10 (+)	CATGCAY	<u>S000100</u>
TATABOX5	site	219 (+)	TTATTT	<u>S000203</u>
TATABOX5	site	301 (+)	TTATTT	<u>S000203</u>
TATABOX5	site	308 (+)	TTATTT	<u>S000203</u>
TATCCAOSAMY	site	844 (+)	TATCCA	<u>S000403</u>
TATCCAOSAMY	site	184 (-)	TATCCA	<u>S000403</u>
TBOXATGAPB	site	571 (+)	ACTTTG	<u>S000383</u>
TRANSINITDICOTS	site	671 (-)	AMNAUGGC	<u>S000201</u>
TRANSINITMONOCOTS	site	500 (-)	RMNAUGGC	<u>S000202</u>
TRANSINITMONOCOTS	site	671 (-)	RMNAUGGC	<u>S000202</u>
WBOXATNPR1	site	932 (+)	TTGAC	<u>S000390</u>

<http://www.dna.affrc.go.jp/sigscan/signal1.pl>

RESULTS OF TRANSFAC SEARCH

Factor	Model	Begin	Length	Sequence
T00133 c-Jun	R00007 ()	597	8	GTGACTCA
T00601 NF-1 (-like proteins)	R00015 ()	748	7	ACACCAC
T00528 myogenin	R00018 ()	972	7	TGCCTGG
T01786 E12	R00019 ()	400	6	CACCTG
	R03330 ()			
T00369 HNF-1	R00077 ()	773	6	GGTTAG
T00599 NF-1/L	R00078 ()	195	6	TTGGCA
T00108 C/EBPalpha	R00103 ()	869	9	CTTGCCNCA
	R01344 ()			
T00108 C/EBPalpha	R00104 ()	579	8	TTNNGTAA
T00825 Tf-LF1	R00113 ()	378	10	AGGTCAaAGG
T00825 Tf-LF1	R00113 ()	812	10	AGGTCAaAGG

T00029 AP-1	R00133 ()	598	7	TGACTCA
	R00196 ()			
	R00805 ()			
	R01037 ()			
	R01776 ()			
	R01777 ()			
	R01946 ()			
	R02573 ()			
	R02666 ()			
	R02676 ()			
	R02887 ()			
	R03439 ()			
	R04288 ()			
	R04348 ()			
	R05093 ()			
T00923 Zta	R00296 ()	262	7	TGTGTCA
	R01412 ()			
T00923 Zta	R00298 ()	262	7	TGTGTCA
	R00299 ()	797	6	AGGACA
T00333 GR	R00429 ()			
	R01030 ()			
	R02993 ()			
	R02994 ()			
T00306 GATA-1	R00566 ()	137	6	CTATCT
	R03491 ()			
	R08264 ()			
	R08265 ()			
	R08276 ()			
T00321 GCN4	R00645 ()	598	6	TGACTC
	R00829 ()			
T00321 GCN4	R00648 ()	598	6	TGACTC
	R00651 ()			
	R00655 ()			
T00321 GCN4	R00656 ()	477	6	CAGTCA
T00360 HiNF-A	R00658 ()	156	7	TGTGTTT
T00360 HiNF-A	R00686 ()	502	7	CATTTCT
T00360 HiNF-A	R00686 ()	829	7	CATTTCT
T00870 USF	R00711 ()	596	6	CGTGAC
T00386 HSTF	R00751 ()	587	11	GAANN TTCNNG
T00386 HSTF	R00752 ()	587	11	GAANN TTCNNG
T00918 Zeste	R00830 ()	129	6	CACTCA
	R04938 ()			
	R04941 ()			
	R04954 ()			
T00321 GCN4	R00831 ()	967	6	TGACTT
T00321 GCN4	R00832 ()	526	6	TGATTC
T00215 muEBP-C2	R00844 ()	354	6	CACATG
