Molecular mechanisms controlling the tissue-specific activity of the nutritionally regulated promoter I of the acetyl-CoA carboxylase-α encoding gene in cattle

Inaugural dissertation for the academic degree

Doctor rerum naturalium

of

Mathematisch-Naturwissenschaftlichen Fakultät

Universität Rostock

By

Xuanming Shi (M. Sc.), born on 17-09-1975, in Anhui, China

From Forschungsinstitut für die Biologie landwirtschaftlicher Nutztiere in Dummerstorf

Rostock (2009)

urn:nbn:de:gbv:28-diss2009-0184-9

Dean: Prof. Dr. Hendrik Schubert

1. Reviewer: Prof. Dr. Hans-Martin Seyfert
   Molecular Biology Research Unit, Research Institute for the Biology of Farm Animals; Wilhelm-Stahl-Allee 2; D-18196 Dummerstorf; Germany.

2. Reviewer: Prof. Dr. Martin Hagemann
   Department of Plant Physiology, Institute of Life Sciences, University of Rostock; Albert-Einstein-Strasse 3, 18051 Rostock, Germany;

3. Reviewer: PD. Dr. Monika Schweigel
   Research Institute for the Biology of Farm Animals (FBN), Department of Nutritional Physiology "Oskar Kellner," Wilhelm-Stahl-Alee 2, D-18196 Dummerstorf, Germany.

Date of defense: 19-Oct-2009

Dedicated to my family

TABLE OF CONTENTS

1 Introduction 1

1.1 Fat metabolism and acetyl-CoA carboxylase (ACC) 1
1.1.1 Fat deposition and mobilization 1
1.1.2 Biosynthesis of fatty acids and ACC 1
1.1.3 Roles of ACC in fatty acid synthesis and oxidation 2
1.1.4 Functional domains of the ACC-α 3

1.2 Regulation of ACC-α 4
1.2.1 Long-term regulation 4
1.2.1.1 Multi-promoters regulate *ACC-α* transcription initiation 5
1.2.1.2 Pre-mRNA splicing 7
1.2.1.3 Stability of mRNA 7
1.2.2 Short-term regulation 7
1.2.2.1 Allosteric regulation 8
1.2.2.2 Reversible phosphorylation 9

1.3 PI is regulated by a bipartite repressor 10

1.4 Transcription factors relevant to regulating *ACC-α* PI activity 11
1.4.1 Roles of CCAAT/Enhancer Binding Protein (C/EBP) 11
1.4.2 Roles of Nuclear Factor-Y (NF-Y) 14

1.5 Goals of this study 14

2 Materials and Methods 17

2.1 Materials 17
2.1.1 Bacterial strains 17
2.1.2 Reagents and antibodies 17
2.1.3 Plasmid vectors 18

2.2 Preparation of DNA 19
2.2.1 Mini-preparation of plasmid DNA 19
2.2.2 Midi-preparation of plasmid DNA 20
2.2.3 Preparation of genomic DNA 21
2.2.4 Preparation of BAC DNA 21

2.3 Preparation of RNA 22
2.3.1 Preparation of RNA from cells and tissues 22
2.3.2 Electrophoresis of RNA through agarose gels containing formaldehyde 23

2.4 Cloning of DNA 23
2.4.1 Digestion by restriction enzyme 23
2.4.2 Detection of DNA in Agarose Gels 24
2.4.3 Purification of DNA fragment from gel 24
2.4.4 Blunting with Klenow enzyme 24
2.4.5 Ligation 25
2.4.6 Transformation of DNA into *E. coli* 25
2.4.7 Screening of the recombinant clones 26

2.5 Polymerase Chain Reaction (PCR) 27
2.5.1 Primer design 28
2.5.2 Standard PCR 28
2.5.3 High-fidelity PCR 28
2.5.4 GC-rich PCR 29

2.5.5 Reverse transcription PCR (RT-PCR) ... 29
2.5.6 Rapid amplification of cDNA ends (RACE) ... 30
2.5.7 Genomic Walking ... 31
2.5.8 PCR- mediated site-directed mutagenesis ... 32
2.5.9 Quantitative Real-Time PCR ... 33

2.6 Cell culture and transfection ... 34
2.6.1 Cell line ... 34
2.6.2 Transient transfection ... 34
2.6.3 Luciferase activity assays ... 35

2.7 Preparation and purification of antigen and antibodies ... 35
2.7.1 Protein expression and preparation (for inclusion bodies) ... 35
2.7.2 Affinity purification via Ni-NTA sepharose ... 36
2.7.3 Measurement of protein concentration ... 37
2.7.4 SDS-PAGE electrophoresis ... 37
2.7.5 Antigen preparation ... 37
2.7.6 Immunization ... 38
2.7.7 Antibody purification ... 39

2.8 Immunological methods ... 40
2.8.1 Western blot ... 40
2.8.2 Protein-protein interaction by immunoprecipitation ... 41
2.8.3 Indirect sandwich ELISA for bovine ACC-α ... 41

2.9 Southern blot ... 43

2.10 Eletrophoretical Mobility Shift Assay ... 45

2.11 Chromatin Immunoprecipitation ... 47

3 Results ... 50

3.1 Developing basic knowledge and tools to the PI repressor ... 50

3.2 PI is nutritionally regulated in different tissues and physiological conditions ... 52
3.2.1 *ACC-α* PI is repressed *in vivo* by starvation ... 52
3.2.2 Starch-enriched diet enhances, but lactation silences *ACC-α* transcription in adipose tissue 53

3.3 Delineation of the repression elements and factors in the distal region of PI ... 54
3.3.1 Elements contributing to PI repression ... 54
3.3.1.1 Delineation of *cis*-relevant DNA-sequence element of the PI repressor ... 54
3.3.1.2 Element A and C, but not B, repress PI activity ... 55
3.3.2 Identification of transcription factors binding to EA and EC ... 56
3.3.2.1 Delineation of the factors binding to EA ... 56
3.3.2.1.1 The left side of EA is relevant for PI repression in HC11 ... 56
3.3.2.1.2 NF-Y and GLI may bind to EA ... 57
3.3.2.1.3 Mutation of the CCAAT binding motif of NF-Y relieves repression ... 59
3.3.2.2 Delineation the factors binding to EC ... 60
3.3.2.2.1 Element C is bound by C/EBP factors ... 60
3.3.2.2.2 C/EBPβ and -ε can bind to the C/EBP binding site in EC ... 61
3.3.2.2.3 Mutation of C/EBP binding site (site1) in EC relieves repression ... 62
3.3.3 Functional analyses of NF-Y and C/EBP factors on regulation of PI ... 63

3.4 Genetic control elements in the proximal PI ... 65
3.4.1 *Cis*-elements in the proximal PI are conserved among species ... 65
3.4.2 NF-Y activates proximal PI by binding to its proximal binding site in PI ... 67
3.4.2.1 Interaction of NF-Y with the its proximal binding site results in activation of PI .. 67
3.4.2.2 NF-Y binds to proximal NF-Y binding motif ... 68

3.4.3 C/EBP factors activate the proximal PI promoter by interaction with the C/EBP site3 of proximal PI 69
3.4.3.1 C/EBP factors bind to the C/EBP site3, but not site2 69
3.4.3.2 C/EBPα, -β and -ε may interact with the C/EBP site3 71
3.4.3.3 Low amounts of C/EBPα and -ε activate PI by dose dependent way..... 72
3.4.3.4 High amounts of C/EBPβ and -δ activate proximal PI..... 73
3.4.3.5 Mutation of the C/EBP site blocks the activation by full length C/EBP proteins... 74
3.4.3.6 DN-C/EBPs block its activation by full length C/EBPα and ε..... 76

3.5 The molecular mechanism of PI repression 77
3.5.1 NF-Y synergistically represses C/EBPβ mediated activation of the proximal PI by specific protein-protein interaction..... 77
3.5.1.1 NF-Y specifically represses C/EBPβ mediated activation on proximal PI..... 77
3.5.1.2 All subunits of NF-Y are indispensable for the repression of C/EBPβ activated proximal PI78
3.5.1.3 NF-Y and C/EBPβ bind to proximal PI by specific protein-protein interaction..... 79
3.5.2 Affinity comparison of the *cis*-elements in different region of PI..... 80
3.5.2.1 The NF-Y binding site in the proximal region has higher affinity than the repressive binding site in the distal region..... 81
3.5.2.2 The C/EBP site in the proximal region has a higher affinity than the repressive site in distal region 82

3.6 The associations of the levels of NF-Y and C/EBP factors with PI activity 84
3.6.1 The proportion of NF-Y over C/EBPβ correlates with the tissue specific level of PI activity 84
3.6.2 mRNA levels of NF-Y and C/EBP factors may not be regulated in the liver 86
3.6.3 C/EBPδ and –ε may be repressors, but C/EBPα and NF-YA may be activators in adipose tissues during the transition period (before and after calving) 86
3.6.4 Bovine C/EBPβ-Thr^{269} (relative mouse Thr^{217}) is phosphorylated in refed liver..... 87

4 Discussion..... 89

4.1 ACC-α is the regulation target of lipid metabolism in cattle..... 89

4.2 PI is the nutritionally regulated promoter 90
4.2.1 PIn is not the principle promoter in lipogenic tissues 90
4.2.2 PI is the key promoter relevant for the nutritional regulation of fatty acid synthesis in lipogenic tissues..... 91

4.3 Characterization of a master control unit for PI promoter 91
4.3.1 NF-Y and C/EBP factors function as repressors via the distal PI promoter..... 91
4.3.2 NF-Y and C/EBP factors function as activators via the proximal PI promoter..... 92
4.3.3 Both NF-Y and C/EBP factors are of key importance for PI regulation 93
4.3.4 Both the driver sites have vastly different binding affinities from the repressor sites ... 94
4.3.5 NF-Y represses the activating capability of C/EBPβ 95
4.3.6 NF-Y and C/EBPβ may physically interact via protein-protein interaction..... 95
4.3.7 The mRNA proportion of NF-YA over C/EBPβ is greatly correlated in a tissue-specific manner with the *ACC-α* PI activity 96
4.3.8 All C/EBP family members potentially regulate ruminant and rodent *ACC-α* PI..... 98
4.3.8.1 C/EBPα is a tissues-specific regulable activator 98
4.3.8.2 C/EBPβ may regulate PI at different levels..... 98
4.3.9 The active NF-Y is regulated in a tissue-specific fashion in the protein level 99
4.3.10 The master control unit controlling PI activity..... 100

5 Summary..... 101

6 Acknowledgements 102

7 References 104

8 Appendix I

A. Isolation and identification of PIn of bovine *ACC-α* I

8.1 Isolation of exon1n and PIn I
8.1.1 Cloning of bovine ACC-α exon1n I
8.1.2 PIn is shared by *ACC-α* and *TADA2L* II
8.1.3 Cloning of the bovine *ACC-α* promoter PIn II
8.1.4 Sequence analysis of PIn IV

8.2 Expression profiles and functional analysis of PIn promoter V
8.2.1 Bovine PIn is prominently active in the brain V
8.2.2 The basal activity of bovine *ACC-α* PIn is significantly repressed in HC11 cells V

B. Cloning of bovine transcriptional factors VI

8.3 Cloning of four members (α, β, δ and ε) of bovine C/EBP family VI
8.3.1 Cloning of bovine C/EBPα VII
8.3.2 Cloning of bovine C/EBPβ VIII
8.3.3 Cloning of bovine C/EBPδ VIII
8.3.4 Cloning of bovine C/EBPε IX

8.4 Cloning of the DN-C/EBP series XI

8.5 Cloning of the three subunits of NF-Y XI

C. Establish an ELISA XIV

8.6 Establishment of an ELISA to measure the expression of bovine ACC-α XIV
8.6.1 Cloning and expression of truncated ACC-α XIV
8.6.2 Two-step purification of antigen: His-tag affinity chromatography and gel purification XV
8.6.3 Preparation and purification of polyclonal antibodies against bovine ACC-α XVI
8.6.4 The measurement sensitivity of ACC was greatly enhanced by ABC-ELISA XVII

D. List of Primers XIX

E. List of Figures XXII

F. List of tables XXII

G. cDNA sequence of bovine C/EBPδ XXIII

H. Exon1n Sequence of bovine ACC-*α* XXIV

I. PIn sequence XXIV

J. mRNA copies of relevant genes in the livers from fed and starved cows XXV

Abbreviation list XXV

1 Introduction

1.1 Fat metabolism and acetyl-CoA carboxylase (ACC)

1.1.1 Fat deposition and mobilization

Fat deposition and mobilization in the dairy cows are intriguing issues in the dairy industry. Chemically, fat generally refers to triester of glycerol and fatty acids. Functionally, fat is one of the major stores of energy and provides ATP for animals. Milk fat is an important energy source for an infant. To produce milk, its mother must store excessive fat than her own needs. This fat is required to be stored during dry standing. When an infant needs it, it is mobilized, transported via blood serum and secreted into milk. However, excessive mobilization might cause metabolic disorders (e. g. ketosis), especially within the first 2 weeks of lactation. Thus, the appropriate energy in diets is a key to avoid excessive mobilization of fat. It is valuable to know about fat metabolism for designing adequate diets.

1.1.2 Biosynthesis of fatty acids and ACC

The biosynthesis of fatty acids is a prominent metabolic process in most organisms. Because higher animals possess the limited capacity to store polysaccharides, if the ingested glucose exceeds immediate energy demands, the surplus will be stored as fat. Firstly, it is converted into pyruvate by glycolysis and then into acetyl-coenzyme A. Fatty acids are synthesized using acetyl-coenzyme A as a substrate and stored in adipose tissues and the mammary gland in triacylglycerol form. Besides serving as an energy storage form, the resulting fatty acids also participate in many important cellular functions, such as a transportable form of metabolic fuel, structural components of cell membranes, protective coating on the surface of the organism and cell signaling.

In higher animals, the biosynthesis of saturated fatty acids from their ultimate precursor acetyl-coenzyme A (CoA) occurs in all tissues, but is especially prominent in the liver, adipose tissues and mammary glands. Biosynthesis fatty acid is opposite to fatty acid oxidation. The former occurs in the cytosol, whereas the latter occurs in the mitochondria. Fatty acid synthase catalyzes one acetyl residue and seven malonyl residues and subsequently undergos successive condensation steps to form a molecule of palmitic acid. Palmitic acid is further converted into various fatty acid forms. The formulation of reaction is:

1 acetyl-CoA + 7 malonyl-CoA + 14 NADPH + 14 $H^+ \rightarrow CH_3(CH_2)_{14}COOH$ + 7 CO_2 + 8 CoA + 14 $NADP^+$ +6 H_2O (Lehninger, 1981).

In the biosynthesis course of palmitic acid, acetyl-CoA only provides one acetyl unit. The other seven units are required in the form of malonyl-CoA. Malonyl-CoA is generated by condensation of acetyl-CoA and HCO_3^-. Hence, acetyl-CoA, derived from carbohydrate or amino acid source, is the ultimate precursor (Figure 1) of all carbon atoms in a fatty acid chain. Acetyl-CoA carboxylase (ACC; EC 6.4.1.2), which catalyzes the synthesis of malonyl-CoA, is a key rate-limiting enzyme throughout fatty acid biosynthesis.

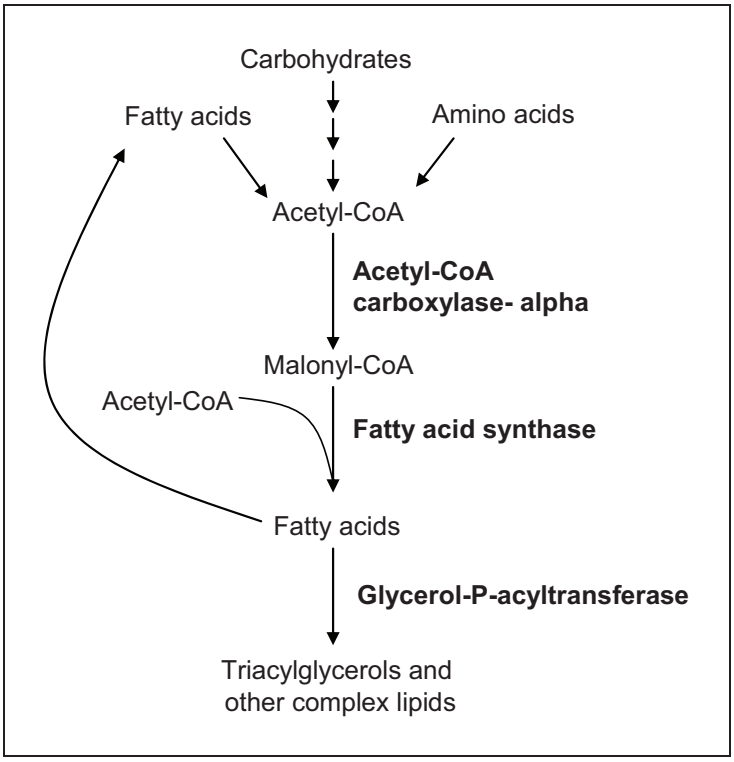


Figure 1 Acetyl-CoA is the key precursor in biosynthesis of lipids.

1.1.3 Roles of ACC in fatty acid synthesis and oxidation

There are two ACC systems to control the amounts of fatty acids in cells. They are ACC-α or ACC1 (ACACA) and ACC-β or ACC2 (ACACB). Though both ACC-α and ACC-β catalyze the synthesis of malonyl-CoA, they play distinct roles in various cellular compartments. ACC-α is necessary to form long chain fatty acids in the cytosol. ACC-α knock out mice are lethal in embryonic period demonstrating that *de novo* fatty acids synthesis is essential for embryonic development (Abu-Elheiga et al., 2005). However, liver-specific ACC-α knock out mice have no obvious health problems under normal feeding conditions. When compared with wild type mice, they exhibit lower ACC activity and lower malonyl-CoA levels. There was a significant decrease in *de novo* fatty acid synthesis and triglyceride accumulation in the liver (Mao et al., 2006).

Conversely, ACC-β catalyzes acetyl-CoA to form malonyl-CoA in mitochondria. The resulting malonyl-CoA is the inhibitor of fatty acid oxidation. It decreases the transport of long-chain fatty acyl-CoA from the cytoplasm to the mitochondrial matrix, by inhibiting carnitine palmitoylacyltransferase I (CPT-I; EC 2.3.1.7) via an allosteric mechanism (Mcgarry et al., 1978a; Mcgarry et al., 1978b; Pripbuus et al., 1990). In ACC-β knock out mice, increases in both fat and carbohydrate oxidation increase total energy expenditure, reduce fat deposits, result in lean body mass and prevents diet-induced obesity (Abu-Elheiga et al., 2003). Furthermore, ACC-β knock out mice were protected from fat-induced peripheral and hepatic insulin resistance (Choi et al., 2007).

Hence, ACC plays a pivotal role in fatty acids synthesis and oxidation. ACC-α is thought more important in keeping a glucose homeostasis which is regulated systemically at the cellular level by energy charges (Cesquini et al., 2008). In this study, I focus on molecular regulation of bovine ACC-α.

1.1.4 Functional domains of the ACC-α

As stated above, ACC-α catalyzes the conversion of acetyl-CoA into malonyl-CoA. However, it plays role not alone, but as a multifunctional enzyme complex. In prokaryotes and most plants, three conserved functional domains, biotin carboxylase (BC), biotin carboxyl-Carrier protein (BCCP) and carboxyl transferase (CT), are distributed in different proteins (Li and Cronan, Jr., 1992a; Li and Cronan, Jr., 1992b). However, in eukaryotes, they are harbored in one long peptide. The bovine ACC-α multifunctional domains and binding sites for ATP, biotin and acyl-CoA are indicated in Figure 2. The overall reaction of biosynthesis of malonyl-CoA by ACC has two steps: Step1, BC catalyzes the carboxylation of biotin which is covalently bound to BCCP. This reaction is ATP-dependent; Step2, CT transfers the carboxyl from biotin to the acceptor, acetyl-CoA. The reactions are listed below (Lehninger, 1981):

Step1: Biotin-BCCP+HCO_3^-+H^++ATP→ carboxybiotin-BCCP+ADP+P_i

Step2: carboxybiotin-BCCP+acetyl-CoA→biotin-BCCP+malonyl-CoA

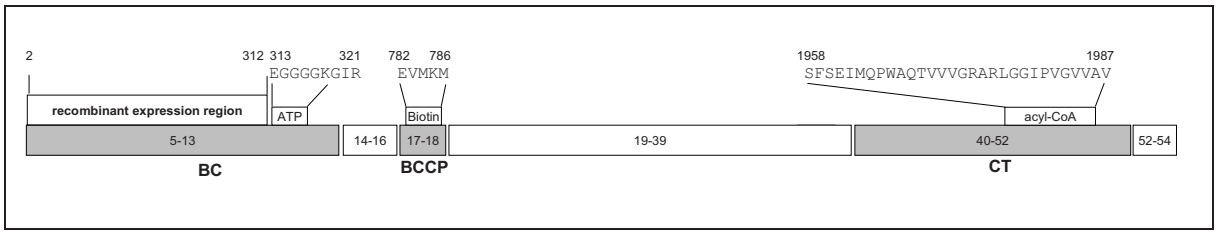


Figure 2 Functional domains of bovine ACC-α.

ACC-α shown in the figure is the 265 kDa isoform derived from PI and PII of bovine *ACC-α*. Numbers within boxes refer to exon numbers and functional domains are shown as grey filled boxes. The corresponding names are given below the exon boxes. The amino acid sequences and positions in the functional domains for ATP, biotin and acetyl-CoA (Kim, 1997) are listed above the exon boxes. BC, biotin carboxylase; BCCP, biotin carboxyl carrier protein; CT, carboxyltransferase. [The correspondence of the functional domain to the exact exon is referred to (Barber et al., 2005)].

1.2 Regulation of ACC-α

In the above reactions, ACC-α enzyme is regulated at different levels. These include the regulation of transcription initiation and subsequent mRNA splicing and stability. Together with the regulation at protein translation, these regulations belong to long-term regulation. Short-term regulation is also implicated in ACC-α regulation, and is consisted of allosteric or metabolic regulation and post-translational regulation of the enzyme. These processes will be discussed further in the following sections.

1.2.1 Long-term regulation

Liver, adipose tissues and mammary glands are the main sites for *de novo* fatty acid synthesis. In the liver, refeeding starved animals causes increased levels of the ACC-α gene (*ACC-α*) mRNA. Pregnancy and lactation dramatically readjust fatty acid synthesis to meet energy requirements, under these physiological conditions, this response of *ACC-α* transcription is tissue-specific (Kim and Tae, 1994). Particularly in white adipose tissue and the mammary gland, great changes of lipid metabolism are the comprehensive results of complex factors including hormones and cytokines (Ros et al., 1990).

Hormones, such as insulin, glucagon, triiodothyronine (T3), dexamethason, can regulate ACC-α activity in a long-term fashion by adjusting the expression level of ACC-α. These hormones are thought to regulate the *ACC-α* transcription initiation via modulation of cytokines. This is the route by which different nutritional statuses regulate *ACC-α* transcription.

Insulin increases *ACC-α* transcription initiation. Six hours after injection of insulin, the mRNA abundance of *ACC-α* was increased from low level to normal in diabetic mice (Katsurada et al., 1990). T3 plays a similar role to insulin, whereas, glucagon decreases the

abundance of *ACC-α* mRNA in avian hepatocytes (Yin et al., 2000).

1.2.1.1 Multi-promoters regulate *ACC-α* transcription initiation

To control the transcription initiation *ACC-α* gene, three promoters (PI, PII, PIII; Figure3) have been found in bovine with specific characteristics (Mao et al., 2001; Mao et al., 2002; Mao and Seyfert, 2002; Molenaar et al., 2003). PI is the main active promoter in the liver, adipose tissues and mammary glands; PII is the house keeping promoter; PIII is the abundantly expressed and induced promoter in the mammary gland (Mao et al., 2001; Mao et al., 2002; Mao and Seyfert, 2002).

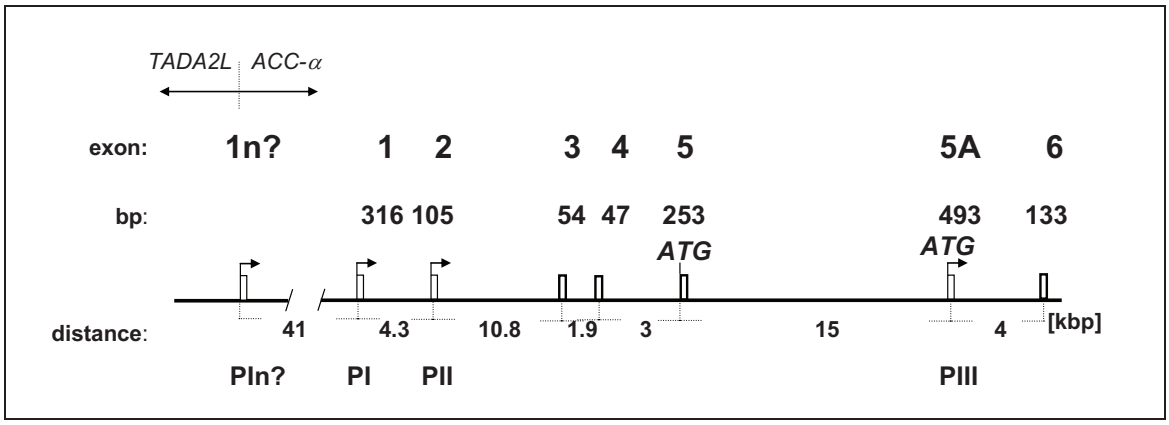


Figure 3 The distribution of exons and bovine *ACC-α* promoters

PIn shown in this figure is the most 5´ promoter of the bovine *ACC-α*, corresponding to PI of human *ACC-α* (Mao et al., 2003), and PI of ovine and mouse *ACC-α* (Travers et al., 2005). PI (Mao et al., 2001) refers to rodent and ruminant specific promoter (PIA) in the recent ACC reviews (Barber et al., 2005). PII refers to the house keeping promoter (Mao and Seyfert, 2002). PIII refers to the mammary gland specific promoter (Mao et al., 2002).The designation of all promoters of the bovine ACC-α gene follows this figure.

PI is a rodent and ruminant- specific promoter (Abu-Elheiga et al., 1995; Kim and Tae, 1994; Lopez-Casillas and Kim, 1991). It has strong activity in the liver, adipose tissues and mammary glands. Unlike rat and ovine PI, bovine PI has no TATA box, where the second 'T' is replaced by an 'A' base. This conserved sequence lies 3´ of the fourth transcription start site (Mao et al., 2001).

PII is evolutionally conserved with the counterparts of human and rat. It has the common characteristics of a house keeping promoter: no TATA box, no CAAT box and is a GC-rich sequence. *In vivo* expression of PII reveals little tissue-specific restrictions. The 133 bp proximal promoter has a strong activity in different cell lines (Mao and Seyfert, 2002).

PIII is primarily expressed and strongly induced (approximately 28-fold) in the lactating mammary gland (Mao et al., 2002). The transcripts of PIII in mammary epithelial cells (MEC)

of bovine and ovine have been identified by in situ hybridization (Barber et al., 2003; Mao et al., 2002). The long PIII promoter driving luciferase reporter was induced by prolactin and dexamethasone in stably transfected mouse MEC cells. Moreover, lactogenic induction was abolished if a binding site of unique signal transducer and activator of transcription (STAT) at position -797 to -786 was mutated by only two nucleotides (Mao et al., 2002). EMSA indicated STAT5A from mammary gland binds strongly to this region. Hence, STAT5 plays an important role in regulating the expression of *ACC-α* in the milk producing cells of the mammary gland (Mao et al., 2002).

PIn is a GC-rich and bidirectional promoter shared with the diversely oriented *TADA2L* gene, which encodes a component of chromatin-modifying complexes in ovine, mouse and human (Travers et al., 2005). PIn was originally identified in human adipose tissue (Ha et al., 1994) and was highly expressed in brain (Travers et al., 2005). The PIn of mouse or rat is located 43 kb upstream of the second regulatory region. PIn is physically, the first promoter of the *ACC-α* gene in ovine, mouse and human designated by the authors as 'PI' (Mao et al., 2003; Travers et al., 2005). To date, there are no reports of a bovine promoter PIn of *ACC-α*. However, I hypothesized that bovine *ACC-α* may harbour this conserved gene structure of PIn. Two reasons were provided. The first, the present bovine proximal PI has high identity with the second promoter of the ruminant and rodent; The second reason is that the most 5′ promoter PIn of human *ACC-α* shows high identity with ovine PIn (The 1st promoter of ovine *ACC-α*) (Barber et al., 2005), but has low identity with bovine PI. Hence bovine PI is distinct from PI of human and ovine *ACC-α*. Hence, the present bovine PI may not physically be the first promoter at 5′ terminus of bovine *ACC-α*. The first promoter of bovine *ACC-α* is hereby named PIn as indicated in Figure 4.

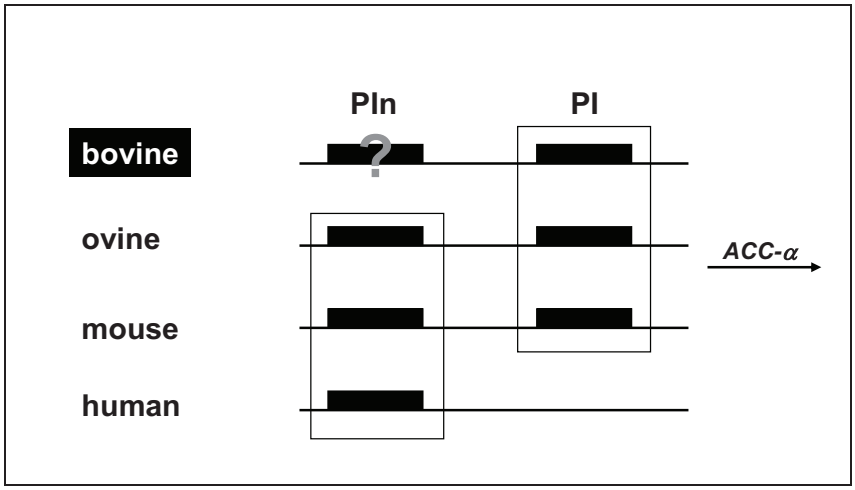


Figure 4 PIn and PI comparisons of different species

1.2.1.2 Pre-mRNA splicing

In *ACC-α*, pre-mRNA splicing likely evolved as an important regulatory mechanism resulting in various mature RNA transcripts. The mature RNA may generate physically related but functionally distinct protein products from the same gene. The diverse 5′-UTRs derived from PI and PII can be synthesized into the *ACC-α* protein using the start codon ATG which lies in exon5 (Mao et al., 2001; Mao et al., 2002; Mao and Seyfert, 2002). Transcripts derived from PIII can be translated into a smaller molecular weight bovine protein using the ATG in exon5A. However, alternative splicing of exon 28, which has been consistently described in pig and sheep (Barber et al., 2001; Gallardo et al., 2008), involves the presence/absence of an 8-aa stretch with the ability to inhibit the phosphorylation of residue Ser-1200. Thus, pre-mRNA splicing, in addition to multi-promoters, contributes to regulate *ACC-α* expression. In fact, in metazoans, the number of genes regulated by pre-mRNA splicing may be comparable to the number regulated by transcription initiation. Despite their obvious importance, alternative splicing mechanisms remain poorly understood (Carey and Smale, 2000; Wang and Manley, 1997).

1.2.1.3 Stability of mRNA

The steady-state abundance of every mRNA is dependent on its rate of synthesis and rate of decay. The intrinsic stability of an mRNA has a profound influence on the abundance of the mRNA and the abundance of the encoded polypeptide. The stabilities of mRNAs in mammalian cells vary widely, with approximate half-lives ranging from 15 minutes to 10 hours. Moreover, mRNA stabilities can be tightly regulated, and in some cases are regulated co-ordinately with transcription initiation (Carey and Smale, 2000). Pape and colleague investigated whether pre-mRNA decay is involved in the decrease of *ACC-α* mRNA synthesis in the presence of tumor necrosis factor (TNF) (Pape and Kim, 1989). They found that the apparent half-lives of *ACC-α* mRNAs (9 h) show no changes with and without induction of TNF. Hence, the regulation mechanism of *ACC-α* mRNA stability is not yet understood.

1.2.2 Short-term regulation

The short-term regulation controls the *ACC-α* enzymatic activity occurring within minutes, including allosteric activators, inhibitors and reversible phosphorylation. Hence, ACC-α activity is a dynamic balance among the various allosteric effectors, specific protein kinase and phophatases (Barber et al., 2005).

1.2.2.1 Allosteric regulation

Several allosteric effectors contribute to control ACC-α activity. They are citrate, isocitrate, glutamate, guanine nucleotide, CoA and long-chain acyl-CoAs. The first four are allosteric activators, and the last two are allosteric inhibitors.

Citrate and isocitrate are classic feed-forward activators of ACC-α activity for the following reasons. First, citrate and isocitrate are intermediates in the tricarboxylic acid cycle (TCA cycle) and are precursor of the main substrate of ACC-α, acetyl-CoA. They are found to activate ACC-α *in vitro* and also stimulate lipogenesis *in vivo* (Abraham et al., 1960; Martin and Vagelos, 1962). Second, citrate activates the polymerization of an inactive enzyme into an active filamentous form composed of 10-20 protomers (Beaty and Lane, 1983a; Beaty and Lane, 1983b). Third, citrate diminishes ACC-α phosphorylation in the hypothalamus, controlling food intake and coordinating a multiorgan network that controls glucose homeostasis and energy uptake through the adrenergic system (Cesquini et al., 2008). Glucagon and dibutyryl cAMP inhibit fatty acid synthesis by decreasing the concentration of citrate in chicken liver. In contrast, pyruvate or lactate activates fatty acid synthesis by increasing the concentration of citrate (Watkins et al., 1977).

Glutamate is a structural analog of citrate. Hence, it can also act as an allosteric activator of ACC-α (Boone et al., 2000b). Glutamate is a common product of amino acid, fatty acid and carbohydrate metabolism. Glutamate was also reported to induce ACC-α activity through complementary action as a phosphatase activator (Boone et al., 2000a).

Guanine nucleotides act as positive intracellular regulators for ACC-α activity (Witters et al., 1981). Photoaffinity labeling techniques indicated an intrinsic or associated GTP binding site on ACC-α (Mick et al., 1998). However, the fact that isolated ACC can not be activated by guanine nucleotides suggests that this is a complex process, requiring an endogenous cell condition (Witters et al., 1981).

CoA is an activator of ACC-α activity (Yeh et al., 1981). ACC-α has a CoA binding motif. The binding of CoA to ACC-α may contribute to polymerize the ACC-α enzyme. However, palmityl-CoA affects the binding of CoA to ACC-α. CoA, together with bovine serum albumin like protein, can block the inhibition by palmityl-CoA. Hence, CoA, palmityl-CoA and bovine serum albumin like protein control ACC-α activity in different physiological conditions (Yeh et al., 1981).

Long-chain acyl-CoAs are classic feed-back inhibitors of ACC-α activity. Palmityl-CoA, stearoyl-CoA and arachidyl-CoA are products of the lipogenic pathway (Faergeman and

Knudsen, 1997; Nikawa et al., 1979; Ogiwara et al., 1978). Long-chain acyl-CoAs were found to activate AMPKK, the kinase of AMPK, and to inhibit ACC-α activity (Carling et al., 1987; Hardie and Carling, 1997). Acyl-CoA binding protein (ACBP) might act as the sensor to monitor the concentration of long-chain acyl-CoAs (Knudsen et al., 1993; Rasmussen et al., 1993).

1.2.2.2 Reversible phosphorylation

Reversible phosphorylations of eight amino acid residues are relevant to the regulation of ACC-α activity by protein kinase A (PKA) and 5´-AMP-activated protein kinase (AMPK) *in vitro* and *in vivo* (Haystead et al., 1990). Interestingly, six (Ser-23, -25, -29, -77, -79, -95) of these residues cluster on the first 100 amino acid residues at the N-terminus of ACC-α, while the remaining two (Ser-1200 and -1215) localize to the central region (Haystead et al., 1988; Munday et al., 1988).

In vitro experiments indicated that AMPK phosphorylates Ser-79, -1200 and -1215 and inactivates ACC-α. Ser-77 and Ser-1200 are phosphorylated by PKA and are accompanied with a moderate loss of ACC-α activity (Davies et al., 1990). Prior phosphorylation of Ser77 and Ser1200 by PKA prevents subsequent phosphorylation of Ser79 and Ser1200, but not Ser1215, by the AMPK. The fact that truncation of the first 200 amino acid residues reversed the phosphorylation of ACC-α by PKA and AMPK implies that the key Ser residue for a regulatory response to these kinases resides in the N-terminus (Davies et al., 1990). In contrast, the phosphorylation of Ser-77 by PKA only results in a small effect on ACC-α activity. Hence, the effects of AMPK on ACC-α activity are mediated chiefly by phosphorylation of Ser-79.

In vivo experiments in isolated adipocytes and hepatocytes manifested that glucagons and adrenaline activate the phosphorylation of Ser-79 and -1200, through PKA decreasing ACC-α activity, but not AMPK. Combining with the evidence *in vitro*, hormones first activate PKA, and then activate AMPK, and results in the phosphorylation of Ser residues. Mutational experiments on rat ACC-α cDNA in HeLa cells showed Ser-1200 was critical for the inactivation of ACC by PKA and that Ser-79 was essential for the inactivation of ACC activity by AMPK. Mutation of Ser-79 also prevented the phosphorylation of Ser-1200 by AMPK, but mutation of Ser-1200, not Ser-77, blocked the phosphorylation by PKA.

Insulin activates ACC-α in adipocytes, hepatocytes and intact liver by inactivating AMPK and decreasing the total phosphorylation of ACC-α. However, insulin stimulates the

phosphorylation of Ser-29 (Haystead et al., 1988) which is mediated by casein kinase II in 3T3 cells and hepatoma cells (Mabrouk et al., 1990). The insulin-activated ACC kinase may play an important role in the activation of ACC by insulin (Heesom et al., 1998). Similarly to insulin, epidermal growth factor (EGF), starvation or refeeding also affect the whole phosphate content of ACC-α.

1.3 PI is regulated by a bipartite repressor

Previous work of our lab showed that a 1.3 kb PI (Figure 4) fragment drives the expression of reporter genes weakly in different model cells HC11, HepG2, and 3T3-L1 adipocyte. The deletion (-1330/-127) of the distal region stimulated reporter gene activity. The activity of bovine long *ACC-α* PI promoter and the subsequently truncated PI (-1045/+84) was approximately as weak as the promoterless (~1-fold pGL3basic). However, the activity of the very short proximal 127 bp fragment of PI (-127/+84) was ~10 -fold of pGL3basic (Mao et al., 2001). Hence, bovine PI is basically repressed, like its homolog in rat (O'Callaghan et al., 2001; Tae et al., 1994).

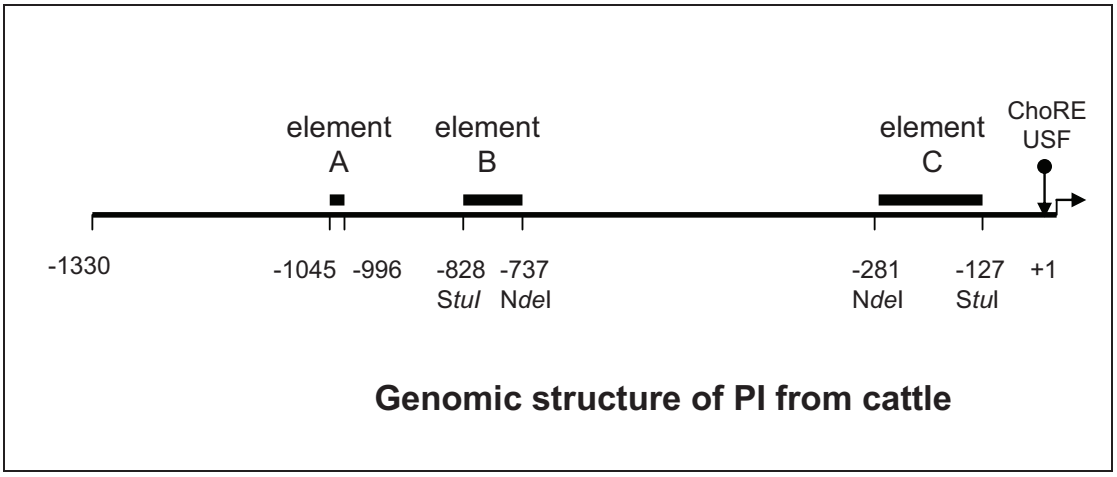


Figure 5 Candidate region for the repressive elements of PI

Serial deletions of PI indicated that two separated regions be present to inhibit bovine PI activity. The distal repressive region resides within a well-preserved Art2 retroposon from position -1297 to position -762 (Mao et al., 2001). The counterpart distal repressive region of rat PI has been identified as a CA-28 microsatellite (Tae et al., 1994). Thus, sequence structure and evolutionary origin of the main repression element of PI in bovine are entirely different from its functional counterpart in rat. Further work found CA-28 together with the downstream CCAAT/enhancer binding protein (C/EBP) element represses the PI activity in rat (Tae et al., 1994). Another report showed that the cotransfection of C/EBPβ expression vectors increased

rat *ACC-α* PI activity (Tae et al., 1995). C/EBPα inhibits the proliferation of liver cells, but activates adipocyte differentiation resulting in adipogenesis (Cao et al., 1991; Millward et al., 2007; Rahman et al., 2007). Observation from C/EBPβ knockout mice demonstrated that lipid accumulation decreased and diet-induced obesity was inhibited (Millward et al., 2007; Rahman et al., 2007). Thus C/EBP factors are relevant to *ACC-α* PI activity and subsequent fat metabolism.

1.4 Transcription factors relevant to regulating *ACC-α* PI activity

1.4.1 Roles of CCAAT/Enhancer Binding Protein (C/EBP)

The C/EBP factors are a large family transcription factors (Schrem et al., 2004), including six members (α, β, δ, ε, γ and ζ). The N-terminus of those proteins is specific and comprises of the activation domain (AD) or repression domain (RD) (Figure 5).

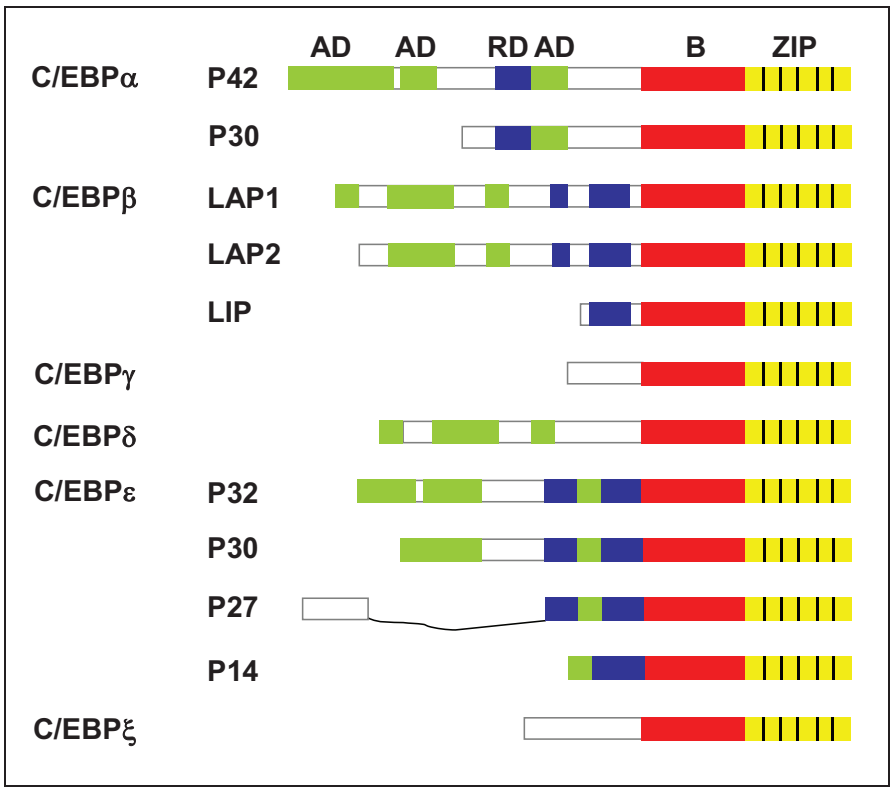


Figure 6 Schematic representation of the C/EBP family members.