	R00886 ()			
	R04432 ()			
T00215 muEBP-C2	R00844 ()	876	6	CACATG
	R00886 ()			
	R04432 ()			
T00563 NF-muE3	R00887 ()	352	8	ACCACATG
T00333 GR	R00973 ()	577	6	TGTTCT
T00696 PR	R00974 ()	22	6	TGTACA
	R01811 ()			
T00696 PR	R00974 ()	22	6	TGTACA
	R01811 ()			
T00798 TBP	R01014 ()	914	6	TATAAA
	R02247 ()			
	R03172 ()			
	R03173 ()			
	R03840 ()			
	R08480 ()			
T00051 ATF	R01053 ()	376	11	TGAGGTCAGgG
T00163 CREB	R01053 ()	810	11	TGAGGTCAGgG
T00033 AP-2alphaA	R01054 ()	207	6	TCCCCA
T00721 RAR-beta	R01063 ()	377	10	GAGGTCAGgG
T00721 RAR-beta	R01063 ()	811	10	GAGGTCAGgG
T00333 GR	R01101 ()	577	6	TGTTCT
	R01103 ()			
	R01106 ()			
	R01107 ()			

	R01108 ()			
	R01110 ()			
	R01117 ()			
	R01120 ()			
	R01121 ()			
	R01122 ()			
	R01312 ()			
	R01547 ()			
	R01549 ()			
	R01550 ()			
	R02995 ()			
	R03890 ()			
T01505 F2F	R01276 ()	310	6	ATTTTA
T00691 Pit-1a	R01280 ()	45	10	ATATATtCAT
T00691 Pit-1a	R01280 ()	517	10	ATATATTCaT
T00335 GR	R01313 ()	255	6	AGTTCT
T00335 GR	R01313 ()	464	6	TCTTCT
T00335 GR	R01313 ()	590	6	TCTTCT
T00108 C/EBPalpha	R01314 ()	869	9	CTTGCNNCA
T00238 EFII	R01342 ()	48	6	TATGCA
T00238 EFII	R01342 ()	50	6	TGCATA
T01379 120-kDa CRE-binding protein				
	R01358 ()	808	11	GCTGAcGTCAG
T00641 Oct-1	R01389 ()	735	10	ATGCAAaGCA
	R01397 ()			
T00918 Zeste	R01518 ()	129	6	CACTCA
	R01519 ()			
	R04939 ()			
	R04942 ()			
	R04943 ()			
	R04944 ()			
	R04945 ()			
	R04953 ()			
	R04955 ()			
T00697 PR	R01556 ()	145	7	TGTTACT
T00697 PR	R01556 ()	229	8	TGTTCCCA
T00034 AP-2	R01563 ()	207	10	TCCCCAGGAg
T00196 DI	R01718 ()	976	10	gGGTTTTCTC
T00759 Sp1	R01761 ()	637	10	GCGGCCCTCc
T00333 GR	R01815 ()	721	6	TGAACT
T00201 DTF-1	R01874 ()	778	8	GCAACATG
T00499 MCBF	R02073 ()	521	6	ATTCCT
	R02074 ()			
T00499 MCBF	R02073 ()	528	6	ATTCCT
	R02074 ()			
T00499 MCBF	R02073 ()	535	6	ATTCCT
	R02074 ()			
T00499 MCBF	R02075 ()	534	73	CATTCCT
	R02200 ()			
T00875 USF	R02079 ()	354	6	CACATG
	R03501 ()			
T00875 USF	R02079 ()	876	6	CACATG
	R03501 ()			
T00684 PEA3	R02095 ()	678	6	TTTCCT
T00869 URTF	R02096 ()	376	8	TGAGGTCA
	R08476 ()			
	R08481 ()			
T00869 URTF	R02096 ()	810	8	TGAGGTCA
	R08476 ()			