The leucine Zipper is shown in yellow with black vertical lines indicating the leucine residues. The basic region is red. AD is the activation domain. RD is the repression domain. [Adapted from Ramji and Pelagia, 2002]

These proteins are highly conserved at their C-terminus, consisting of the DNA binding domain (B) and a leucine zipper domain (ZIP). Normally, C/EBP proteins work as homodimers or heterodimers via the leucine zipper domain dimerizing to regulate gene

transcription (Ramji and Foka, 2002). C/EBPβ and -δ are expressed early in the differentiation program, but are not immediately active. After a long lag, C/EBPβ and -δ become competent to bind to the C/EBP regulatory element in the C/EBPα gene promoter, C/EBPα being a transcriptional activator of numerous adipocyte genes (Cao et al., 1991; McKnight, 2001; Tang and Lane, 1999; Wu et al., 1998). Here, I focus on C/EBPα and C/EBPβ due to limited information for C/EBPδ and ε.

C/EBPα (previously named as C/EBP)

C/EBPα plays an important regulatory role in energy metabolism in the liver (Crosson et al., 1997). Disruption of the C/EBPα gene in mice causes hypoglycaemia associated with impaired expression of gluconeogenic enzymes.

Thyroid hormone positively regulates C/EBPα expression

Serial analyses of rat C/EBPα promoters indicated that the TRE-1 element represents a functional T3 response element regulated by thyroid hormone (Menendez-Hurtado et al., 2000). Moreover, some peptides of C/EBPα were stimulated to autoregulate (Jurado et al., 2002).

Possible regulatory functions of alternative C/EBPα translational products

Based on the same mRNA, C/EBPα can be translated into two isoforms: P42 and P30 (Figure 5). A previous report showed the ratio of these two major proteins changes during differentiation of 3T3-preadipocyte and hepatocyte development (Lin et al., 1993). Hence, alteration of the ratio may be a regulatory event.

The transactivation domain of C/EBPα

It is reported that three domains in the N-terminus (corresponding to amino acid position 1-107, 107-170 and 171 -245) contribute to regulate the transcriptional activity of the target gene (Pei and Shih, 1991). *In vitro,* the first two elements cooperatively mediate binding of C/EBPα to TBP and TFIIB. This might eventually regulate the transcription of the target gene.

Tissue-specific expression of C/EBPα

The human C/EBPα is differently expressed in tissues, expression levels in placenta being the highest of all other tested tissues. High expression was also found in the liver, lung, skeletal muscle, pancreas, small intestine, colon and in peripheral blood leukocytes. In brain, kidney, thymus, testis and ovary, C/EBPα is also expressed, although to a lower extent (Antonson and

Xanthopoulos, 1995).

C/EBPβ (also named as LAP, IL-6DBP, CRP2 or NF-IL6)

Expression of C/EBPβ gene can be stimulated by glucocorticoid and glucagon in primacy-cultured rat hepatocytes (Matsuno et al., 1996). C/EBPβ has been linked to the metabolic and gene regulatory responses to diabetes and implicated as an essential factor underlying gulcocorticoid-dependent activation of insulin-like growth factor binding protein-1 (IGFBP-1) and phosphoenolpyruvate carboxykinase (PEPCK) gene transcription *in vivo*. Isoforms of C/EBPβ were found to participate in gluconeogenesis via insulin and glucocorticoids (Ghosh et al., 2001).

C/EBPβ isoforms are generated by alternative translation initiation and/or proteosomal regulation

Four isoforms of C/EBPβ have been found. They are the full-length 39 -kDa C/EBPβ protein (LAP1), the 36-kDa Liver enriched transcriptional activator protein (LAP2), the 20-kDa Liver enriched transcriptional inhibitory protein (LIP) (Descombes et al., 1990; Descombes and Schibler, 1991) and the 14-kDa isoform (Schrem et al., 2004). Additionally different isoforms are generated by using various in frame starting ATGs. They may also be regulated by proteosomal cleavage (Burgess-Beusse et al., 1999). The cleavage activity is specific to prenatal and newborn livers, and is sensitive to chymostatin. The production of C/EBPβ protein isoforms in the neonatal mouse liver is regulated by C/EBPα. In C/EBPα knockout mice, the predominant C/EBPβ proteins are the larger 39-kDa and 36-kDa isoforms, whereas wild-type animals primarily possess the smaller 20-kDa and 14-kDa isoforms. These C/EBPα-dependent differences are liver specific, present at day 18 of development, but absent from lung or adipose tissues, (Burgess-Beusse et al., 1999).

Tissue-specific regulation of C/EBPβ

Sequence analysis and experimental evidence indicated that the rat C/EBPβ gene features two cAMP responsive elements (CRE) within its promoter. Subsequently, it was found that a C/EBP binding site overlaps with a CRE site. Moreover, in human hepatocellular liver carcinoma cells (HepG2), NF-κB stimulated the autoregulation of C/EBPβ during inflammatory response (Niehof et al., 2001). Transcription factor C/EBP, CRE or NF-κB may control C/EBPβ activity via interaction with their respective binding sites in different physiological conditions and tissues.

Phosphorylation of C/EBPβ

Post-translational site-specific phosphorylation of C/EBPβ is an essential mechanism

regulating the transactivating potential of C/EBPβ factors (Buck et al., 1999; Buck and Chojkier, 2007; Mahoney et al., 1992; Trautwein et al., 1993a; Trautwein et al., 1993b; Trautwein et al., 1994). Several phosphorylated residues have been found in mouse C/EBPβ factor. Phosphorylated Ser-239 stimulates C/EBPβ nuclear translocation. Phosphorylation of Thr-217 mediated by p90 ribosomal S kinase (RSK) contributes to the survival of stellate cells (Buck and Chojkier, 2007). Protein kinase C (PKC) phosphorylates Ser-105, -277 and -299, and PKA phosphorylates Ser-240. Only phosphorylation of Ser-105 is known to activate the expression of target gene at present. The others repress transactivating activity of the factor in PKC signalling pathway.

1.4.2 Roles of Nuclear Factor-Y (NF-Y)

Nuclear factor-Y (NF-Y, this study), also named as CCAAT binding factor (CBF), is composed of three subunits: NF-YA (CBF-B), NF-YB (CBF-A) and NF-YC (CBF-C) (Mantovani, 1999). Only after NF-YB and NF-YC form a dimer, can NF-YA recognize and bind to the complex resulting in a trimer. It is reported that NF-Y also recognizes the CCAAT binding motif. However, the protein structure and function are completely different from C/EBP factors (Celada et al., 1996; Romier et al., 2003). NF-Y represses or activates transcription during different physiological conditions thus acting as a bifunctional regulator (Bernadt et al., 2005). NF-Y can also recruit histone deacetylase (HDAC) to repress promoter activity (Peng et al., 2007). Compaction of DNA by NF-Y (Guerra et al., 2007) is another event that may conceivably contribute to the repression of transcription. Moreover, it is reported that NF-Y can cooperate with C/EBPα to up-regulate transcription in adiponectin and amelogenin genes (Park et al., 2004; Xu et al., 2006).

1.5 Goals of this study

Studying the regulation of fat metabolism plays an essential role in the control of milk and meat quality of the agricultural industry. Since ACC is the rate-limiting enzyme in fatty acid synthesis, and its main active promoter PI is nutritionally regulated in the liver, adipose tissues and mammary glands of ruminant and rodent, it is of importance to understand the regulation mechanism controlling PI of bovine ACC-α-encoding gene. It was previously reported that PI is basically repressed. However, the underlining molecular mechanism keeps unknown. The goals of this thesis are:

1) To characterize more 5′ promoter PIn expressing bovine *ACC-α*. Sequence analyses manifest that the present bovine PI show identity to ovine and mouse PI, which is physically

the second promoter of *ACC-α*. Travers and colleagues (2005) reported that PIn is physically the first promoter of ovine, mouse and human *ACC-α*. Hence, bovine *ACC-α* may harbour physically similar PIn at more 5′ region, initiating the transcription of *ACC-α* in lipogenic tissues, such as the liver, adipose tissues and mammary glands. The identification and characterization of bovine PIn will act as a basis to confirm that PI is the major promoter in lipogenic tissues.

2) To identify the *cis*-elements and the binding factors repressing PI activity. Results from the investigation of the PIn promoter confirmed that PI is the principally and nutritionally regulated promoter in lipogenic tissues. Previous work reported that PI is basically repressed by an upstream repressive element (Mao et al., 2001). O′Callaghan and colleagues found the distal repressive region of rat PI reduces the extent of the response to hormone, and they concluded that defining the repressors of PI is paramount to understanding the regulatory mechanism (O'Callaghan et al., 2001). Hence the elucidation of the repressive mechanism will contribute to understanding the regulation mechanism of fat metabolism.

3) To identify the regulatory mechanism of the basal PI activity. Previous work manifested that the basal PI promoter has strong activity in model cells HC11, HepRI and 3T3-L1 adipocyte. Based on the primary results of this study, C/EBP and NF-Y activate both the long and proximal PI. Hence, it is necessary to investigate how these factors regulate basal PI activity. Identification of the basal control *cis*-elements and the binding factors is the basis to finally resolve the regulation mechanism, and hence know how the repressors and activators synergistically modulate PI.

4) To characterize the molecular mechanism controlling *ACC-α* PI activity. After discovering the *cis*-elements and the relevant factors in both the repression and activation region, I cast interests to how these factors cooperate to control the PI promoter activity in model cells-HC11. It is necessary to examine the effects of these factors separately or collaboratively to control PI promoter. These works will be the basis by which I finally delineate the molecular mechanism controlling PI expression and regulation.

5) To discern the relationship of the relevant endogenous factors to *ACC-α* PI activity. After identification of the master control unit of PI, the interest is to know how the relevant factors *in vivo* cooperate to regulate PI promoter and control fat metabolism. Therefore I aim to probe the mRNA levels of the relevant transcriptional factors in different lipogenic tissues.

Finally, to develop a method for the measurement of ACC-α expression by an **a**vidin-**b**iotin **c**omplex indirect sandwich ELISA (ABC-ELISA). The present measurements of bovine

ACC-α mass are mostly dependent on the measurement of mRNA level, enzyme activity analysis. The enzyme activity assay is easily affected by the measuring conditions. Firstly, the different isoenzymes (ACC-α and ACC-β) can not be easily discriminated in crude protein sample or after purification procedures. Secondly, the loss of phosphorylation of ACC will make the apparent enzyme mass more than the real value due to the activation by dephosphorylation of ACC-α. The measurement by quantitative real-time PCR is in the mRNA level. It can not tell more information about the regulation of ACC-α in the protein level and post-translational level. ABC-ELISA will produce specific, accurate and sensitive information of the regulation of ACC-α enzyme. Hence, two antibodies for ACC-α from rabbit and goat, and the recombinant antigen will be raised in the study to establish the ABC-ELISA.

2 Materials and Methods

2.1 Materials

2.1.1 Bacterial strains

Strains	Genotype	Source
E. coli XL1-Blue	*SupE*44, *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*,*relA*1, *lac*$^-$ [F', *pro*AB+, *lacI*^{q}YΔM15, ::Tn10(*Tet*^{r})]	Stratagene
E. coli BL-21	*E. coli* B F$^-$ *dcm ompT hsdS* (r_B^- m_B^-) *gal* λ(DE3)	Stratagene

2.1.2 Reagents and antibodies

Table 1 Reagents

Reagents	Source
Restriction enzymes	MBI Fermentas
DNA polymerase I Large (Klenow) Fragment	Promega
Superscript II Reverse Transcriptase	Invitrogen
T4 DNA ligase	Invitrogen
RNase A	Serva
RNase T1	Sigma-Aldrich
Proteinase K	Serva
Synthetic oligonucleotides	Sigma-Genosys
dNTPs	Roche
Poly(dIdC)	Roche
^{32}P-dCTP	Amersham Pharmacia
Anti-Digoxigenin-AP, Fab fragments	Roche
Roti®-Block (10 ×)	ROTH
TRIzol	Invitrogen
Sephadex G-25	Amersham Pharmacia
CNBr-activated Sepharose 4B	Amersham Pharmacia
Protein A agarose	Sigma-Aldrich
DANSYL Chlorid	Sigma-Aldrich
AHT (anhydrotetracycline)	IBA
Folin-cicocalteu's phenol	MERCK
Freund's adjuvant complete	Sigma-Aldrich
Freund's adjuvant incomplete	Sigma-Aldrich
Insulin	Sigma-Aldrich
Trypan blue	Sigma-Aldrich
G418 sulfate	Serva
RPMI 1640 medium	Biochrom AG
Dulbecco's MEM (DMEM)	Biochrom AG

Hank's salt solution	Biochrom AG
Trypsin/EDTA solution	Biochrom AG
Fetal calf serum (FCS)	Biochrom AG
Antibiotic antimycotic solution (100×)	Sigma-Aldrich
FuGENE 6 Transfection Reagent	Roche
Lipofectamine™2000 Reagent	Invitrogen
Ni-NTA sepharose (50 % suspension)	IBA
Strep-tag Protein Ladder (lyophilized)	IBA
BenchMark™ Pre-Stained Protein Ladder	Invitrogen
Ultra-Sensitive ABC Peroxidase Rabbit IgG Staining Kit	Pierce

Table 2 Antibodies

	Antibody	origin	No.	Source
1	anti-CBFB (NF-YA, H-209)	Rabbit	sc-10779×	Santa Cruz
2	anti-CEBPα	Goat	sc-9315×	Santa Cruz
3	anti-His tag [His-probe (H-15)]	Rabbit	sc-803	Santa Cruz
4	anti-C/EBPβ (Δ 198)	Rabbit	sc-746×	Santa Cruz
5	anti-p-C/EBPβ (Thr217)	Rabbit	sc-16993×	Santa Cruz
6	anti-C/EBPβ (C-19)	Rabbit	sc-150	Santa Cruz
7	anti-FLAG ® M1 Monoclonal Antibody	Mouse	F3040	Sigma
8	mono anti-actin	Mouse	A2547	Sigma
9	anti-(bovine) ACC-α	Rabbit	X7	This study
10	anti-(bovine) ACC-α	Goat	G7	This study
11	anti-mouse IgG (Fab specific) HRP	Goat	A3682	Sigma
12	anti-Rabbit IgG AP	Goat	A-3687	Sigma
13	anti-mouse IgG AP	Goat	A-2429	Sigma
14	Anti-Goat IgG (whole molecule)–AP	rabbit	A4187	Sigma
15	Strep-Tactin AP conjugate		2-1503-001	IBA
16	anti-Goat IgG HRP	Rabbit	3140	Pierce

2.1.3 Plasmid vectors

Table 3 Plasmid vectors

Plasmids	Source
pBR328	Our lab
pGEM-T easy	Promega
pGEM-T	Promega
pGL3-basic	Promega
phRL-TK	Promega
pASK-IBA43+	IBA
pPR-IBA101	IBA

pFlag.cmv-1	Sigma
pcDNA3.1+	Invitrogen
pEGFP-N1	Dr. Wei Yang
pAsRed2-L1	As above
pCMV-CHOP	Prof. Dr. Gerald Thiel, University of the Saarland Medical Center
pCMV-flag-C2/CEBPβ	As above
pCMV-Flag-C/EBPβΔN	As above
A-C/EBP	As above
SREBPIA	Dr. Jianqiang Mao
TRB	As above

2.2 Preparation of DNA

2.2.1 Mini-preparation of plasmid DNA

1) Inoculate 3 ml of LB medium containing appropriate antibiotics with a single bacterial colony. Incubate at 37 °C overnight with vigorous shaking.
2) Harvest the bacterial cells from 1.5 ml of the overnight culture by centrifugation at 13,000 rpm for 30 sec. Discard the supernatant.
3) Resuspend the bacterial pellet in 100 µl of ice-cold Solution I. Incubate on ice for 5 min.
4) Add 200 µl of freshly made Solution II, and mix by gently inverting the tube 6 times. Let stand on ice for 5 min.
5) Add 150 µl of ice-cold Solution III and mix the contents by inverting the tube 6 times. Let stand on ice for 10 min.
6) Centrifuge at 13,000 rpm for 5 min at room temperature. The cell DNA and bacterial debris should form a tight pellet on the bottom of the tube.
7) Transfer 450 µl of the supernatant into a fresh tube.
8) Add 450 µl of isopropanol to the tube. Mix well and let stand at room temperature for 15 min.
9) Recover the DNA by centrifugation at 13,000 rpm for 5 min at room temperature.
10) Discard the supernatant. Wash the pellet with 70 % ethanol at room temperature. Discard as much ethanol as possible, and then dry the DNA pellet briefly in vacuum desiccator.
11) Dissolve the DNA pellets in 40 µl of TE (pH8.0, containing 20 µg/ml RNase A) and store the DNA at –20°C.

Solution I	50 mM Glucose, 25 mM Tris-Cl pH8.0, 10 mM EDTA (autoclaved)
Solution II	0.2 NaOH, 1 % SDS (Prepare and use freshly)
Solution III	60 ml of 5 M KAC, 11.5 ml of HAC, 28.5 ml of H_2O (autoclaved)
TE	10 mM Tris-Cl pH8.0, 1 mM EDTA pH8.0 (autoclaved)

2.2.2 Midi-preparation of plasmid DNA

1) Harvest the bacterial cells from 50 ml of the overnight culture by centrifugation at 4000 rpm for 10 min. Discard the supernatant.
2) Resuspend the bacterial pellet in 10 ml of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).
3) Collect the bacterial cells by centrifugation at 4000 rpm for 10 min.
4) Resuspend the washed bacterial pellet (from step 3) in 2 ml of Solution I. Let stand on ice for 5 min.
5) Add 4 ml of freshly made Solution II, and mix gently by inverting the tube 4-6 times. Let stand on ice for 5 min.
6) Add 3 ml of Solution III, and mix the contents by inverting the tube for 6 times. Let stand on ice for 10 min.
7) Centrifuge the bacterial lysate at 4000 rpm for 15 min at 4°C.
8) Filter the supernatant through four layers of cheesecloth in a 50 ml centrifuge tube. Add 0.6 volume of isopropanol, mix well and let stand at room temperature for 15 min.
9) Recover the DNA by centrifugation at 4000 rpm for 15 min at room temperature.
10) Decant the supernatant and rinse the pellet with 70 % ethanol.
11) Air-dry the pellet and dissolve the DNA pellet in 1.4 ml TE (pH8.0). The nucleic acids are purified further by the following steps.
12) Add 5 µl of RNase A (10 mg/ml) and 0.3 µl of RNase T1 (100 U/µl), incubate for 5 min at 37°C.
13) Add 2.5 µl of Proteinase K (20 mg/ml) and 1.88 µl of 20 % SDS, incubate for 30 min at 37°C.
14) Extract with 1.4 ml of phenol/chloroform/isoamylalcohol (25:24:1).
15) Centrifuge at 13,000 rpm for 5 min at room temperature.
16) Extract with 1.4 ml of chloroform/isoamylalcohol (24:1).
17) Centrifuge at 13,000 rpm for 5 min at room temperature.
18) Add one-tenth volume of 3 M NaOAC (pH 5.2) and 1 volume of isopropanol, mix well.
19) Centrifuge at 13,000 rpm for 5 min.
20) Wash the pellet with 70 % ethanol. Air-dry the pellet and dissolve the DNA in 200 µl of TE buffer (pH8.0). The purified DNA is ready for sequencing and transient transfection.

2.2.3 Preparation of genomic DNA

1) One gram of tissue is ground into powder with or after freezing with liquid nitrogen in a motar.
2) Add 250 mg of tissue powder into 3 ml of proteinase K buffer (containing 0.5 mg/ml proteinase K and 1 % SDS).
3) Mix gently and incubate overnight at 56 °C.
4) Cool down to room temperature, and add an equal volume of phenol into above solution. Mix gently by inverting the tube for 10 min.
5) Remove the upper aqueous phase to a new tube. Add one-tenth volume of 3 M NaOAC (pH 5.2), mix and add 1 volume of isopropanol and mix gently but thoroughly.
6) Centrifuge at 3000 g for 10 min at 4°C.
7) Wash the DNA pellet once with 70 % ethanol. Remove as much of the 70% ethanol as possible, using an aspirator. Store the pellet of DNA in an open tube at room temperature until the last visible traces of ethanol have evaporated. Note: Do not allow the pellet of DNA to dry completely; desiccated DNA is very difficult to dissolve.
8) Add appropriate amount of 1 mM Tris-HCl (pH 8.0). Place the tube on a rocking platform and gently rock the solution for 12-24 hours at 4°C until the DNA has completely dissolved. Store the DNA solution at 4°C.

Buffer	150 mM EDTA, 50 mM Tris-HCl (pH 8.0), 100 mM NaCl
Proteinase K solution	Add the following sequentially: 400 ml of buffer, 6 mM DTT, 0.5 mg/ml proteinase K, 1 % SDS

2.2.4 Preparation of BAC DNA

1) Inoculate 10 ml of LB medium containing 125 μg/ml Chlorampenicol with a single bacterial colony. Incubate at 37 °C overnight with vigorous shaking.
2) Inoculate 500 ml of LB medium containing 125 μg/ml Chlorampenicol appropriate antibiotics with 5 ml of the overnight culture. Incubate at 37 °C overnight with vigorous shaking.
3) Harvest the bacterial cells by centrifugation at 6000 g for 15 min at 4 °C. Discard the supernatant.
4) Homogeneously resuspend the bacterial pellet in 10 ml Buffer P1.
5) Add 10 ml of Buffer P2, mix thoroughly by vigorously inverting 4-6 times, and incubate at room temperature for 5 min.
6) Add 10 ml of Buffer P3, mix thoroughly by vigorously inverting 4-6 times, and let stand on ice for 20 min.

7) Centrifuge at 4000 rpm for 10 min at 4 °C and filter the supernatant.
8) Equilibrate a QIAGEN-tip by applying 10 ml of Buffer QBT and allow column to empty by gravity flow.
9) Apply the supernatant to the QIAGEN-tip and allow it to enter into the resin by gravity flow.
10) Wash the QIAGEN-tip with 2×30 ml Buffer QC. Allow Buffer QC to move through the QIAGEN-tip by gravity flow.
11) Elute DNA with 15 ml of Buffer QF pre-warmed to 65 °C into clean 50 ml vessel.
12) Precipitate DNA by adding 10.5 ml of isopropanol to the eluted DNA. Centrifuge at 15000 g for 30 min at 4 °C. Carefully decant the supernatant.
13) Wash the DNA pellet with 5 ml of 70 % ethanol and centrifuge at 15,000 g for 10 min. Carefully discard the supernatant.
14) Air-dry the pellet for 5-10 min and dissolve DNA in a suitable volume of H_2O.

Buffer P1	50 mM Tris-Cl, pH8.0; 10 mM EDTA, 100 µg/ml Rnase A 2-8 °C
Buffer P2	0.2 M NaOH, 1 % SDS 15-25 °C
Buffer P3	3.0 M KAC pH5.5 15-25 °C
Buffer QBT	750 mM NaCl, 50 mM MOPS pH7.0, 15 % isopropanol, 0.15 % Triton x-100 15-25 °C
Buffer QC	1 M NaCl, 50 mM MOPS pH7.0, 15 % isopropanol 15-25 °C
Buffer QF	1.25 M NaCl, 50 mM MOPS pH8.5, 15 % isopropanol 15-25 °C
TE	10 mM Tris-Cl, pH8.0; 1 mM EDTA

2.3 Preparation of RNA

2.3.1 Preparation of RNA from cells and tissues

1) a. Add 1 ml of TRIzol reagent to 100 mg of tissue and homogenize with Ultra-Turrax T25 for 10 s.
2) b. Aspirate the medium and then lyse cells directly in a culture dish by adding 1 ml of TRIzol reagent to a 3.5 cm diameter dish. Pipette the cell lysate up and down several times.
3) Incubate the homogenized samples for 5 min at room temperature.
4) Add 0.2 ml of chloroform per 1 ml of TRIzol reagent. Shake tubes vigorously for 15 s and incubate for 2-3 min at room temperature.
5) Centrifuge at 11,500 rpm (Eppendorf 5417R) for 15 min at 4°C.
6) Transfer the aqueous phase to a fresh tube. Precipitate the RNA from the aqueous phase by mixing with 1 volume of isopropanol.
7) Incubate for 10 min at room temperature, and centrifuge at 11,500 rpm for 10 min at 4°C.

8) Discard the supernatant and wash the RNA pellet once with 70 % ethanol, adding 1 ml of 70 % ethanol per 1 ml of TRIzol reagent used for the initial homogenization.
9) Centrifuge at 11,500 rpm for 5 min at 4°C.
10) Briefly air-dry the pellet and dissolve RNA in DEPC-treated water.
11) Stored at -70 °C.

2.3.2 Electrophoresis of RNA through agarose gels containing formaldehyde

Separation of RNAs according to size is the first stage in quality analysis. The method described in this protocol uses formaldehyde to denature the RNA, ethidium bromide to stain it, and electrophoresis through agarose gels containing formamide to separate the resulting formaldehyde-RNA-ethidium adducts.

1) Dissolve 0.72 g of agarose in 43.3 ml of DEPC-treated water.
2) Cool to 70 °C, and add 10.7 ml of 37 % formaldehyde and 6 ml of 10× MOPS buffer. Mix thoroughly and pour onto gel apparatus.
3) Add 1/5 volume of 5× RNA loading buffer to RNA sample, heat to 65°C for 5 min, and chill on ice.
4) Load the sample onto the gel, and run at a voltage of 5 V/cm in 1× MOPS buffer.

10× MOPS buffer	400 mM MOPS, 100 mM NaOAC, 10 mM EDTA
RNA loading buffer	15 % Glycerin, 0.4 % Bromophenol blue, 0.4 % Xylencyanol, 1mM EDTA
5× Loading buffer	10 µl Formamid, 3.5 µl Formaldehyde, 1 µl 10× MOPS buffer, 2 µl RNA loading buffer, 0.1 µl EtBr

2.4 Cloning of DNA

2.4.1 Digestion by restriction enzyme

To construct a recombinant vector, 5-10 µg empty plasmid DNA is digested with 10 unit of restriction enzyme in a 100 µl digestion system. Incubate overnight at 37°C and analyze the digestion efficiency by agarose gel electrophoresis. To prepare the insertion DNA fragment, digest 500 ng-2 µg of the original DNA with 5 units of restriction enzyme depending on the length of the original material in 50 µl. For restriction enzyme analysis of the recombinant vector, 100 ng of plasmid DNA and 1 unit of restriction enzyme of each are enough for one digestion for a unique restriction site for 30-60 min at optimal temperature. If it is the insertion fragment that should be checked, 20 ng of the smallest band has to be visible. So the DNA amount used for digestion should be adjusted accordingly.

2.4.2 Detection of DNA in Agarose Gels

These sites of DNA fragment that were assessed by comparing the residual restriction enzyme digests towards either of two `DNA ladders´ (Table 4).

Table 4 DNA molecular weight ladder

λDNA/*Hin*dIII (bp)	pBR328/*Hin*fI+../*Bgl*I (bp)
23,130	2,176
9,416	1,766
6,682	1,230
4,361	1,033
2,322	653
2,027	517
564	453
	394
	298
	234
	220
	154

2.4.3 Purification of DNA fragment from gel

Load the PCR reaction mixture of RE digestion mixture on a 0.8-2 % agarose gel using 1× TBE as running buffer. Run gel until the DNA band of interest could be separated from adjacent contaminating fragments. Purify the excised gel according to the manufacturers' protocol. Add appropriate amount of elution buffer to the upper reservoir of the filter tube. Centrifuge for 1 min at maximum speed then store the eluted DNA at +2 to +8°C for short time or -20°C for later analysis.

2.4.4 Blunting with Klenow enzyme

DNA Polymerase I Large (Klenow) Fragment consists of a single polypeptide chain (68 kDa) that lacks the 5´→3´ exonuclease activity of intact *E. coli* DNA polymerase I but retains its 5´→3´ polymerase, 3´→5´ exonuclease and strand displacement activities. The 5´→3´ polymerase activity of the Klenow Fragment can be used to fill in 5´-protruding ends with dNTPs; The 3´→5´ exonuclease activity can be used to generate blunt ends from a 3´-overhang.

DNA fragment	x µl
10× Klenow buffer	5 µl
Klenow enzyme (5 U/ µl)	0.5 µl
dNTPs (10 mM each)	1 µl
Add water to total volume	50 µl

Incubate the reaction for 30 min at 37 °C, then stop the reaction by heating the mixture for 10 min at 75°C.

2.4.5 Ligation

For the highest ligation efficiency, the molar ratio of vector: insertion should be optimized. In most cases, a 1:3 molar ratio works well. Set up the following reaction.

Insertion DNA fragment	x µl
Vector DNA fragment	y µl
2× ligation buffer	5 µl
T4 DNA ligase (1 U/µl)	0.5 µl
Add water to total volume	10 µl

Incubate the reaction for 1-2 h at room temperature or overnight at 4 °C. Following the ligation reaction, transform the ligated DNA into competent cells.

2.4.6 Transformation of DNA into *E. coli*

Preparation of competent *E. coli* cells and transformation are performed as described (Sambrook et al., 1989).

Preparation of competent cells

1) Plate *E. coli* XL1-1 blue on Tetracycline plate (LB+15 µg/ml Tet).
2) Inoculate 3 colonies into 5 ml of SOB-medium, grow overnight at 37 °C.
3) In the morning, inoculate 5 ml hereof into 50 ml (at 1:10) of PSI medium in 1 L flask.
4) Grow until OD_{600}=0.45-0.55 (need 2 h).
5) Transfer into a 50 ml Falcon tube, and let stand on ice for 15 min.
6) Spin down for 15 min at 3000 rpm. Discard the supernatant and invert. Dry with paper towel.
7) Carefully resuspend in 15 ml of RF1 medium (ice cold), and let stand on ice for 10 min.
8) Spin down for 15 min at 3000 rpm, remove supernatant, invert dry with paper towel.
9) Resuspend pellet in 4 ml of RF2 medium (ice cold). Let stand on ice for 15 min.
10) Snap freeze 80 µl (or 40 µl) aliquots in liquid N2.
11) Store at -70 °C.

SOB-Medium	2 % Bacto-tryton, 0.5 % Bacto-yeast extract, 20 mM of NaCl, 2.5 mM KCl, 10 mM $MgSO_4$, 10 mM MgCl
PSI-Medium	2 % Bacto-tryton, 0.5 % Bacto-yeast extract, 10 mM of NaCl, 5 mM KCl, 20 mM $MgSO_4$
RF1	100 mM RbCl, 50 mM $MnCl_2$, 30 mM KAC pH7.5, 15 % Glycerol. adjust pH value to 5.8, sterile by filtration.
RF2	100 mM MOPS (pH 6.8), 10 mM RbCl, 75 mM $CaCl_2$, 15 % Glycerol. pH 6.8 adjusted by NaOH, sterile by filtration.

Transformation

12) Add ligated DNA into the tube containing 40 µl of competent cells. Mix the contents of

the tubes by swirling gently. Let to stand on ice for 30 min.

13) Incubate the tube at 42 °C in a water bath for 90 s.
14) Chill the tubes on ice for 1-2 min. Add 0.4 ml of LB medium to each tube. Incubate the culture at 37 °C for 45 min with gentle shaking.
15) Transfer the appropriate volume of transformed competent cells onto LB/X-gal/Amp plates.
16) Leave the plates at room temperature until the liquid has been absorbed. Invert the plates and incubate overnight at 37 °C.

2.4.7 Screening of the recombinant clones

α-complementation

α-complementation occurs when two inactive fragments of *E. coli* β-galactosidase associate to form a functional enzyme. Many plasmid vectors such as pGEM-T easy and -T vector carry a short segment of DNA containing the coding information for the first 146 amino acids of β-galactosidase. Vectors of this type are used in host cells that express the carboxy-terminal portion of the enzyme. Although neither the host nor the plasmid-encoded fragments of β-galactosidase are themselves active, they can associate to form an enzymatically active protein. Lac^+ bacteria that result from α-complementation are easily recognized because they form blue colonies in the presence of the chromogenic substrate X-gal. However, insertion of a fragment of foreign DNA into the polycloning site of the plasmid almost invariably results in production of an aminoterminal fragment that is no longer capable of α-complementation. Bacteria carrying recombinant plasmids therefore form white colonies.

Normally, 20 mg of X-gal and 40 µl of IPTG are added into 500 ml of LB medium with agar. Substitutively, 40 µl of 40 mg/ml X-gal and 7 µl of 0.5 M IPTG were added to 100-200 µl transformed cells for one plate. Plate on LB agar and incubate overnight under 37°C.

Fast extraction

In order to quickly identify the positives transformants with about 1 kb insertion in 3-5 kb vector, fast extraction is used to screen the correct transformants. The bacteria cells were lysed with phenol, and at the same time RNA was decomposed by adding Rnase A. Plasmid DNA can be visualized by EB staining on an agarose gel.

1) Inoculate 3 ml LB medium containing appropriate antibiotics with a single bacterial colony. Incubate at 37 °C overnight with vigorous shaking.
2) Transfer 100-500 µl of overnight bacterial cultural cells into a 1.5 ml of centrifuge tube.
3) Spin down for 30 s at max speed.

4) Discard the supernatant and add 50 µl of TE buffer, 50 µl of phenol into the pellet. Add 2 µl of Rnase A and 2 µl of 10× DNA loading buffer in the cover of 1.5 ml centrifuge tube.
5) Vortex well. Gently spin down the Rnase A and add 10× loading buffer into the tube bottom and mix well. Spin down for 6 min at maximal speed at room temperature.
6) Load 5-10 µl of supernatant onto agarose gel.

Colony PCR

1) Inoculate 3 ml LB medium containing appropriate antibiotics with a single bacterial colony. Incubate at 37 °C overnight with vigorous shaking.
2) Transfer 50 µl of overnight bacterial culture and spin for 30 s at max speed to collect the cell pellet. Discard the supernatant.
3) Add 50 µl of water into the pellet. Boil for 5 min.
4) Spin at max speed for 5 min. 1 µl of the supernatant will be used as template for normal PCR.

Sequencing

250 ng of DNA in 8 µl of water is necessary for the construct of pGEM-T easy or pGEM-T vector series. T7 and SP6 primers are used for sequencing. For others constructs, specific primers from our lab are used for sequencing. The primers for different vectors are listed in Table 5.

Table 5 Sequencing primers used in this study

vectors	Sequencing primers	
pGEM-T easy (or T vecter)	SP6	T7
pGL3 basic	BASENDf (S92)	5_Lucr (S72)
pAskIBA43+	pAIBA3f (S497)	IBA_r (S1576)
pFlag.cmv-1	pFLAG-f (S1221)	
pcDNA3.1	pcDNA3.1_f (S1557)	pcDNA3.1_r (S1558)

2.5 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a common and indispensable technique in molecular research. In principle, PCR has three reactions: denaturation, annealing and chain extentions. The detailed procedure is adjusted for various requirements such as Touch-Down, high-fidelity and GC-rich templates.

2.5.1 Primer design

Basically, the primers are designed by Primer Premier 5.0 (PremierBiosoft). And make the annealing temperature about 64-66 °C resulting in real annealing temperature will be 60 °C. The Tm of oligonucleotides is calculated according to the following formula (Thein et al., 1986): Tm=2°C × (A+T) + 4°C × (G+C).

2.5.2 Standard PCR

All the PCR reactions are carried out in 0.5 ml PCR tubes. The thermocycler is a trio-thermoblock (Biometra). High quality water is always used for all the PCR applications. For normal PCR, FastStart Taq DNA polymerase is used. The specific polymerase for specific PCR is desribed in the following section. Generally for subcloning, the reaction is prepared in a total 50 µl volume as the following:

1	water	42 µl
2	10 × incubation buffer	5 µl
3	dNTPs (10 mM each)	1 µl
4	Forward primer (50 µM)	0.5 µl
5	Reverse primer (50 µM)	0.5 µl
6	Template (10-40 ng of DNA)	1 µl
7	Taq DNA polymerase	0.1 µl
	Total volume	50 µl

Place the tubes in the thermocycler and start cycling immediately using the following Touchdown program (Don et al., 1991):

	Temperature	Time	Cycles
Initial denaturation	95°C	3 min	1
Denaturation	95°C	30 sec	
Annealing	70°C (-1°C per cycle)	30 sec	10
Elongation	72°C	1-3 min	
Denaturation	95°C	30 sec	
Annealing	60°C	30 sec	30-35
Elongation	72°C	1-3 min	
Final elongation	72°C	7 min	1

2.5.3 High-fidelity PCR

For cloning of protein expression and point mutation, Expand High-Fidelity PCR System kit (Roche) is used for PCR. Due to the inherent 3′-→5′- exonuclease or “proofreading” activity of *Taq* DNA polymerase, the fidelity of DNA synthesis with this is higher than that of the normal *Taq* DNA polymerase. Usually, prepare two reaction mixes in microfuge tubes on ice:

Mix1		Mix2	
PCR grade water	x µl	PCR grade water	19.25 µl
dNTPs	1 µl	5× buffer vial 2	5 µl
Primer1	0.3 µl	Enzyme system	0.75 µl
Pimer2	0.3 µl		
DNA template	x µl		
Total volume	35 µl	Total volume	25 µl

Combine Mix 1 and Mix 2 in a PCR tube, and mix. Place samples in the preheated thermocycler, and start cycling using Touch-Down program.

2.5.4 GC-rich PCR

Amplification of GC-rich nucleic acids as well as uniform amplification of a mixture of DNAs with varying GC content is still problematic. Several reagents have been used to disrupt base pairing of isostabilize DNA like DMSO, formamide, glycerol, TMAC, Betaine and 7-deaza dGTP. The GC-RICH PCR System (Roche) is composed of a unique enzyme blend of thermostable enzyme with a proofreading activity. This polymerase mixture by itself outperforms *Taq* DNA polymerase in respect to yields, fidelity and specificity beside the possibility to amplify fragments up to 5 kb in length. The GC-RICH reaction buffer in combination with the separately included GC-RICH solution allows one to amplify very efficiently difficult templates such as GC-rich targets. The GC-solution works perfectly together with adjusting DMSO to amplify high GC content DNA target.

Mix1		Mix2	
PCR grade water	22.5 µl	PCR grade water	4.5 µl
dNTPs	0.5 µl	5× buffer vial 2	10 µl
Primer1	0.5 µl	Enzyme system	0.5 µl
Primer2	0.5 µl		
DNA template	0.5 µl		
GC-RICH solution vial3	5 µl		
DMSO 100%	5 µl		
Total volume	35 µl	Total volume	15 µl

Pipette together on ice Mix1 and Mix2 in a thin-walled PCR tube and mix well by pipetting up and down. Run Touch-Down program.

2.5.5 Reverse transcription PCR (RT-PCR)

First-Strand cDNA is synthesized using SuperScript II (Invitrogen). The following 20 µl reaction volume can be used for 10 pg-5 µg of total RNA or 10 pg-500 ng of mRNA.

1) Add the following components to a nuclease-free microcentrifuge tube: 1 µl of oligo

$(dT)_{20}$ (50 μM); *or* 200-500 ng of oligo$(dT)_{12-18}$; *or* 50-250 ng of random primers; *or* 2 pmol of gene-specific primer and 5 μg of total RNA and 1 μl 10 mM dNTP Mix (10 mM each), add distilled water to 13 μl.

2) Heat mixture to 65°C for 5 min, and incubate on ice for at least 1 min. Collect the contents of the tube by brief centrifugation and add 4 μl of 5× First-Strand Buffer, 1 μl of 0.1 M DTT, 1 μl of RNaseOUT and 1 μl of SuperScript II RT (200 U/μl).

Note: When using less than 50 ng of starting RNA, the addition of RNaseOUT is essential.

3) Mix by pipetting gently up and down. If using random primers, incubate tube at 25°C for 5 min. Incubate at 42°C for 30-60 min. Increase the reaction temperature to 42°C for gene-specific primer. The reaction temperature may also be increased to 55°C for difficult templates or templates with high secondary structure.
4) Inactivate the reaction by heating at 70°C for 15 min.

Note: Amplification of some PCR targets (those >1 kb) may require the removal of RNA complementary to the cDNA. To remove RNA complementary to the cDNA, add 1 μl (2 U) of *E. coli* RNase H and incubate at 37°C for 20 min.

5) The cDNA is purified with High PCR Pure Kit and eluted with 50 μl of water. Subsequent PCR amplification is performed using this purified cDNA as the template to obtain the specific gene sequence.

2.5.6 Rapid amplification of cDNA ends (RACE)

The GeneRacer™ (Invitrogen) method is based on RNA ligase-mediated and oligo-capping rapid amplification of cDNA ends (RACE) methods, and results in the selective ligation of an RNA oligonucleotide to the 5´-ends of decapped mRNA using T4 RNA ligase. The kit ensures the amplification of only full-length transcripts via elimination of truncated messages from the amplification process. The truncated mRNA and non-mRNA is eliminated by dephosporylation with calf intestinal phosphatase (CIP) to remove the 5´phosphates. Hence these RNA can not be ligated with RNA oligo. Whereas, the full-length RNA only can be ligated after decapping with tobacco acid pyrophosphatase (TAP) to remove the 5´cap structure from them. This treatment leaves a 5´phosphate required for ligation to the GeneRacer™ RNA Oligo.Upstream primers are derived from the RNA oligonucleotide and the downstream primers are devised based on the target sequence for 5´RACE.

For amplification of 3´ RACE, the first-strand cDNA is reversely transcribed with GeneRacer

™. The upstream primers are derived from the target sequence, but the downstream primers are designed to the Oligo $(dT)_{18}$ primer. Using nested PCR, specific and productive target sequence can be amplified.

2.5.7 Genomic Walking

GenomeWalker System (Clontech) is a PCR based method that facilitates cloning of unknown genomic regions upstream or downstream from a known DNA sequence. The first step is to construct genome Walker "libraries" – separate aliquots of DNA are digested to complete with different restriction enzymes (*Dra*I, *EcoR*V, *Pvu*II, *Sca*I and *Stu*I) that leave blunt ends and then are ligated to the Genome Walker Adaptor. Two steps of PCR are used to amplify the DNA sequence of interest; the first or primary PCR uses the outer adaptor primer (AP1) and an outer gene-specific primer1 (GSP1). The primary PCR mixture is then diluted and used as a template for a second or "nested" PCR with the nested adaptor primer (AP2) and a nested, more 5′ located the GSP1, gene-specific primer2 (GSP2). The amplified product can then be cloned and further analyzed. The following procedure has been used for amplification of DNA sequence from BAC DNA.

1) Digestion of BAC DNA: Set up five blunt-end digestions. For each reaction, combine the following in a separate 0.5 ml tube and then incubate at 37°C for 3 h.

2 µl	BAC DNA (50 ng/µl)
0.2 µl	Restriction enzyme (10 U/µl)
1.0 µl	10 × RE buffer
6.8 µl	H_2O
10 µl	Total volume

2) Purification of digested DNA: Add 90 µl water to each reaction tube, and extract with 100 µl of phenol, and then 100 µl of chloroform. Precipitate with isopropanol and dissolve the DNA pellet in 10 µl of water.
3) Ligation of digested Genomic DNA to Genome Walker Adaptors: For each library construction, set up a total of five ligation reactions. From each tube, transfer 4 µl of digested, purified DNA to a fresh 0.5 ml tube. To each, add the following. Incubate overnight at 4 °C. To stop the reactions, incubate at 70 °C for 5 min. To each tube add 72 µl of H_2O.

1.9 µl	Genome Walker Adaptor (25 µM)
1.6 µl	5× ligation buffer
0.5 µl	T4 DNA ligase (1U/µl)

4) Procedure for PCR-based DNA walking in Genome Walker Libraries: Expand ™ Long Template PCR System (Boehringer Mannheim) has been chosen for the amplification of

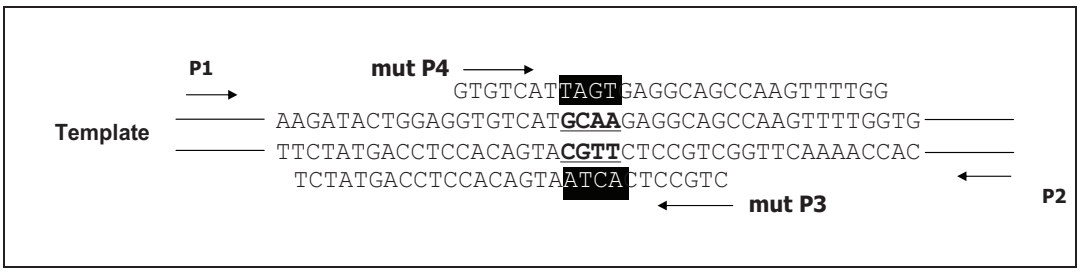
appropriate PCR product from the Genome Walker libraries. For primary PCR (primer: AP1/GSP1), use 1 µl of each library. The reaction is prepared and carried out as the following.