	R08481 ()			
T00029 AP-1	R02120 ()	598	7	TKASTCA
T00027 AP-1	R02120 ()	598	7	TGASTMA
T00115 c-Ets-1 68	R02153 ()	688	8	RCWTCCKS
T00017 C/EBPbeta	R02170 ()	231	7	TTYCCAG
T00017 C/EBPbeta	R02170 ()	446	7	CTGGRAA
T00505 MEF-2	R02201 ()	301	10	YTATTTWWaR
T00991 PPAR	R02233 ()	378	6	AGGTCA
	R03961 ()			
T00991 PPAR	R02233 ()	812	6	AGGTCA
	R03961 ()			
T00803 TCF-2alpha	R02249 ()	688	8	RCTTCCTS
T00119 CF2-I	R02316 ()	75	9	TATAATATA
T01945 NF-ATc	R02348 ()	677	6	TTTTCC

T00774 su(Hw)	R02385 ()	731	12	RRRtATGCARYR
T00137 c-Myb	R02392 ()	192	6	CAGTTG
	R02893 ()			
	R04182 ()			
T00759 Sp1	R02446 ()	659	11	CCCcTCCCCTC
T00922 Zmhox1a	R02476 ()	245	11	AGCTGCTCcTA
T00395 Hb	R02500 ()	301	10	TTATTTTTTt
T00395 Hb	R02500 ()	308	10	TTATTTTTTT
T00395 Hb	R02554 ()	304	10	TTTTTTATgT
T00395 Hb	R02606 ()	176	10	TTTTTTATKS
T00395 Hb	R02606 ()	304	10	TTTTTTATKs
T00395 Hb	R02607 ()	254	13	TANTTtTTTGNGT
T00018 AhR	R02656 ()	964	1	YNSNRNSTNGCgTGNNW
T00572 NF-GMa	R02684 ()	584	10	gAAGAATCTT
T00572 NF-GMa	R02684 ()	585	10	AAGAItCTTC
T00019 AhR	R02720 ()	321	12	WWGCGTGWNWNG
T00627 NIT2	R02725 ()	28	6	TATCTA
	R02728 ()			
T00627 NIT2	R02725 ()	138	6	TATCTA
	R02728 ()			
T00029 AP-1	R02734 ()	598	7	TGACTCA
	R03059 ()			
	R03440 ()			
	R03540 ()			
	R03910 ()			
	R04261 ()			
	R08301 ()			
	R08470 ()			
	R08472 ()			
	R08473 ()			
	R08488 ()			
	R08489 ()			
T00691 Pit-1a	R02789 ()	6	7	CATTCAT
T00196 DI	R02854 ()	976	10	TGGTTTTCCc
T00989 CREB	R02863 ()	376	8	TGAGGTCA
T00989 CREB	R02863 ()	810	6	TGAGGTCA
T00305 GATA-1	R02883 ()	137	6	CTATCT
	R04294 ()			
T00306 GATA-1	R02885 ()	904	6	TGATCA
T00590 NF-kappaB	R02909 ()	892	10	AGgAAATTCC
T00160 CPC1	R02976 ()	597	9	GTGACTCAT
T00698 PR	R02996 ()	797	6	AGGACA
T00517 myc-CF1	R02999 ()	672	6	CCATGT
T00092 CCAAT-binding factor	R03016 ()	618	10	GCAGATtGGC
T00395 Hb	R03023 ()	159	10	ITTTTCAAGG
T00108 C/EBPalpha	R03069 ()	869	9	CTTGCCCCA
T00913 Yi	R03154 ()	874	10	CCNCNINNCT
T00691 Pit-1a	R03191 ()	45	10	ATtTATGCAT
T00535 NF-1	R03309 ()	457	6	AGCCAG
T00923 Zta	R03344 ()	497	7	TGAGCCA
T00923 Zta	R03345 ()	775	7	TTAGCAA
T00960 ANF-1	R03413 ()	466	10	TTgTGCAAGA
T00641 Oct-1	R03417 ()	731	10	GgGCATGCAA