Master Mix 1 (25 µl)		Master Mix 2 (25 µl)	
H_2O	20.25 µl	H_2O	19.25 µl
dNTPs (10 mM each)	1.75 µl	Expand PCR buffer (10×, 17.5 mM $MgCl_2$)	5 µl
AP1	1 µl	Enzyme Mix (3.5 U/µl)	0.75 µl
GSP1	1 µl		
DNA library	1 µl		

5) If there are any bands or smearing obtained, continue the secondary PCR (primer: AP2/GSP2) with Touch-Down program, using 1 µl of a 50-fold dilution of the primary PCR products as the primary PCR.

2.5.8 PCR- mediated site-directed mutagenesis

Site-directed mutagenesis is produced by Fusion PCR which named also overlapping PCR. Generally, three steps of PCR are performed to introduce a point mutation, or to delete or to insert a short DNA fragment in the target region by overlapping PCR fragment. Due to that the PCR product can be checked in each step ensuring that, this technique is safe and quick to mutate (including point mutation, deletion and insertion) a target sequence by normal PCR. The first step is to design suitable primers, especially the mutation primer pairs, insuring enough overlapping temperature. In the following example, four nucleotides **GCAA** will be mutated into TAGT (relative to the forward sequence) by fusion PCR. P1 and P2 primer pairs are derived based on the vector; P3 and P4 primer pairs are devised for mutation. P3 and P4 primer pairs are overlapped (≥18 bp) and the mutation sequences are included. Three PCR reactions are performed to result in the final mutation sequence. The procedure is described in the following:



1) Amplify with the template and P1 and P3 primers to obtain product 1. Amplify with P2 and P4 to retrieve product 2. These two reactions can be completed in the same PCR program but in different vials. The standard Touch-Down PCR program, and purify

product 1 and product 2 with Kit.

2) Mix equal molar of Prod1 and Prod2 in PCR system, and run the same PCR program for only 5 cycles.
3) After the additional extension time is over, complement the reaction with P1, P2 and run another 30 cycles.
4) The aim product (Prod3) can be detected by agarose gel electrophoresis.
5) Excise the aim band and clean with kit, and the product can be cloned into pGEM-T easy vector or digested for ligation if enough products were obtained.

2.5.9 Quantitative Real-Time PCR

Real-Time PCR is a rapid and sensitive way to measure the transcription level of genes. In this study all real-time PCRs were carried out with LighterCycler system (Roche). SYBR Green® I Dye is used in real-time PCR because it can bind to the dsDNA and emit fluorescent signal. This signal is captured by the combined microvolume fluorimeter and analyzed by computer. The cycle number is defined as Cot in which the fluorescence signal exceeds certain threshold intensity. The melting curve analysis of the PCR products is performed to evaluate the specificity of the PCR products.

In this study, 1-5 μg of total RNA is first primed in reverse with oligo $(dT)_{20}$ and a gene specific primer. Normally, β-actin is used to normalize transcription data. After the first-strand synthesis, products are purified with the High Pure PCR Product Purification Kit. Real-time PCR are carried out with the LightCycler FastStart DNA Master SYBR Green I Kit, which includes the fluorescent dye and the reagents for PCR. A dilution series (10^1-10^6 copies) of appropriate cDNA subclones are included in each run and serve to calculate the copy numbers of transcripts.

Normal reactions are carried out in 10 μl. The reaction containes 5 μl of DNA template, 2.6 μl of water, 0.2 μl of forward primer (25 μM), 0.2 μl of reverse primer (25 μM) and 2 μl of master (prepared by adding 14 μl of tube a into one tube of b). For GC-rich gene transcripts, the reaction system is composed of 3 μl of template, 2.6 μl of water, 0.2 μl of forward primer (25 μM), 0.2 μl of reverse primer (25 μM), 1 μl of 5× GC-solution (Roche), 1 μl of 100 % DMSO and 2 μl of master. The basic amplification profile is as desribed:

	Temperature: °C	Time	Slope: °C/s	Cycles
Pre-incubation	95	10 min	20	1
Amplification	95	15 s	20	40
	60	5 s	20	
	72	20 s	20	
	85	5 s	20	
Melting	95	0 s	20	1
	65	15 s	20	
	95	0s	0.1	
Cooling	40	60	20	1

2.6 Cell culture and transfection

2.6.1 Cell line

The cell line and relevant cell media used in the study are as described:

Cell line		Growth Medium
HC11	murine mammary epithelial cell	RPMI1640 supplemented with 10 % FCS, 5 µg/ml insulin, 1 ng/ml murine epidermal growth factor (EGF), and 50 µg/ml gentamicin
HEK293	human embryonic kidney cell	DMEM supplemented with 10 % FCS, 1 mM sodium-pyruvate and 50 µg/ml gentamicin
HepG2	human hepatocellular liver carcinoma cell	Minimum essential medium (Eagle) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, 90 %; FCS, 10 %
pbMEC	primary bovine mammary epithelial cells	RPMI1640 supplemented with 0.1 mM L-methionine, 0.4 mM L-lysine, 0.01 mM sodium pyruvate, 10 % FCS, and the hormones prolactin, hydrocortisone, and insulin all at 1 µg/ml. pbMEC cells were always cultured in the dishes coated with Collagen R (Serva)
3T3-L1	murine 3T3-L1 pre-adipocyte cell line	Growth medium: DMEM with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 10 % FCS

2.6.2 Transient transfection

1) The day before transfection, seed ~3×10^5 cells in growth medium without antibiotics to result in 60-80 % confluence in the next day for transfection. Change cell numbers and total DNA amount proportionally according to Table 6 for different cell plate.

Table 6 Growth area of culture plates

Culture vessel	Growth Area (cm^2)	Relative Area (fold)
96-well	0.32	0.2
24-well	1.88	1
12-well	3.83	2
6-well	9.4	5
35 mm	8.0	4.2
60 mm	21	11
100 mm	55	29

2) Prepare DNA in 100 μl of serum-free medium (RPMI or DMEM). For transfection with Fugene6, add 3 μl of Fugene6 reagents into the DNA tube. Gently mix and collect the reagent mix to the bottom by short time spinning. Incubate for 20 min at room temperature and add into the 6-well-plate. For transfection with lipofactamine, prepare lipofactamine in 100 μl of medium (serum-free) and incubate for 5 min at room temperature.
3) Add lipofactamine-medium mix into the diluted DNA tube and incubate for 20 min at room temperature, and add into the 6-well-plate with optimal confluent growing cells.
4) 24 h later after transfection, split the cells into 3 wells of 24-well-plate and continue to incubate for another 24 h.
5) 48 h later after transfection, the reporter gene activity is assayed with Dual-Luciferase® Reporter Assay system (Promega).

2.6.3 Luciferase activity assays

The Dual-Luciferase® Reporter Assay system (Promega) is used to assay the activities of firefly and renilla luciferases sequentially in the same assay tube. The former activity is measured first by adding 100 μl of luciferase assay reagent II to generate a stabilized signal. Their activities are quenched by adding 100 μl of Stop & Glo® Reagent (0.25× SG), and the renilla activities are measured simultaneously.

	Water	Original reagent	Substrate
0.25× LARII	3 ml	LAR: 1 ml	
0.25× SG	3 ml	SG buffer: 1 ml	16.6 μl

2.7 Preparation and purification of antigen and antibodies

The molecular weight of native ACC-α is about 265 kDa or 259 kDa. ACC-α has conserved domains such as ATP binding domain, Biotin binding domain and acyl-CoA binding domain (Figure 2). The technique to raise antibodies relied on the full-length purified ACC will be laborious and non-specific. In order to handle easily to raise anti-bovine ACC-α, the recombinant strategy is performed in the study. I truncate and recombinantly express the specific N-terminus bovine ACC-α with His-tag at the N-terminus in *E.coli*. Subsequently, the recombinant ACC-α is purified by Ni-NTA sepharose for antigen.

2.7.1 Protein expression and preparation (for inclusion bodies)

1) Inoculate 5 ml of LB medium containing 100 μg/ml ampicillin with a fresh colony

harboring the expression plasmid. Incubate overnight at 37°C with vigorous shaking.

2) Inoculate 250 ml of LB medium containing 150 μg/ml ampicillin with 5 ml of the overnight culture. Incubate for 3~4 h at 37°C in a shaking incubator until the cells reach mid-log growth (A_{550}=0.45-0.55).
3) Transfer 1 ml of the un-induced culture to a tube, which acts as a negative control in future analysis.
4) Induce the remained culture by adding 25 μl of 2 mg/ml anhydrotetracycline (AHT) solution and continue incubation at 37°C for 3~5 h.
5) Harvest the cells by centrifugation at 6500 rpm (rotor JA20) for 15 min at 4 °C.
6) Wash the cell pellet with 20 ml of 1× His-Buffer.
7) Resuspend the pellet in 10 ml of Equilibration Buffer (8 M urea is for solublization of inclusion body) and sonicate for 5 min in 15 ml centrifuge tube on ice-cold water bath.
8) Centrifuge at 3750 rpm for 15 min at 4°C and collect the supernatant.

1×His-Buffer	50 mM sodium phosphate, pH 8.0, 0.3 M sodium chloride
Equilibration Buffer	0.1 M sodium phosphate pH 8.0, 8 M urea;

2.7.2 Affinity purification via Ni-NTA sepharose

Ni-NTA sepharose provides a powerful and versatile technique for isolating recombinant, 6×His-tagged proteins. Protein purification is based on the high affinity and specificity of unique Ni-NTA (nickel-nitrilotriacetic acid) resin for proteins with a 6×His tag. The interaction between the metal ion and target His-tagged proteins is steady in denaturing conditions (8 M urea, pH8.0), but is decreased by the decreasing of pH value. Hence, His-tag affinity purification provides an efficient and specific purification for recombinant inclusion bodies. The procedures are as follows:

1) Prepare 1 ml (1×) of Ni-NTA affinity column and equilibrate with 5× Equilibration buffer.
2) Load clear supernatant sample onto the column, wash with 3× Equilibration buffer.
3) Wash with 3× volume of Wash buffer.
4) Elute the target proteins with 3× volume of Elution buffer.
5) Dialyze against water and condensed against PEG20000.
6) Collect the dialyzed inclusion body (target protein) by centrifugation and dissolve in appropriated buffer.

Equilibration buffer	0.1 M sodium phosphate pH8.0, 8 M urea;
Wash buffer	0.1 M sodium phosphate pH6.3, 8 M urea;
Elution buffer	0.1 M sodium phosphate, pH 4.5, 8 M urea;

2.7.3 Measurement of protein concentration

Bradford method is used to measure protein concentration in the research. 1-20 μl of serial dilutions of BSA is used as standard proteins. Assay reagent is diluted by mixing 0.2 ml of Bio-Rad protein reagent with 0.8 ml of water. The protein is added into the diluted Assay reagent. A_{595} is measured after 5 min. The absorption of the standard protein is used to calculate the protein concentration of the sample.

2.7.4 SDS-PAGE electrophoresis

1) Recipe of the separating gel and the stacking gel.

	10 % Separating gel	5 % Stacking gel
H_2O	3.5 ml	2.2 ml
40 % Acrylamide	2.5 ml	0.5 ml
2 % N,N'-methylene-bis-acrylamide	1.4 ml	0.27 ml
1.5 M Tris	2.5 ml (pH8.8)	1 ml (pH 6.8)
20 % SDS	50 μl	20 μl
20 % APS (ammonium persulfate)	50 μl	20 μl
TEMED	8 μl	4 μl
Total volume	10 ml	4 ml

2) Assemble the electrophoresis device Mini-PROTEAN 3 cell (Bio-Rad). Pipette 3.3 ml of the separating gel, and seal immediately with H_2O. After the polymerization is completed, discard the overlay water and add the stacking gel, and then insert a comb.
3) Prepare samples using 2× Loading buffer. Heat to 95 °C for 5 min to denature the proteins.
4) Loading up to 20 μl each of the samples into the wells. Run the gel at a voltage of 80 V for 10 min in the Running buffer, and followed at 200 V for 35 min.
5) For Laemmli gel, submerge the gel in staining buffer for 20 min with gentle shaking. Wash the gel with H_2O and incubate the gel in Destaining buffer for 1-4 h or overnight with gentle shaking. To make a permanent record, dry the gel in gel drier machine at 75 °C for 45 min.

10× Running buffer	25 mM Tris base, 250 mM Glycine, 0.1 % SDS (Tris: 32g; Glycine: 188g; SDS: 10g; total=1L)
2× Loading buffer	100 mM Tris-Cl pH6.8, 4 % SDS, 0.2 % bromophenol blue, 20 % glycerol, 5 % 2-mercaptoethanol
Staining buffer	40 % Methanol, 10 % Galcial acetic acid, 0.1 % Coomassie brilliant bule R-250
Destaining buffer	10 % Methanol, 7 % Galcial acetic acid

2.7.5 Antigen preparation

The purified protein is then coupled with the fluorogenic reagent Dansyl chloride, which

emits green light after activation by UV. This facilitates the purification of the antigen from SDS-PAGE gels (without previous staining) which always includes denaturation. This in turn hinders subsequent elution of proteins.

1) Concentrate the purified protein to 2 ml by dialyzing against PEG20000. Measure the final concentration and determine the protein contents. Aliquot 100 µg of concentrated protein to be dansylated.
2) Precipitate the 100 µg of protein by adding 3× volume of ice-cold acetone. Incubate for 30 min at -20 °C, and centrifuge at 14000 rpm for 15 min at 4 °C.
3) Dry the pellet and dissolve in 100 µl of 2 % SDS solution.
4) Add 25 µl of Dansylation buffer and 2 µl of 10 % DANSYL chloride in acetone. Mix vigorously.
5) Boil for 3 min, and then add 1 µl of 2-mercaptoethanol and boil for another 1 min.
6) Separate unreacted products via the sephadex G-25 column equilibrated with Tris-Glycine buffer by collecting the fast fraction (orange fluorescence).
7) Unit the dansylated protein solution with the residual sample (from Step 1), and add 2–mercaptoethanl to 2.5 %. Boil for 3 min.
8) Dialyze against Tris-Glycine buffer for 3 h.
9) Add 1 volume of 2× protein Loading buffer, mix and boil for 3 min.
10) Separate the samples on SDS-PAGE gel. Check the green fluorescence bands with UV light and excise the desired band as the antigen. Store at -20 °C for future use.

Dansylation buffer	0.4 M Na_2HPO_4 pH8.2, 10 % SDS
Tris-Glycine buffer	2 ml of 5× SDS-running buffer, 0.5 ml of 20 % SDS, 97.5 ml H_2O

2.7.6 Immunization

1) Prior to injection, pre-bleed the rabbit in order to get a pre-immunization control serum.
2) Crunch the gel slice containing 100-200 µg of antigen by pressing it through 2 syringe several times. For the first injection, mix the crunched antigen with complete Freund's adjuvant, while for boost injection, mix antigen with incomplete Freund's adjuvant.
3) Two muscular injections on the thigh were done every 4 weeks.
4) Bleed 7-10 days after the third injection, and then bleed every 7-10 days after each boost injection for three times. Each time collect 20-40 ml of blood form one rabbit, 50-100 ml from one goat.
5) Place the collected blood at 4 °C overnight to clot. Clean the supernatant (serum) by centrifugation at 300 g for 10 min.

6) Incubation at 56 °C for 30 min to inactivate complement. Store at -20 °C for future purification.

2.7.7 Antibody purification

General procedures: Follow Pharmacia booklet ´Affinity Chromatograph´ about CN-Br activated Sepharose.

Preparations of antigen

1) Dissolve ~40 mg of crude extract (containing >10 % of desired protein; eventually pre-extract with ~4 M urea) in ~3 ml of 2 % SDS in 1/10 coupling buffer.
2) Press through syringe (yellow needle) several times to completely resuspend the pellet.
3) Heat to 100 °C for 3 min.
4) Spin at 13 000 rpm (Epp Centrifuge) to remove clear supernatant.
5) Salt up by adding 300 µl of 5M NaCl and 300 µl of 1 M $NaHCO_3$.
6) Fill up the final volume to 8 ml with coupling buffer.
7) This will be the ligand for next step.

Coupling the ligand to CN-Br activated Sepharose

1) Take 1 g of CN-Br activated Sepharose powder (yields 3 ml of swollen gel).
2) Wash and re-swell on a sintered glass filter; take 200 ml ice-cold (!) 1 mM HCl, apply in several aliquots, resuspend on filter and suck off (take ~15 min minutes). This is the ready activated Sepharose gel.
3) Mix ligand immediately with activated gel, swirl slowly for 1 h in an Erlemeyer flask at room temperature.
4) Wash away excess ligand with coupling buffer.
5) Block unreacted groups for 1 h at room temperature with 0.1M Tris pH 8.0.
6) Wash with 5 fold volume of 1 % triton X-100, 8 M urea and 2 % SDS and water sequentially.
7) Wash again with coupling buffer followed by 0.1M HAC pH 4, 0.5 M NaCl and binding buffer.

Purification of polyclonal antibodies

1) Pour column and store in binding buffer (may wish to add NaAzide: Na_3N_3).
2) Load clear serum supernatant into the column.
3) After the serum is loaded, wash the medium with binding buffer until the base line is stable.
4) Subsequently the specific antibody is eluted off using 8 ml of Elution buffer from affinity

column into 2 ml of Neutralization buffer.

5) Dialyze the eluted antibody solution against 3×500 ml of 1× PBS for 48h. Concentrate the antibody by dialyzing against PEG 20000. After determining the concentrations of the affinity purified antibody (usually the final concentration is 1~2 μg/μl), store aliquots at –20°C.

Binding buffer	Tris buffer (0.05 M Tris pH 7.8, 0.15 M NaCl, 0.1 % NaAzid)
Elution buffer	1 M Propionic acid (pH 2.3)
Neutralisation buffer	1 M Tris (unbuffered = pH 11.2)

2.8 Immunological methods

2.8.1 Western blot

1) After SDS-PAGE electrophoresis, incubate the gel in Transfer solution for 5 min with gentle shaking.
2) Prepare a sandwich device using one layer of PROTRAN nitrocellulose membrane (Whatman® Schleitcher & Schull). Electrotransfer for 1 h for 30-65 kDa protein in 10 % SDS-PAGE with 200 mA current. If for 265 kDa native ACC-α, blot for 3 h in 5 % SDS-PAGE with 200 mA current. Pre-stained marker was used to test the transfer efficiency.
3) Block the transferred membrane with Blocking buffer for 1 h with shaking at room temperature or overnight at 4 °C.
4) Wash the membrane 3 times with 20 ml of Washing buffer, each 5 min.
5) Incubate the membrane with primary antibody diluted (normally 0.1 μg/ml) in Washing buffer for 1 h at room temperature with gentle shaking.
6) Wash as in step 4.
7) Incubate the membrane with secondary antibody diluted (1:10,000) in Washing buffer for 1 h with shaking.
8) Wash as in step 4
9) Equilibrate the membrane in Detection III solution for 5 min with gentle shaking.
10) Develop the membrane in freshly prepared substrate solution (NBT/BCIP) in a sealed bag in dark. No shaking during development.
11) When the intensities of desired bands are achieved, stop the colour reaction by rinsing the membrane with TE buffer.

10× PBS	1.37 M NaCl, 78.10 mM Na_2PO_4, 26.80 mM KCl, 14.70 mM KH_2PO_4
Transfer buffer	10 mM Tris pH8.2, 2 mM EDTA, 50 m M NaCl, 0.1 mM DTT
Transfer buffer-wet	1 L: 50 ml of 1 M Tris pH8.5, 200 ml methanol, 15 g of Glycine

Blocking buffer	1× Roti-Block in 1× PBS pH8.2
Washing buffer	1× Roti-Block, 1× PBS pH8.2, 0.2 % Triton X-100, 0.1 % SDS

2.8.2 Protein-protein interaction by immuno-precipitation

The immuno-precipitation was performed as described (Xu et al., 2006).

1) Add 1 ml of ice-cold 1× PBS to cells and collect to a 1.5 ml tube. Spin at max for 1 min at 4 °C to collect cells again.
2) Discard the supernatant and add 0.4 ml of IP buffer into the cell pellet.
3) Lyse the cells by passing six times through a 22-gauge needle and centrifuge at 3000 rpm for 15 min at 4°C. Aliquot 100 µl for SDS-PAGE analysis.
4) Add 0.5 ml of IP buffer into the rest cell lysate. Pre-clean with 40 µl of Protein A-agarose (Upstate Biotechnology, Lake Placid, NY, USA) for 2 h at 4°C.
5) Add 2 µg of antibody into the 300 µl of pre-cleaned cell lysate and incubate overnight at 4°C in 0.5 ml centrifuge tube.
6) Add 20 µl of Protein A-agarose and incubated for 2 h at 4°C.
7) Centrifuge at 5000 rpm for 1 min at 4°C.
8) Wash with 0.3 ml of ice-cold 1× PBS 3 times.
9) Centrifuge at 5000 rpm for 1 min at 4°C.
10) Add equal volume of 2× LP buffer, boil for 5 min and spin for 1 min at 5000 rpm. Load to gel or stored at -70°C

IP buffer (1 ml)	0.9 ml of 1× PBS; 100 µl of 10 % NP-40; 10 µl of 100 mM PMSF; 5 µl of cocktail inhibitor (Sigma); 5 µl of 200 mM $NaVO_3$

2.8.3 Indirect sandwich ELISA for bovine ACC-α

The Ultra-sensitive ABC Peroxidase Staining Kits use the avidin-biotin complex (ABC) method to amplify signal intensity (Hsu et al., 1981). The multiple binding sites between the tetravalent avidin and biotinylated antibodies (bound to the antigen) are ideal for improving sensitivity by increasing the number of enzyme molecules at the target. This method uses a biotinylated horseradish peroxidase (HRP) that is pre-incubated with avidin at a specific ratio, forming large complexes. An aliquot of this solution is then added to the biotinylated antibody that is already bound to the target. The result is a greater concentration of enzyme (three enzyme molecules to one avidin molecule) at the antigenic site and, therefore, an increase in signal intensity and sensitivity in ELISA. This signal amplification method may also be used for immunohistochemistry, Western blotting, in situ hybridization and electron microscopy procedures.

1) Coat plate with 100 µl of coating buffer (antibodies) o/n at 4 °C.
2) Rinse each well with 150 µl of washing buffer
3) Add antigen in 100 µl of washing buffer.
4) Rinse 3 times with 150 µl of washing buffer.
5) Add primary antibodies in 100 µl of washing buffer at RT for 1 h.
6) Rinse 3 times 150 µl of washing buffer.

For normal method, continue step 7-9), then continue step13-end

7) Add secondary antibodies in 100 µl of washing buffer at RT for 1 h.
8) Rinse 3 times with 150 µl of washing buffer.
9) Rinse with 150 µl of water.

For ABC method, continue from step6 then step10-end

10) Add 100 µl of Biotinylated Secondary Antibody to each well and incubate plate for 30 minutes at room temperature. Note: The ABC Reagent may be prepared during this incubation step (see Materials Required Section).
11) Rinse each well three times with 200 µl of Wash Buffer. Add 200 µl of Wash Buffer to each well and incubate at room temperature for 5 min.
12) Empty plate and tap residual liquid on a paper towel. Add 100 µl of the ABC Reagent to each well and incubate at room temperature for 30 min at room temperature.
13) Immediately before use mix equal volumes of the TMB solution and the Peroxide Solution.
14) Add 100 µl of TMB Substrate solution to each microplate well.
15) Incubate plate at RT for 15-30 min or until the desired color develops.
16) Stop reaction with 100 µl 2 M sulfuric acid or 8 M acetic acid with 1 M sulfuric acid.
17) Measure the absorbance of each well at 450 nm.

Coating buffer	1.59 g Na_2CO_3; 2.93 g $NaHCO_3$; 0.2 g NaAzid and 1 L water, pH9.6.
Washing buffer	1× PBS pH8.2; 0.2 % Triton X-100; 0.1 % SDS; 0.5 % BSA (1× Roti-block)
anti-goat IgG HRP	Conjugated (1: 5 k-100 k for ELISA) (horseradish peroxidase)
Substrate buffer	Prepare before use with TMB solution and peroxide solution
TMB solution	3, 3, '5, 5'-tetramentylbenzidine solution: 0.4 g/L
Peroxide solution	contains 0.02 % hydrogen peroxide in citric acid buffer
Stop buffer	2 M sulphuric acid
for ABC method	
Biotinylated Secondary Antibody	Add 3 drops (135 µl) of Blocking Serum and 1 drop (45 µl) of the Biotinylated Secondary Antibody from the Ultra-Sensitive ABC Staining Kit to 10 ml of PBS.
ABC Reagent	Prepare reagent 30 minutes before use (see Step 6). Add 4 drops (180 µl) of Reagent A to 10 ml of PBS followed by 4 drops (180 µl) of Reagent B.

Immediately mix solution and allow 30 minutes for the complex to form.

2.9 Southern blot

The Southern blot hybridization is used to locate and identify a specific DNA segment within a complex mixture of molecules. DNA (genome or other source) is digested with restriction enzymes and separated by gel electrophoresis according to size. The separate DNA molecules are transferred from the agarose gel onto a nitrocellulose membrane or Hybond–N nylon membrane. The blot is then incubated with a labelled single–stranded probe of the target DNA. This probe will hybridize with its complementary DNA sequence and form a double-stranded DNA molecule. The probe must be labelled before hybridization either radioactively or non-radioactively (e.g., DIG-labelled).

- Gel blotting

1) Digest the DNA sample with restriction enzymes and separate the DNA fragments on an agarose gel.
2) Submerge the gel in 0.25 M HCl for 10 min with gentle shaking.
3) Denature the dsDNA molecules to form a single stranded (ss) DNA molecule by immersing the agarose into Denaturation solution for 30 min.
4) Rinse the gel once with H_2O and submerge it in the Neutralization solution for 30 min.
5) Set up a sandwich device to transfer the DNA fragments from the gel onto Hybond –N nylon membrane (Amersham Pharmacia) by the capillary action in 20×SSC buffer. Transfer overnight to ensure the sufficient transfer of DNA.
6) After transfer, submerge the membrane in 6×SSC for 5 sec. Air dry briefly and fix the DNA to nylon membrane by UV crosslink for 3 min.

Denaturation solution	0.5 N NaOH, 1.5 M NaCl
Neutralization solution	1 M Tris-Cl pH7.5, 1.5 M NaCl
20×SSC buffer	3 M NaCl, 0.3 M Sodium citrate, pH7.0

- Probe labeling

The DIG Oligonucleotide 3′–End labelling Kit is used to non-radioactively label the probe in this application. Oligonucleotide probes are enzymatically labelled at their 3′–end with terminal transferase by the incorporation of a single digoxigenix-labelled dideoxyuridine-triphosphate (DIG-dd UTP).

1) Add the following components on ice:

Probe (100 pmol)	x µl
5× Reaction buffer	4 µl
$CoCl_2$ solution	4 µl
DIG-ddUTP solution	1 µl
Terminal transferase (2.5 U / µl)	1 µl
H_2O	x µl
Total volume	20 µl

2) Mix and incubate at 37°C for 30 min. Place on ice.
3) Stop the reaction by adding 2 µl of 0. 2 M EDTA (pH8.0).

- Hybridization

1) Prewarm the Prehybridization buffer and add it to a heat sealable bag with the blot membrane. Seal the bag. Incubate for 1-2 h with gentle shaking in 60 °C bath.
2) Replace the solution with about 0.2 ml/cm^2 membrane prehybridization buffer containing the 100 pmol DIG-labelled probe. Incubate the blot overnight in 60 °C water bath with gentle agitation.
3) Wash the blot twice for 5 min in 2× SSC, 0.1 % SDS at RT.
4) Wash the blot twice for 15 min in 0.1× SSC, 0.1 % SDS at 60 °C with shaking.

Prehybridization buffer 5× SSC, 0.1 % SDS, 1 % Blocking reagent (Roche)

- Immunological detection

The DIG-labelled probes can be detected by the antibody conjugated to the enzyme alkaline phosphatase (AP), which catalyzes the deposition of dyes at the site of the enzyme in the presence of nitroblue tetrazollium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

1) After hybridization and stringency washing, rinse the membrane briefly with Detection I solution.
2) Incubate for 30 min with Detection II solution.
3) Dilute Anti-DIG-AP Fab fragments to 150 mU/ml (1:5000) in Detection II solution and incubate the membrane for 30-60 min with diluted antibody solution.
4) Wash twice for 15 min with Detection I solution with gentle shaking.
5) Equilibrate the membrane in Detection III solution.
6) Incubate the membrane with freshly prepared Colour solution in a sealed bag in the dark. Do not shake during colour development.
7) When desired intensities of the band are achieved, stop the colour reaction by rinsing the membrane with TE buffer.

Detection I solution	0.1 M Tris-Cl pH7.5, 0.15 M NaCl
Detection II solution	Dection I solution with 1 % Blocking reagent
Detection III solution	0.1 M Tris-Cl pH9.5, 50 mM $MgCl_2$, 0.1 M NaCl
Color solution	337.5 μg/ml NBT, 175 μg/ml BCIP in Detection III solution

2.10 Eletrophoretical Mobility Shift Assay

Eletrophoretical mobility shift assay (EMSA) is used to identify transcriptional factor that bound to the target DNA element. Normally the DNA probe is labelled with α-^{32}P-ATP at the end by T4 polynucleotide kinase or α-^{32}P-NTP by DNA polymerase large fragment (Klenow). The protein-DNA complex is visible by the labelled probe.

The specificity of the shifted band can be resolved based on two different criteria. One can use a molar excess of the unlabelled double stranded oligonucleotide to compete with the labelled for the binding proteins. The sequence specific radioactively labelled band will eventually disappear proving sequence identity. The site specific band will be not be affected by mutational unlabelled double stranded oligonucleotide. Alternatively, or augmenting the competition analysis, one can apply an antibody know to bind to the transcription factor of interest. If the antibody recognizes and binds to the factor having bound to the radioactive probe, then the molecular weight increases. The shifted band appears as heavier and new is called ``supershifted´´. This assay verifies the identity of the DNA binding factor. Using sensitive scanning system, this technique can also quantity the candidate factors.

- **Step1: Probe labeling**

The probe labelling can be done either by end labelling using T4 polynucleotide kinase or using filling-in of 5´-overhangs with the Klenow enzyme. In our lab, the latter method is preferred due to its greater reproducibility and better labelling efficiency.

1) Combine 5 μl of 50 μM long forward primer (-25 mer) with 10 μl of 50 μM short reverse primer (10 mer) into 30μl of H_2O. Mix well. The primers used for EMSA in the study are listed in the following table.

No.	Probe	long	short	No.	Probe	long	short
1	EA	1396	1395	15	Eccmu	1420	1418
2	NFY_CBF	1369	1371	16	C/EBP3mu	1346	1244
3	Ecc	1419	1418	17	SP1mu	1422	1421
4	C/EBP3	1245	1244	18	GLI	1375	1374
5	SP1	1226	1421	19	Fast	1373	1372
6	EAm6	1401	1395	20	STAT	1450	1451
7	EAm1	1397	1395	21	STATmu	1452	1451
8	EAm3	1398	1395	22	C/EBP3mu2	1575	1574
9	EAm4	1399	1395	23	ECcmu3	1572	1418
10	EAm5	1400	1395	24	NFY5	1633	1634

11	EAm6	1401	1395	25	CEBP_con	1487	1486
12	EAm7	1402	1395	26	CEBP_conmu	1489	1488
13	EAm8	1403	1395	27	LDLR	1510	1511
14	EAm9	1404	1395	28	C/EBP2	1243	1242

2) Boil the mixture in a water bath for 10 min and immediately chill on ice.

3) Transfer 1 μl of mixture to a new tube containing 99 μl H_2O, and set up the following two reactions in a total volume of 50 μl to synthesize cold and hot probe:

Table 7 Labeling of hot probe

Cold probe reaction		Hot probe reaction	
36 μl	Original primers mix	36 μl	diluted primers mix
5 μl	10× Klenow buffer	5 μl	10× Klenow buffer
1 μl	dNTPs	1 μl	dNTPs (A G T)
1 μl	Klenow enzyme	1 μl	dCTP(^{32}P)
7 μl	H_2O	1 μl	Klenow enzyme
		6 μl	H_2O
50 μl	Total volume	50 μl	Total volume

4) Incubate at 30°C for 1 h. 75 °C 10 min and then place the tubes on ice.

5) Proceed to purify with the Mini Quick Spin Column. Briefly, prepare the spin column by evenly resuspending the matrix and subsequently removing excess buffer. Spin for 1 min at 1000 g. Slowly and carefully, apply the samples to the center of the column bed, and finally collect the purified probe by centrifugation of 1000 g 4 min. The molecular concentration of labelled probe is 40 fmol/μl, while that of unlabelled probe, which is used in competition reaction, is 4 pmol/μl.

- **Step2: EMSA reaction**

DNA-protein binding reactions are normally conducted in a total volume of 20 μl and always work on ice except indicted elsewhere. To set up the binding reactions, add the components in the order given below.

Binding reaction (μl)	**Competition**	**Standard**	**Supershift**
dd H_2O	X	X	X
Poly-dIdC (1 μg/μl)	1	1	1
2× binding buffer	10	10	10
Nuclear extract (2-10 μg)	X	X	X
Hot probe (40 fmol/μl)	1	1	1
Cold or mutant probe (4 pmol/μl)	1		
antibody (2 μg/μl)			0.5-1
Total volume	20	20	20

After the addition of all components, spin the reaction tubes briefly in a microfuge, to bring all fluid down to the bottom of the tube. Incubate 20 min at RT. But the supershift reaction

should be kept for 10 min under RT before adding the hot probe. Then load all the samples on the 4 %-6 % non-denaturing PAGE gel.

2× binding buffer	10 mM $MgCl_2$; 0.2 mM EDTA pH8; 2 mM DTT; 0.1 M NaCl; 20 % Glycerol; 20 mM HEPES pH7.9, 0.4 mM PMSF.

Step3: Electrophoresis and autoradiography detection

Prepare a 4-6 % non-denaturing PAGE (0.5× TBE) gel for running the EMSA samples according to the following protocols.

4 %	5 %	6 %	PAGE gel
0.5 ml	0.5 ml	0.5 ml	10×TBE
1 ml	1.25 ml	1.5 ml	40 % Acrylamide
0.5 ml	0.62 ml	0.75 ml	2 % N,N'-methylene-bis-acrylamide
7.9 ml	7.52 ml	6.9 ml	H_2O
100 µl	100 µl	60 µl	20 % APS (ammonium persulfate)
10 µl	10 µl	15 µl	TEMED
	10 ml		Total volume

Pre-run the gel for 1 h in 0.5× TBE at a voltage of 5 V/cm. Load the EMSA samples into the wells of the gel. Run the gel for around 2 hours. Transfer the gel to a double layer of Whatman paper and dry the gel on a gel dryer for 45 min at 75 °C with vacuum. Put the dried gel in the cassette with screen on top for overnight exposure. Screens are qualitatively and quantitatively analyzed with a Storm 840 PhosphoImager (Molecular Dynamics).

2.11 Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) is a powerful tool for the study of protein-DNA interactions *in vivo* (Weinmann et al., 2002; Weinmann et al., 2001). In this method, protein is fixed on DNA *in vivo*. After the chromatin is sheared either by sonication or by enzymatic digestion, the protein-DNA complex could be captured by immunoprecipitation using antibodies. Subsequently, the DNA is purified by digestion of the protein with proteinase K and the copies of target DNA can be quantified by real-time PCR. Using specific antibodies against different transcriptional factors, the native recruitment of relevant factors is known from copies of DNA. ChIP assays were performed according to manufacturer's protocol (Upstate Biotechnology, Lake Placid, NY, USA).

- **Step 1: Prepare cell lysate**

1) Cells are washed with 1× PBS.
2) Add 40 µl of 0.5 M DSG freshly made (fc is 2 mM) in 10 ml of PBS/Mg^{2+}, pH8.0. Cells are fixed with DSG for 45 min at room temperature by gentle rotation.
3) Aspirate the PBS/Mg^{2+} from plate and wash the cells with 1×PBS pH8.0 twice.
4) Add 10 ml of PBS/Mg^{2+} pH8.0 containing 270 µl of 37 % formaldehyde and incubate at

room temperature for 10min with rotating.

5) Add 1 ml of 1.25 M Glycine (10×) to quench formaldehyde.
6) Wash cells three times with ice cold 1× PBS pH8.0 and transfer the plates on the ice. Add 1 ml of ice-cold 1× cold PBS pH8.0 containing 0.5 % Protease Inhibitor Cocktail II (Sigma) and 1 mM PMSF to scrape the cells into a new tube.
7) Collect cells by centrifugation at 1000 rpm for 5 min at 4 °C.
8) Add 0.35 ml of SDS lysis buffer (Upstate) containing 0.5 % Protease Inhibitor Cocktail II and 1 mM PMSF to tube, suspend cell pellet.
9) Let stand on ice for 10 min.

1× PBS pH8.0	Adjust 1× PBS pH7.5 to 8.0 with 137.5 µl of 5 M NaOH
1× PBS/Mg^{2+}	Add 5 µl of 2 M $MgCl_2$ into 10 ml of the above 1× PBS pH8.0
0.5 M DSG	Add 6.5 mg of DSG in 40 µl of 100 % dimethyl sulfoxide

- **Step 2: Sonication to Shear DNA**

1) Shear chromatin with Branson sonifier (setting 2, 40 %) for 20 s.
2) Transfer 40 µl aliquot for agarose gel analysis.
3) Add 1.6 µl of 5 M NaCl and 1 µl of 20 % SDS, 0.4 µl of 20 mg/ml Protease K, 65°C for 1h.
4) Extract with equal volume of phenol. Transfer the supernatant and add one-tenth of 3 M NaAC pH5.2. Add 1 volume of isopropanol to precipitate DNA.
5) Rinse with 70 % ethanol and air dry at room temperature.
6) Dissolve the DNA in TE buffer and analyze by 2 % agarose gel.
7) If desired, spin lysate at 14,000 rpm at 4°C for 10 min to remove insoluble material.
8) Sheared crosslinked chromatin can be stored at -80 °C.

- **Step 3: Immunoprecipitation (IP) of Crosslinked Protein/DNA**

1) Add 900 µl of Dilution buffer containing 0.5 % Protease Inhibitor Cocktail II and 1 mM PMSF into the tube containing 100 µl of chromatin (one IP).
2) Add 25 µl of Protein A agarose (Upstate) for each IP, incubate for 2 h at 4 °C with rotation.
3) Supplement 3 µg of rabbit normal IgG and continue to incubate for another 1 h.
4) Pellet agarose by brief centrifugation 3000-5000 g for 1 min.
5) Collect the supernatant. Transfer 100 µl of the supernatant as 10 % Input and save at 4°C.
6) Add 4 µg of the target antibody to the rest supernatant fraction. Incubate overnight at 4°C

with rotation.

7) Add 50 µl of Protein A agarose and incubate for 3 hour at 4 °C with rotation.
8) Pellet Protein A agarose by brief centrifugation (3000-5000 g for 1 min) and discard the supernatant fraction.
9) Wash the Protein A agarose-antibody-chromatin complex by resuspending the beads in 1 ml of the cold buffers in the order listed below and incubating for 5 min on a rotating platform followed by brief centrifugation (3000-5000 g for 1 min). Remove the supernatant fraction:
    a. Low Salt Immune Complex Wash Buffer, one wash
    b. High Salt Immune Complex Wash Buffer, one wash
    c. LiCl Immune Complex Wash Buffer, one wash
    d. TE Buffer, two washes

- **Step 4: Elution of Protein/DNA Complexes for analyses**

1) Elute twice with Elution buffer and incubate for 15 min at room temperature. Combine all elutes (~300 µl).
2) Add 12 µl of 5 M NaCl and 6 µl of 0.5 M EDTA pH8.0, 15 µl of 1 M Tris-Cl pH6.5 and 3 µl of 20 mg/ml Proteinase K and incubate at 65°C for 4-5 h or overnight to reverse the DNA-Protein crosslinks. After this step the sample can be stored at -20°C and continued the next day.
3) Extract with phenol/chloroform. Transfer the upper aqueous layer.
4) Add one-tenth of 3 M NaAC pH5.2, and add glycogen 0.2 µg/µl. Mix well.
5) Add 1 volume of isopropanol and let stand at -20 °C > 20 min. Pellet the target DNA by centrifugation for 5 min at 13,000 rpm at room temperature. Rinse with 70 % ethanol. Air-dry and dissolved into 36 µl H_2O.
6) Run quantitative real-time PCR to measure the copy number of the candidate region of target genes

Elution buffer (1 ml)	50 µl of	20 % SDS, 100 µl of 1M $NaHCO_3$ and 850 µl of dH_2O

3 Results

3.1 Developing basic knowledge and tools to the PI repressor

Our lab's previous work demonstrated that the bovine ACC-α gene is located on bovine chromosome 19. The full length bovine ACC-α cDNA comprises 7381 bp (Sequence ID AJ132890). Seven different transcripts have been found to be transcribed by the three promoters (PI, PII, PIII). The transcripts derived from PI and PII can be translated into the ACC-α 265-kDa isoform. In mammary gland PIII abundantly transcribes mRNA for translation to the ACC-α 259-kDa isoform. Among the three promoters, PI promoter is abundantly expressed and nutritionally regulated in the mammary gland, adipose and liver tissues. Due to the importance of PI in fatty acid synthesis, research on PI will shed light on the regulation mechanism of fat metabolism.

It has been reported that another promoter PIn locates more 5′ than PI as a bifunctional and GC-rich promoter expressing *ACC-α* abundantly in brain among sheep, mouse and human (Travers et al., 2005). The presence of a similar promoter possibly exists in the bovine ACC-α gene. If so, this promoter may contribute to *ACC-α* expression, warranting its investigation in bovine lipogenic tissues. In the study, bovine PIn was isolated and identified as a brain-specific promoter, barely contributes to the ACC expression in the lipogenic tissues (appendix A). Hence, PI, but not PIn, is the key promoter for ACC expression in these tissues.

Sequence analysis demonstrated that PI (Sequence ID AJ276223): a TATA-box is absent and multi-transcription start sites exist. The proximal promoter has strong activity when the repression region is deleted in reporter gene analysis, but the long promoter has retained marginal activity compared to pGL3basic. Thus, PI promoter is basically repressed by a distal repression region. Interestingly, a repression region was also found in rat PI promoter. The repression region hampers the response of PI to induction by hormone (O'Callaghan et al., 2001). Hence, the regulation of PI promoter can be better understood after the elucidation of the repression mechanism.

Further analysis manifested that the repression region is from position -1045 to -127. Restriction analysis divides the region into three possible elements: A, B and C. Removing element A or element B and C relieves the repression of PI. Hence, the repression region is bipartite, similar as the counterpart of the rat repressive element. Tae *et al.* 1994 discovered

that the upstream repressive element is a CA-28 microsatellite, unlike the bovine upstream element, which is included in a retroposon. The rat downstream repressive element of PI is a C/EBP binding site. Mutation of the C/EBP binding site relieves the repression of PI. Unlike bovine PI, a TATA box is present in rat PI promoter, which suggests that the bovine PI repression mechanism maybe different from mouse PI. The fine mapping of the exact elements and the *cis*-elements within these elements remain unknown.

To unravel the responsible elements controlling PI activity, computer-aided database analyses were performed on bovine PI. Results indicated that a great many putative binding sites of transcription factors were present both in the distal and proximal regions (Figure 7). Hence, fine mapping of the repressive elements and the repressors is a necessary and efficient means to define the molecular mechanism of PI regulation.

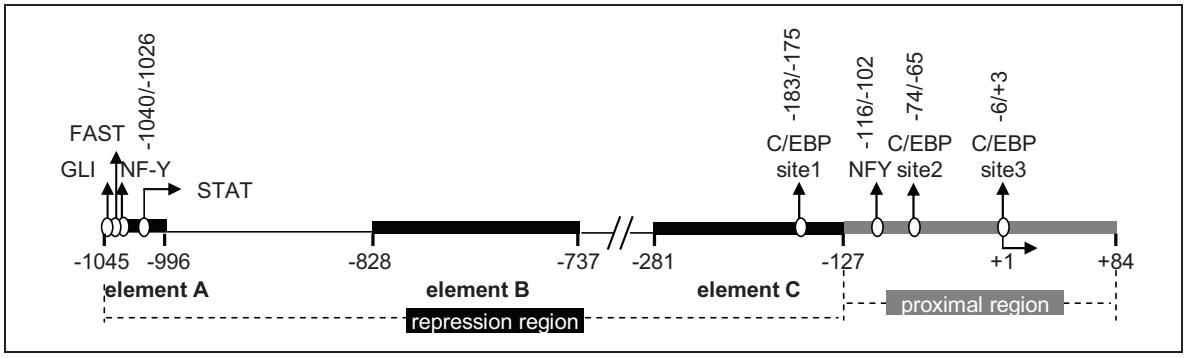


Figure 7 Putative attachment sites for transcription factors in bovine *ACC-α* PI

The corresponding putative factors are shown above the promoter. The positions are indicated below the promoter. Repressive and proximal regions are determined based on previous studies. The repressive region lies between position -1045 to -127 and the proximal region extends from -127 to +84. +1 indicates the transcription start site. Element A, -B & -C are candidate repression elements.
GLI: Glioma associated oncogene; NF-Y/CBF: Nuclear Factor-Y/The CCAAT-Binding Factor (or CP1); C/EBP: CCAAT/Enhancer Binding Protein;

Species-specific transcriptional factors are indispensable to study gene regulation. Functional studies have been well documented in human and mouse models. However, no many tools are available to study bovine gene regulation. Hence, it is necessary to clone and construct the expression vectors of the relevant transcription factors. In the current study, this includes the C/EBP and NF-Y factor families (appendix B).