T00196 DI	R03463 ()	282	10	KGKwTYTCCC
T00196 DI	R03463 ()	976	10	KGKWTYTCCc
T00333 GR	R03537 ()	262	6	TGTGTC
T00264 ER	R03566 ()	378	6	AGGTCA
	R03567 ()			
	R03569 ()			
T00264 ER	R03566 ()	812	6	AGGTCA
	R03567 ()			
	R03569 ()			
T00997 SRY	R03579 ()	259	7	CTTTGTG
T00997 SRY	R03579 ()	572	7	CTTTGTG
T00997 SRY	R03581 ()	175	7	ATTTGTT
T00505 MEF-2	R03584 ()	301	10	TTATTTTTaA
T01040 Olf-1	R03617 ()	207	10	
T01040 Olf-1	R03621 ()	207	11	TCCCCAGGgAG
T00105 C/EBPalpha	R03657 ()	384	8	GAGGAGGG
T00003 AS-C T3	R03718 ()	400	6	CACCTG
	R03719 ()			
	R04144 ()			
T00715 RAP1	R03739 ()	125	10	CANCCNNNCA

T00715 RAP1	R03739 ()	264	10	TGNNNGGNTG
T00715 RAP1	R03739 ()	933	10	TGNNNGGNTG
T00096 CCBF	R03740 ()	592	12	TTTCGTGRNNY
T01246 TBF1	R03755 ()	107	6	CCCTAA
T01085 abaA	R03759 ()	534	6	CATTCC
	R03760 ()			
	R03761 ()			
	R03765 ()			
	R03766 ()			
	R03768 ()			
	R03769 ()			
	R03770 ()			
	R03771 ()			
	R03772 ()			
	R03777 ()			
	R03784 ()			
T01085 abaA	R03771 ()	534	6	CATTCC
	R03774 ()			
	R03775 ()			
	R03776 ()			
T00798 TBP	R03841 ()	300	7	TTTATTT
T00798 TBP	R03841 ()	307	7	TTTATTT
T01035 Isl-1	R03914 ()	141	6	CTAATG
	R03915 ()			
	R04227 ()			
T01351 T3R-alpha	R03947 ()	711	15	GGAAcNNNNNTGAAC
T00719 RAR-alpha1	R03949 ()	379	15	GGTCANNNNNNGGtCA
T01351 T3R-alpha	R03951 ()	711	15	GGACCNNNNNTGAAC
T01345 RXR-alpha	R03980 ()	378	16	AGGTCANNNNAGGtCA
	R04508 ()			
T01649 HES-1	R03990 ()	594	6	CTCGTG
	R04469 ()			
	R04470 ()			
T00794 TBP	R04010 ()	304	7	TTTTTTA
T00115 c-Ets-1 68	R04065 ()	687	10	TGCTTCCTGc
T01422 Ste11	R04089 ()	257	10	TTCTTTGTtY
T00557 NF-E2	R04115 ()	494	11	YGCTGAStCAY
T00137 c-Myb	R04174 ()	398	6	CTCACC
T00137 c-Myb	R04181 ()	699	6	
T00137 c-Myb	R04183 ()	192	6	CAGTTG
T01463 MAF	R04194 ()	868	9	MCTTSCYYC
T01469 Ik-1	R04204 ()	447	7	TGGGAAT
	R04205 ()			
	R04206 ()			
	R04207 ()			
T01469 Ik-1	R04209 ()	231	4	TTCCCA
T01469 Ik-1	R04209 ()	447	6	TGGGAA
T01469 Ik-1	R04210 ()	448	6	GGGAAT
T00183 DBP	R04248 ()	578	10	RTTaYGTAAR
T01498 IL-6 RE-BP	R04252 ()	232	6	TCCCAG
T01498 IL-6 RE-BP	R04252 ()	446	6	CTGGGA
T01498 IL-6 RE-BP	R04252 ()	550	6	CTGGGA
T00305 GATA-1	R04289 ()	136	6	TCTATC