3.2 PI is nutritionally regulated in different tissues and physiological conditions

In mammals, liver, adipose and mammary gland are main sites of *de novo* fatty acid synthesis. Previous data showed that *ACC-α* PI is the prominent promoter which contributes to bovine total *ACC-α* transcription in these tissues (Mao et al., 2001). It was reported that PI activity was regulated in adipose and liver tissues during starvation and refeeding in the rats (Kim and Tae, 1994). The present study concentrated on probing the regulation of bovine *ACC-α* PI. Hence, PI derived mRNA copies were measured by real-time PCR during different physiological conditions in bovine liver and adipose tissues.

3.2.1 *ACC-α* PI is repressed *in vivo* by starvation

Liver biopsies were taken from four dry standing cows. The cows were subsequently starved for 43 h, and liver biopsy was repeated for each cow. Then the cows were refed with their normal diet and 29 h later liver biopsies were taken once again.

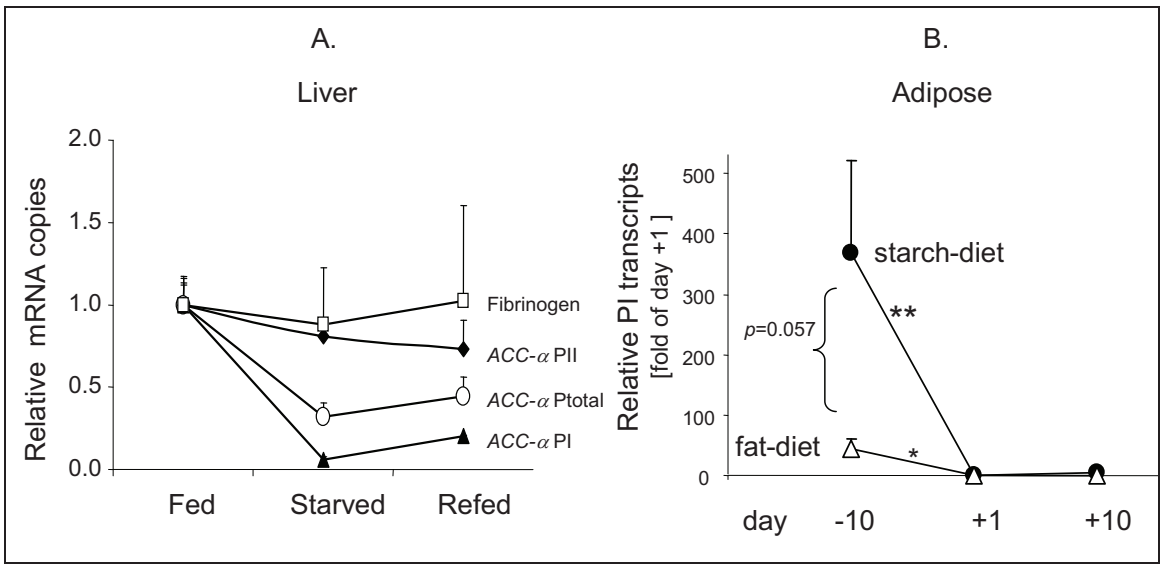


Figure 8 Expression patterns of bovine *ACC-α* in the liver and adipose tissues during different nutritional conditions by real-time PCR

A) Transcripts of genes or derived promoters in the liver during nutritional regulation.
B) PI expression by feeding changes and calving in adipose tissue.

For A, mean (error bars, SEM., n=4) of mRNA level of normally fed cows (with maximal PI activity) is set to 1. The different symbols represent various genes. Both *ACC-α* total (all promoters) and PI transcripts decreased significantly after starving ($p<0.01$), but only *ACC-α* PI transcripts increased significantly when refed for 29 h ($p<0.01$). For B, total RNA was from the subcutaneous adipose biopsy on day –10 (ten days before calving), day + 1(the first day after calving) and +10 (the tenth day after calving). Mean (error bars, SEM., n=8) of *ACC-α* PI mRNA level on day +1 is set to 1. The black round (●) and empty triangle symbols (Δ) represent the diets enhanced with starch and fat respectively before calving. * represents $p<0.05$, ** represents $p<0.01$.

In the liver as indicated in Figure 8A, the transcripts derived from *ACC-α* PI and all promoters were decreased significantly and synchronously after starvation for 43 h. The concentration of mRNA copies derived from *ACC-α* PI in the fed condition was 16 fold that found after starvation. The concentration of all *ACC-α* transcripts increased in tendency as well after refeeding, but the increase was statistically not significant. In contrast, the concentration of ACC PII derived transcripts decreased only slightly by 19 % not significantly. The concentration of transcripts encoding Fibrinogen did not change during the experiments. These were assessed as references for liver function. The measurements of PI derived transcripts thus reconfirm that PI activity in the liver is indeed nutritionally regulated.

3.2.2 Starch-enriched diet enhances, but lactation silences *ACC-α* transcription in adipose tissue

In order to evaluate the nutritional regulation of PI activity in adipose tissue, a different experimental setting had to be chosen. Groups of eight cows each were fed with two different diets for 3 months before calving. The diets consisted of either the ordinary diets (carbohydrate-enriched) or were enriched with fat components. Adipose tissue was collected from a skin area on the back behind the withers, 10 days before calving and subsequently at day1 and 10 after calving.

The same assay technique was used to measure *ACC-α* PI activity in adipose (Figure 8B). When the mean level of *ACC-α* PI derived transcripts after calving (at day +1) was set to 1, the concentration of *ACC-α* PI derived transcripts of starch-enriched group was remarkably higher (368 -fold) than that of fat-enriched group (43 -fold). Ten days after calving (until day +10), the concentration of PI derived transcripts did not change significantly in either group. These data demonstrate that the onset of lactation after calving silences the *ACC-α* PI transcription in bovine adipose tissue. Specifically they show that the starch-enriched diet elevated ACC-α PI activity ~9 fold compared to the fat-enriched group before calving (p=0.057, n=8). Hence, bovine *ACC-α* PI transcription is nutritionally and physiologically regulated in adipose tissue.

3.3 Delineation of the repression elements and factors in the distal region of PI

Previous work indicated that the activity of long PI promoter is repressed (Mao et al., 2001). Hitherto, the knowledge about the molecular nature of the repressor is limited, except that it is overlapped with a retroposon.

3.3.1 Elements contributing to PI repression

3.3.1.1 Delineation of *cis*-relevant DNA-sequence element of the PI repressor

In order to delineate and finely map the PI elements contributing to the PI repressor, PI reporter constructs (Figure 9) were established harboring serial deletion of the wild type promoter. All vectors were constructed by restriction digestion or PCR.

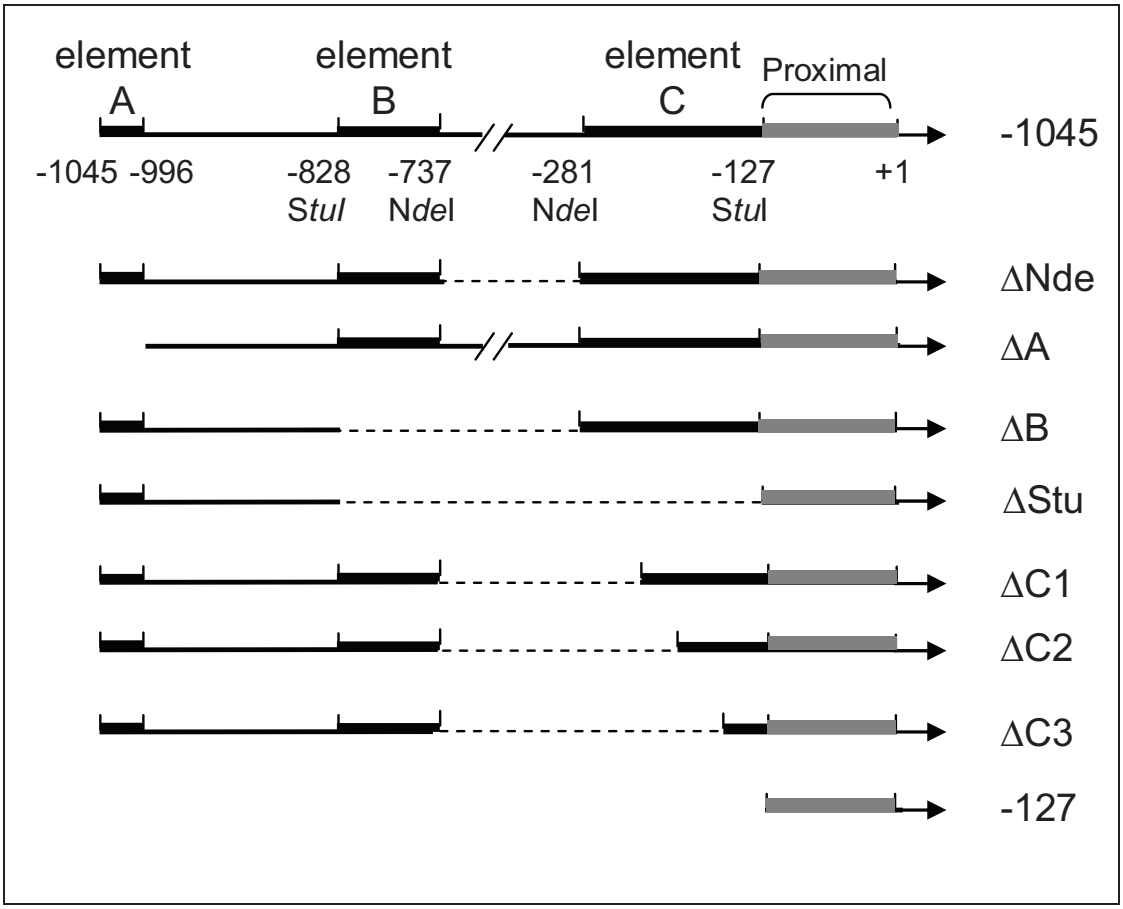


Figure 9 Structure of PI and its deletion series in reporter constructs

The wild type promoter region was cloned into the multi cloning site of pGL3basic. The derived constructs were either shortened with restriction enzyme or recloned by PCR. Bold lines indicate the sequence elements included into the respective construct.

To delete element B (EB), PCR was carried out with Ac_pI_f 9 and PI_eb_r using ΔNde as a template. The amplified product was inserted into *Kpn*I/*Nde*I (-281) of ΔNde and resulted in

ΔB. EC was deleted by three steps: ΔC1, ΔC2 and ΔC3. Three substituent forward primers PI_ec_1f, PI_ec_2f and PI_ec_3f respectively (introduced a *Nde*I site) and the same reverse primer 5_lucr were used for PCRs to amplify the insertion fragments. ΔNde was also used for the templates. The amplified fragments were inserted into the *Nde*I(-737)/*Hind*III site of ΔEB. The other vectors were constructed by according restriction enzyme as indicated in the scheme. All clones were confirmed by digestion or colony PCR, followed by sequencing.

3.3.1.2 Element A and C, but not B, repress PI activity

Serial constructs were transiently transfected into HC11 cells. The long promoter -1045 was close (repressed) and the proximal promoter (-127) was open (data not shown), just as previously reported (Mao et al., 2001). Therefore, the repression region resides between position -1045 and position -127 including elements (E)A, EB and EC as indicated Figure 10. When EA was deleted, the activity [ΔA (-996/+84)] was ~2.9 -fold of that of the wild type promoter (Figure 10). Thus, PI was derepressed by deletion of EA.

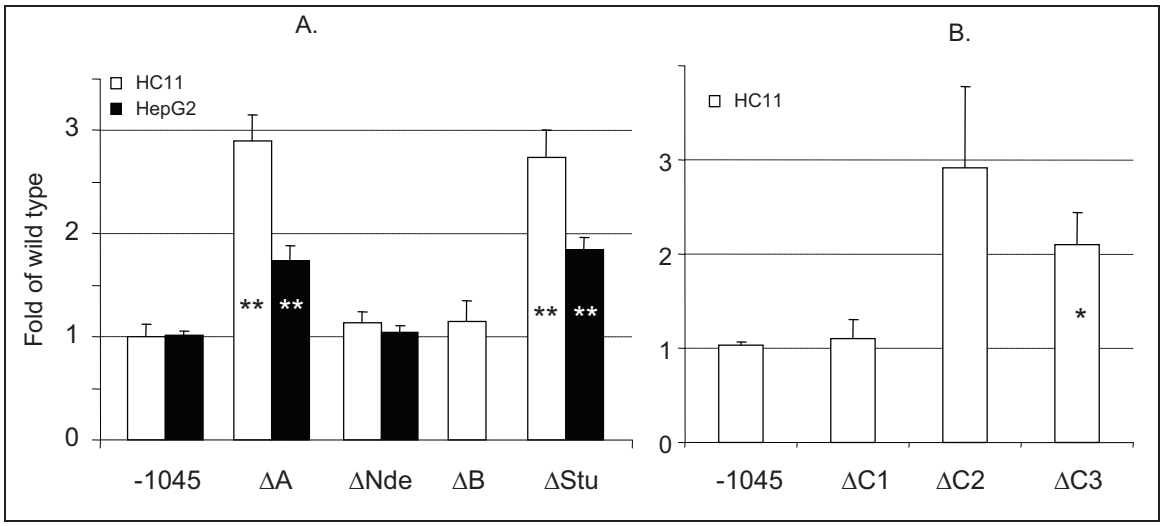


Figure 10 Mapping of the repressive elements of PI promoter by deletion in HC-11 and HepG2 cells

The map of the reporter constructs as described in Figure 9. Reporter construct and pRL-TK vectors were transient cotransfected into HC11 cells. The transfection efficiency was normalized with pRL-TK activity. The activity of wild type promoter (-1045) was set as 1.0. Empty column represents the data from HC11 cells and filled column indicates the data from HepG2. Error bars: SEM. Data indicated were the summary of at least three independent experiments. * $p<0.05$, ** $p<0.01$.

Similarly, PI was fully active in the clone ΔStu (~2.7 -fold of wild type) in which the region -828/-127 comprising EB and EC had been deleted. However, the deletion of position -737 to position -281 between two *Nde*I sites in ΔNde reporter, together with EB (-828/-281) deletion in the ΔB reporter kept the deleted PI close, indicating that EB does not contribute to PI repression (Figure 10A). Similar results were found using human hepatocellular liver

carcinoma (HepG2) cells, but the effect is moderate. Hence, the repressor is associated with two elements: EA and EC. EB does not contribute to repression. To confirm the role of EC, EC was deleted by three steps in further experiments (Figure 10B). The deletion ΔC3 derepressed the PI activity. This result indicated that the region had been deleted in ΔC3 contributes to PI repression.

To examine if the repression elements and repressor can also control heterogonous promoters, the PI driver (from -127 to +84) in -1045 was replaced with the mini-TK promoter and a reporter construct (-1045TK) was established. TK-promoter is the thymidine kinase promoter from herpes simplex virus. Subsequently, the EB and EC were deleted based resulting in ΔStuTK. These two constructs were transfected into HC11 cells. Results indicated that when the repressive elements were disrupted by deleting EB and EC, the deleted promoter was open (data not shown). Hence, PI repressive elements and repressor can also repress the heterologous TK-promoter.

3.3.2 Identification of transcription factors binding to EA and EC

3.3.2.1 Delineation of the factors binding to EA

Since element EA comprises 50 base pairs of DNA segment, it is still a large region for fine analysis of the potential transcriptional factors. Narrowing the key region of EA relevant to PI repression facilitates the eventual search for the relevant binding factors.

3.3.2.1.1 The left side of EA is relevant for PI repression in HC11

EA extends from position -1045 to -996. In order to narrow down the key repressive region of EA, EA was split by two deletions: ΔA-L and ΔA-R (Figure 11). In ΔA-L, the left part (5´) of EA from position -1045 to -1019 was deleted based on the clone -1045. Amplification was performed with the forward primer PI_A_f4 and the reverse primer 5_lucr. The PCR product was blunted and inserted into the *Kpn*I(blunted with Klenow)/*Hind*III of clone -1045. In ΔA-R, right part (3´) was deleted by PCR-mediated mutagenesis. The mutational primer pairs (P2 and P3) were PI_ec_CEBP_B_fm and PI_ec_CEBP_B_rm, and P1 and P4 were BASENDf and 5_lucr respectively. Results indicated that the deletion of the left side (in ΔA-L, -1045/-1019) resulted in derepression, similar to the deletion of the entire EA (Figure 11) in my previous experiments. In contrast, the deletion of the right side of element A (ΔA-R, -1019/-996) did not result in derepression. Hence, the left side of element A conveys repression of PI activity (Figure 11).

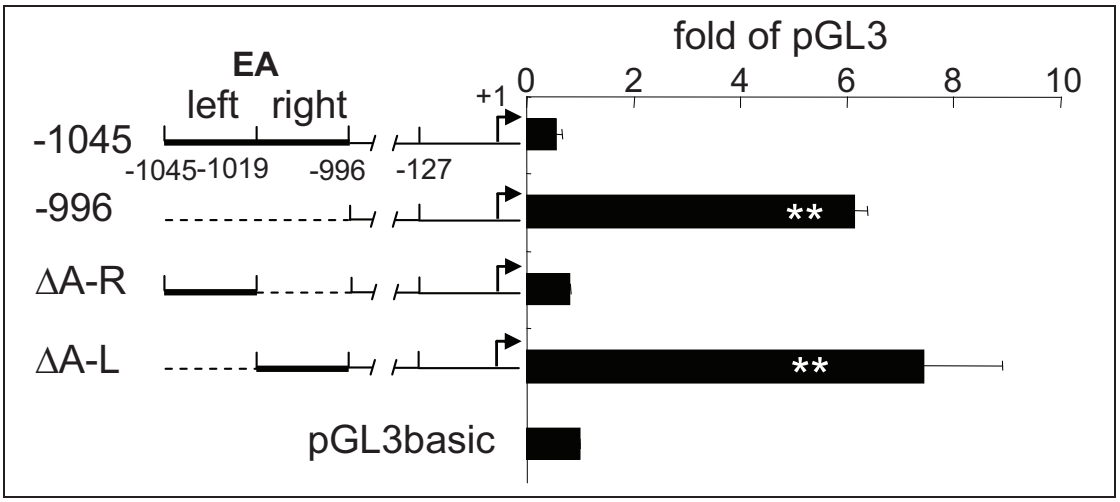


Figure 11 The fine mapping of element A repressing the PI activity

L or R: the left or right side of EA. Data indicated were from assays measured in triplicate. Error bars: SEM. **: p <0.01.

3.3.2.1.2 NF-Y and GLI may bind to EA

Computer analysis demonstrated that the left side of element A contains putative attachment sites for the factor NF-Y, Glioma associated oncogene (GLI), signal transducers and activators of transcription (STAT) and FAST (Figure 12A). To identify which of these transcription factors do indeed bind here, EMSAs were performed using the labelled element left side (EA-L) as a probe. Two main DNA-protein complexes (named C1 and C2) were formed using nuclear extracts from HC11 cells, pbMEC and mammary gland (Figure 12B). C1 could be competitively inhibited by the unlabelled GLI consensus competitor (Figure 12D: Lane 3). When the core sequence GGG in oligonucleotide of EA-L was mutated into ttt (named m3), the unlabelled mutation probe could not compete for C1 (Figure 12D: Lane 9). This indicates that GLI can bind to EA through the original sequence of m3.