T00305 GATA-1	R04296 ()	136	6	TCTATC
T01517 Twi	R04317 ()	174	6	CATTTG
T01517 Twi	R04317 ()	717	6	CAAATG
T00630 N-Oct-3	R04327 ()	175	10	ATTWNNNATK
T00630 N-Oct-3	R04327 ()	303	10	ATTWNNNATK
T00630 N-Oct-3	R04327 ()	315	9	ATTWNNATK
T00630 N-Oct-3	R04327 ()	414	10	ATTWNNNATK
T00113 c-Ets-2	R04343 ()	205	6	CTTCCC
T00113 c-Ets-2	R04343 ()	661	6	CTTCCC
T00113 c-Ets-2	R04343 ()	926	6	CTTCCC
T00702 PU.1	R04413 ()	529	6	TTCCTC
T01067 AML1	R04441 ()	352	6	ACCACA
T01067 AML1	R04441 ()	713	6	ACCACA
T01067 AML1	R04441 ()	750	6	ACCACA
T01067 AML1	R04441 ()	789	6	TGTGGT
T01649 HES-1	R04468 ()	787	6	CTTGTG
T00851 T3R-beta	R04771 ()	147	6	TtACTT
T00278 delta factor	R04898 ()	173	6	CCATTT
T00278 delta factor	R04898 ()	501	6	CCATTT
T00278 delta factor	R04898 ()	828	6	CCATTT

T00278 delta factor	R04898 ()	995	6	CCATTT
T00918 Zeste	R04947 ()	545	6	TGAGTC
T00918 Zeste	R04947 ()	599	6	GACTCA
T02416 SpOtx	R04993 ()	216	6	AGATTA
	R08290 ()			
T02457 AML1	R05028 ()	763	6	CCCACA
T02457 AML1	R05028 ()	874	6	CCCACA
T00301 GAGA factor	R05037 ()	920	8	CTCTCTCT
T00291 FosB	R05052 ()	66	7	TATTACA
T00537 NF-1	R05057 ()	126	10	RRCCAaTSRG
T00084 CBF (2)	R05057 ()	292	10	CYSATtGGYY
T00084 CBF (2)	R05057 ()	944	10	CYSATtGGYY
T01945 NF-ATc	R05080 ()	677	6	TTTTCC
T01944 NF-ATp	R05082 ()	455	6	GGAGCC
T00029 AP-1	R05098 ()	404	7	TGAAACT
T02521 SpRunt-1	R08077 ()	712	7	GACCACA
T02521 SpRunt-1	R08077 ()	789	7	TGTGGTC
T00590 NF-kappaB	R08108 ()	893	10	GAAAATTCCc
T00138 c-Myb	R08124 ()	900	6	CCGTTG
T00112 c-Ets-1	R08152 ()	210	8	CCAGGAAG
T00112 c-Ets-1	R08152 ()	689	8	CTTCCTGG
T00111 c-Ets-1	R08168 ()	211	7	CAGGAAG
T00111 c-Ets-1	R08168 ()	689	7	CTTCCTG
T00702 PU.1	R08195 ()	591	6	CTTCTC
T00702 PU.1	R08195 ()	821	6	GAGAAG
T00702 PU.1	R08195 ()	957	6	CTTCTC
T00311 GATA-3	R08270 ()	27	6	ATATCT
T02711 GATA-4	R08277 ()	27	6	ATATCT
T02690 Dof2	R08282 ()	956	10	GCTTCTtTTT
T02786 RITA-1	R08319 ()	375	10	CTGAcGTCAG
T02786 RITA-1	R08319 ()	375	10	CTGAcGTCAG
T02786 RITA-1	R08319 ()	809	10	CTGAcGTCAG
T02808 REB	R08325 ()	434	10	GCCACGTAAg
	R08452 ()			
	R08453 ()			
T02803 LIP15	R08439 ()	435	6	CCACGT
T02807 OSBZ8	R08450 ()	436	6	CACGTA