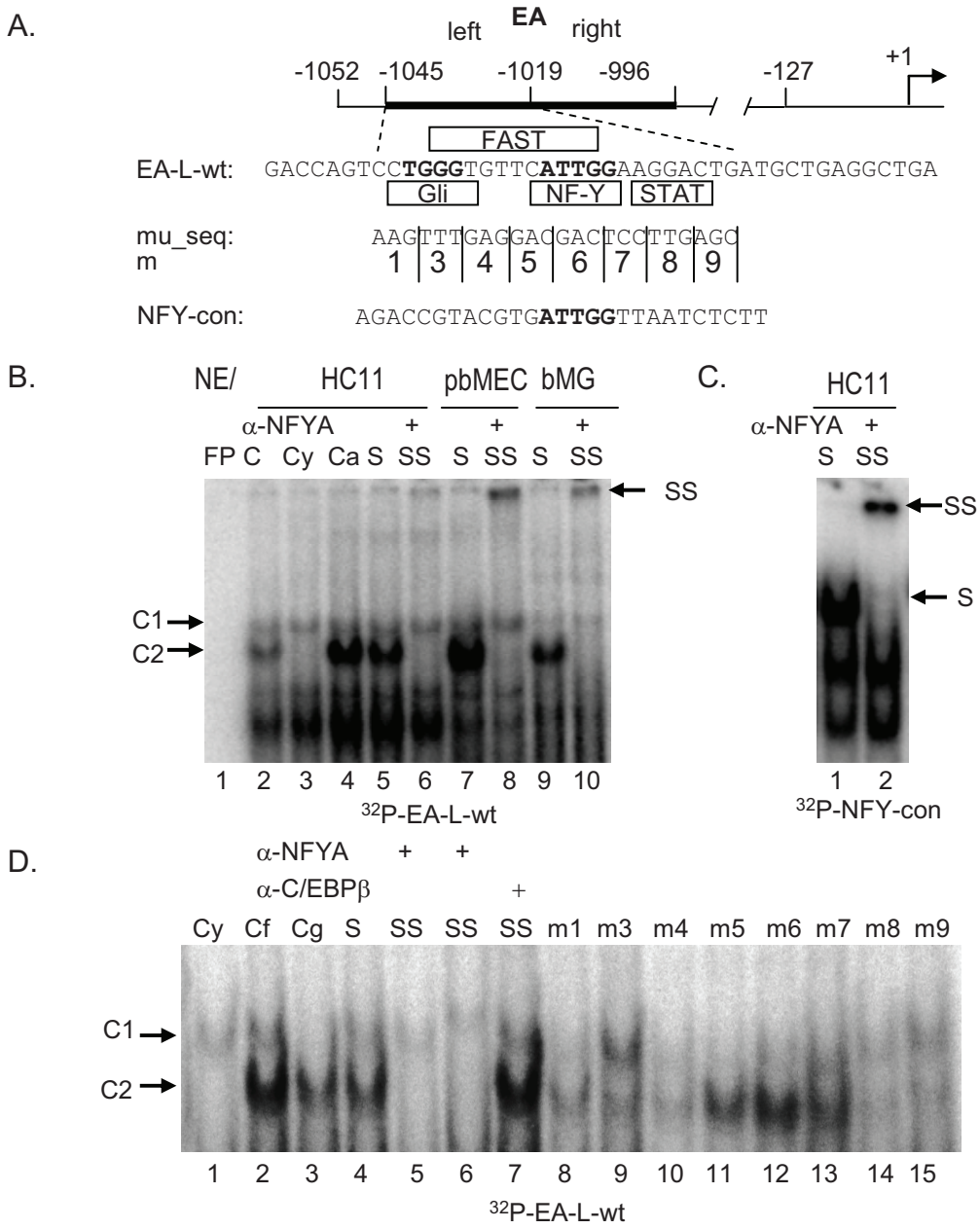


Figure 12 Comprehensive EMSA analyses of the factors binding to element EA

A) Diagram summarizing the sequences used as probes in EMSA assays; the putative binding sites for transcription factors are depicted. `m´ represents is the alteration of wild type promoter sequence (wt) introduced into the wt sequence, as used for the EMSA-analyses.
B) EMSA analyses of the putative factors interacting with the left side of element A with nuclear extracts from mouse HC11, bovine pbMEC (primary bovine mammary epithelial cells) and MG (mammary gland). FP: free probe. C: competition of self. Cy: **C**ompetition of consensus NF-**Y** binding motif. Ca: **C**ompetition of consensus ST**A**T6 binding motif (GTATTTCCCAGAAAAGGAAC). S: shift. SS: supershift with anti-NF-YA. ^{32}P-EA-L is the left side of EA. C1: DNA-protein complex 1; C2: complex 2.
C) NF-Y binding to its consensus binding motif. ^{32}P-NF-Y-con is the consensus NF-Y binding motif. `S´ represents the DNA-protein complex formed with nuclear extract from mouse HC11 cells. `SS´ represents the supershift band.
D) Competition analyses indicated that NF-Y and GLI bind to the contiguous sites of EA-L. Cf: **C**ompetition of **F**AST (GCTGCCCTAAAATGTGTATTC-CATGGAAAT). Cg: **C**ompetition of **G**LI (TCGACTCCCGAAGACCACCCACAATGATGGTTC (Yoon et al., 2002)). SS is the lane with antibody. m1-9 are unlabelled mutational series of EA-L for mutation competition.

Results also indicated that in the presence of 100 fold molar excess unlabelled NF-Y consensus probe, C2 is efficiently competitively inhibited using nuclear extracts from HC11 (Figure 12 B: Lane 3, D: Lane 1). Addition of anti-NFY antibodies always supershifted C2, no matter if nuclear extracts from mouse or bovine mammary epithelial cells or from udder were used (Figure 12B, lane 6, 8, 10). The unrelated rabbit anti-C/EBPβ did not affect C2 (Figure 12D, lane 7). Taken together, the data show unambiguously that NF-Y binds to the left side of EA. Supporting these data, the mutated probes (m5, 6 and 7) did not affect C2 in mutational competition analyses (Figure 12D: Lane 11, 12, 13). Figure 12 also suggests both FAST and STAT can not bind to EA-L. The data together show that element A may eventually be bound by the factors GLI and NF-Y.

3.3.2.1.3 Mutation of the CCAAT binding motif of NF-Y relieves repression

In order to further unravel the functional role of NF-Y interaction with the reverted CCAAT element in EA-L, the core NF-Y binding sequence was mutated from A**TTG**G to A**gac**G by PCR mediated site-directed mutagenesis. It was reported that the CCAAT nucleotides mutations dramatically decreased NF-Y binding (Dorn et al., 1987). For the PCR, P2 and P3 for EA-m6 were replaced by the primers PI_eA_m6f and PI_eA_m6r. The resultant mutant reporter construct (EA-m6) was transiently transfected into HC11 cells.

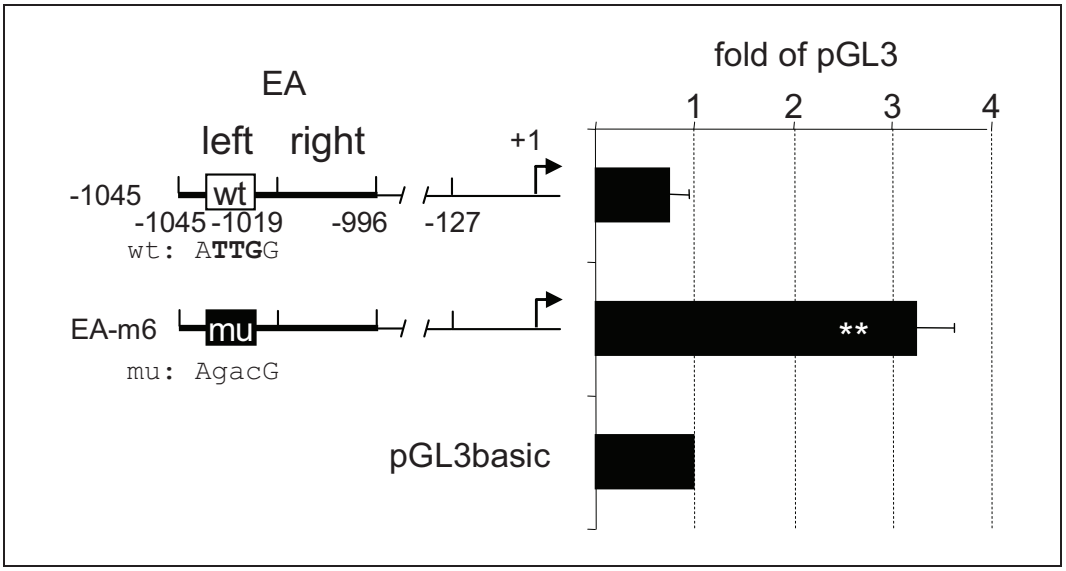


Figure 13 Derepression of PI promoter activity by mutation of the NF-Y binding site in the left side of element EA

The results were the average of four independent experiments. Error bars: SEM. ** represents $p<0.01$.

Results indicated that the relative activity of the mutant promoter is 3.2 -fold of that of pGL3basic, in contrast to the wild promoter activity which was 0.8 -fold of pGL3basic in this case (Figure 13). Hence, the mutation of three nucleotides in the reverted CCAAT binding motif resulted in significant derepression of PI. Since I have determined that the identified mutation can block the binding of NF-Y (Figure 12D), NF-Y interacting with the reverted CCAAT binding motif in EA acts as a repressor. Hence, mutation of the NF-Y binding motif alone, without altering the GLI attachment sites, relieves repression of PI in mammary epithelial cells.

3.3.2.2 Delineation the factors binding to EC

3.3.2.2.1 Element C is bound by C/EBP factors

Subsequently, research is focused on the repressive motif and the repressor in EC. Computational analysis manifested a putative C/EBP binding motif (site1) in EC (Figure 14A). In order to confirm the possibility that C/EBP interacts with its binding motif in EC, EMSA analysis was performed using the labelled oligonucleotide of EC as a probe. Results demonstrated that two protein-DNA complexes were formed using the nuclear extracts prepared from HC11. These two complexes (Figure 14B ´a´ S) were competitively inhibited by unlabelled wild type oligonucleotide competitor (Figure 14B ´a´ C), but the unlabelled mutant competitor can not affect them (Figure 14B, ´a´ Cm). After adding anti-C/EBPβ (Δ198), shift bands formation was hindered in the extent, and retarded bands are visible (Figure 14B ´a´ SS). Hence, C/EBP factors can bind to the C/EBP binding motif in the repressive element (EC) of PI.

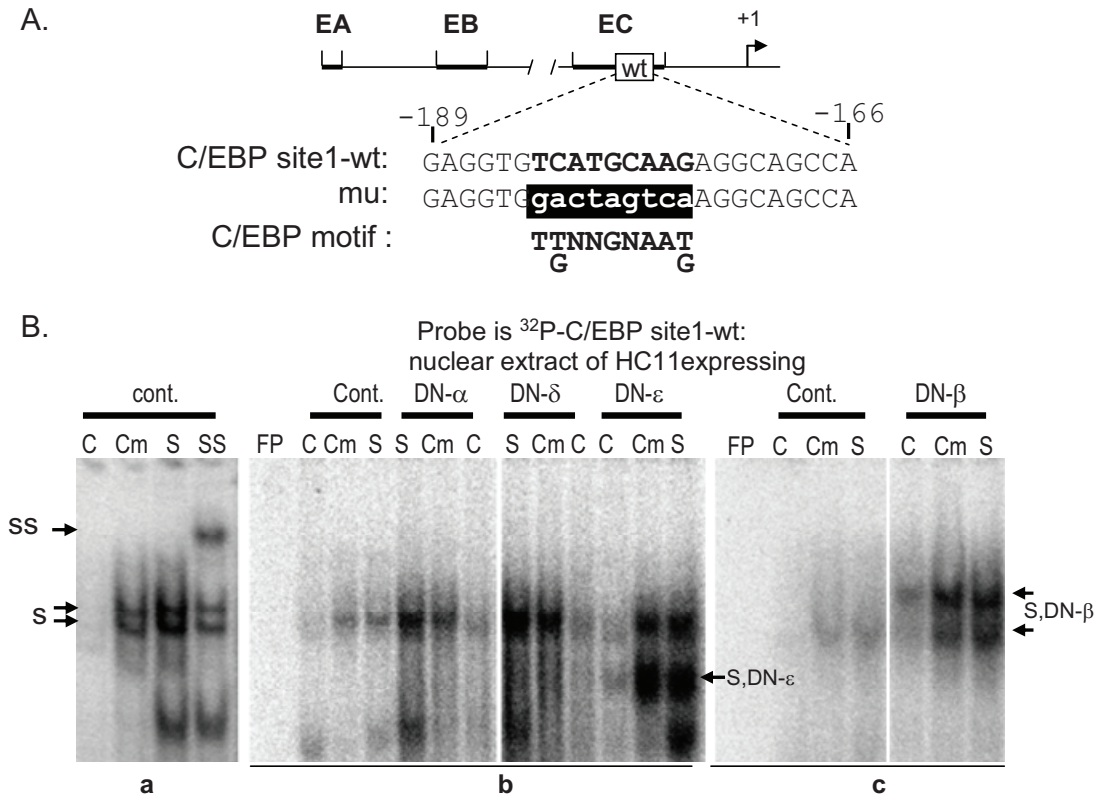


Figure 14 Determination of the C/EBP factors binding to the repressive element EC

A) Diagram summarizing the sequences used as probes in EMSA assays; Putative wt DNA sequence for C/EBP binding is shown in black bold-face. The mutated DNA sequence is shown in white lower-case with black background.
B) The probe was labelled with ^{32}P-dCTP. Nuclear extracts are from HC11 cells of control or transiently transfected DN-C/EBP factors as indicated. **FP**: Free Probe; **C**: Competition with cold C/EBP site1-wt; **Cm**: Competition with cold C/EBP site1-mu. **S**: Shift; **SS**: Supershift with anti-C/EBPβ. S and SS with arrows indicate the shift and supershift bands. `a´, `b´, `c´ represent three independent experiments each from one gel.

3.3.2.2.2 C/EBPβ and -ε can bind to the C/EBP binding site in EC

It is uncertain which member directly binds to the C/EBP binding motif in EC, because there are many members in C/EBP family, and the epitope recognized by anti-C/EBPβ (Δ198) is the conserved C-terminus. Expression constructs were constructed, including C/EBPα, -β, -δ and –ε. However, since the factors are all of about the same molecular weight, it is hard to differentiate the shift bands. They were truncated by deleting the N-terminal transactivation domain resulting in so called trans-dominant ("DN"-) factors. Their binding to a relevant probe can easily be distinguished from binding of the native factor, due to their low molecular weight. Also, these DN-factors were engineered to carry tags, such as the six-His residue comprised His-tag. Application of anti-His-tag antibodies can permit identification of DN-factor binding to the probe in EMSA-supershift analyses, providing adequate binding exists between the probe and the DN-factor.

To differentiate which member of the C/EBP family bound to element EC, nuclear extracts were prepared from HC11 cells transiently transfected with DN-C/EBPα, -β, -δ or –ε. DN-C/EBP have only DNA binding and leucine zipper domains, thus the lower molecular weight results in the lower, or stronger shift band, differs from the native shift band. For DN-C/EBPβ, 154 amino acids were deleted, and a His-tag and a Strep-tag were fused into the protein resulting in a similar molecular weight as native full length C/EBPβ. Results derived from the nuclear extract of HC11 cells over-expressing DN-C/EBPε indicated that a specific DNA-DN-C/EBPε complex was formed at the C/EBP binding motif in EC. However, not DN-C/EBPα or -δ. The binding activity of DN-C/EBPα and -δ were confirmed using the labelled C/EBP consensus binding motif (data now shown). Regarding C/EBPβ, the intensities of DNA-protein complexes formed by the nuclear extract of HC11 cells over-expressing DN-C/EBPβ were much higher than by the control nuclear extract. Hence, C/EBPβ and -ε may directly bind to the C/EBP binding motif (site1) in EC (Figure 14B, gel `b´ and gel `c´).

3.3.2.2.3 Mutation of C/EBP binding site (site1) in EC relieves repression

The conserved core site (**GNAA**) in the conserved C/EBP binding motif (TT$_{/G}$NN**GNAA**T$_{/G}$) is very important for C/EBP binding (Ryden and Beemon, 1989). In order to examine the functional role of C/EBP factors binding to the C/EBP site1 of EC, the four core nucleotides: **GCAA** of the C/EBP site1, were substituted by **tagt** in a site1mu reporter construct by PCR-mediated site directed mutagenesis. The P2 and P3 used for C/EBP site1 mutation were PI_ec_CEBP_B_fm and PI_ec_CEBP_B_rm respectively. Results demonstrated that the mutation relieved the repression of PI (Figure 15), just similar to the role of NF-Y binding motif in EA. The promoter activity was significantly higher (3.7 fold) than that of the wild type reporter (1 fold). Hence, C/EBP interacting with the C/EBP site1 contributes to PI repression.

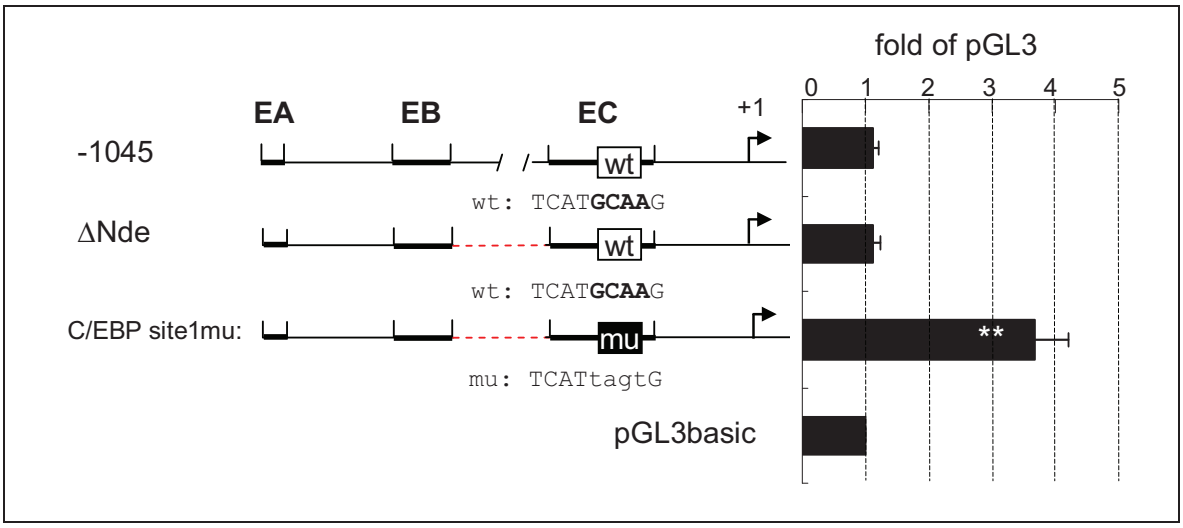


Figure 15 Derepression of PI promoter activity by mutation of the repressive C/EBP binding site in element C

The C/EBP binding site in the mutated reporter was mutated from GCAA into tagt based on the reporter ΔNde. 1.5 µg of reporter construct was transfected into HC11 cells, the transfection efficiency was normalized by phRL-TK and the data were normalized with pGL3basic. Data shown were from ten independent experiments. Error bars: SEM. **:$p<0.01$.

3.3.3 Functional analyses of NF-Y and C/EBP factors on regulation of PI

EMSA and mutational techniques described so far have demonstrated that NF-Y and C/EBP factors may be the repressors of PI regulation. Hence, to further evaluate the function of candidate factors in regulation of PI, the relevant expression vectors of NF-Y factors and various C/EBP factors were cotransfected together with the wt PI promoter reporter construct (-1045) into HC11 cells. This resulted in intact NF-Y factors (NFYA+NFYB+NFYC) activating, but not repressing the PI promoter (-1045). In contrast, the single subunit of NF-YA had no effect (Figure 16A). Results also manifested that 100 ng of C/EBPα and -ε expression vectors can significantly activate the PI promoter in HC11 cells (Figure 16B). Equal amounts of -β and -ε expression vectors could only moderately activate the promoter PI. Taken together, NF-Y and C/EBP factors both activate PI promoter, playing no part in PI promoter repression.

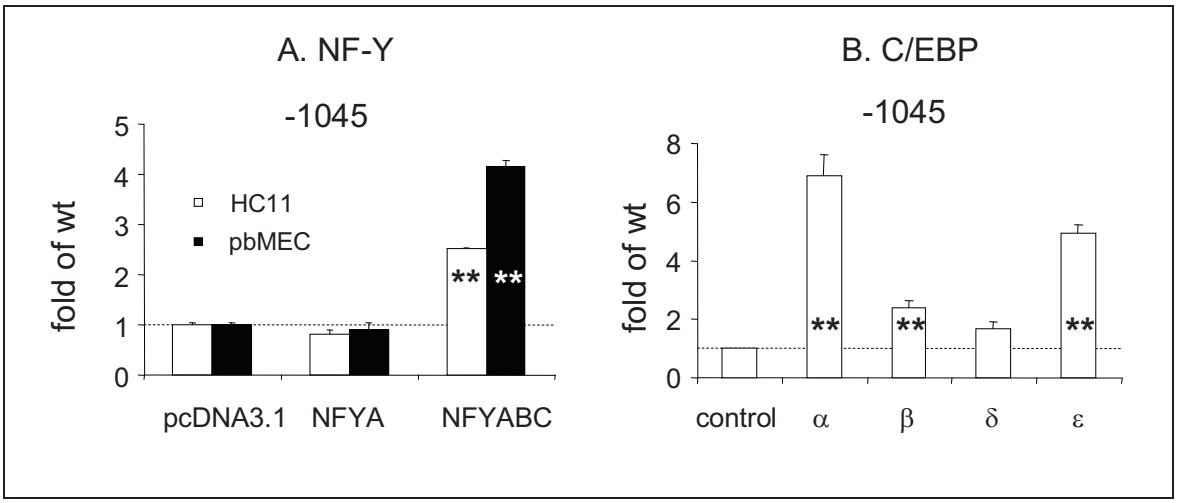


Figure 16 Cotransfection analyses of NF-Y and C/EBP factors on the PI promoter

The wild type reporter (-1045) was used for cotransfection analyses. A) 1.5 µg of reporter construct was cotransfected with 200 ng of NF-YA, or 600 ng of NF-YABC (each 200ng) expression vector constructed in pcDNA3.1. B) 100 ng of C/EBPs expression vector was cotransfected as described in the methods, with 1.5 µg of -1045 reporter in HC11 cells. Transfection efficiency was normalized by phRL-TK renilla luciferase. Activity was the fold change relative to mock transfection, in which only -1045 was transfected. Error bars: SEM. Data were from at least three independent experiments. Black columns represent transfection data in pbMEC. α, β, δ and ε are C/EBPα, -β, -δ and –ε respectively. ** $p<0.01$

3.4 Genetic control elements in the proximal PI

In order to understand the paradox that the addition of the factors previously presented repressing rather than activating the wt-PI promoter, systematic analyses were carried out on the functional elements relevant for activity of the short, proximal promoter segment. Computer analysis against databases indicated that there are several conserved attachment sites of transcription factors in the proximal PI promoter, including NF-Y & C/EBP (Figure 17A).

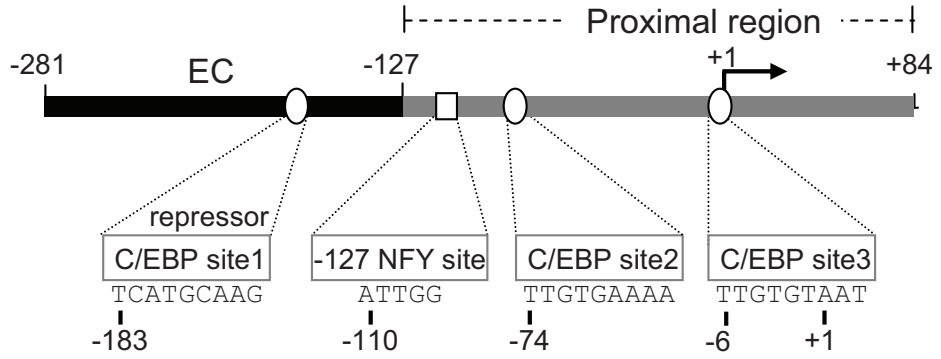
3.4.1 *Cis*-elements in the proximal PI are conserved among species

The distal parts of *ACC-α* PI are species-specific, being found in rodents and ruminants (Barber et al., 2001; Kim, 1997; Mao et al., 2001). However, sequence comparisons of proximal PI suggested that bovine PI (-127/+84) shows 93 % identity with ovine PI (-186/+29), and 75 % identity with murine PI in Figure 17B (-200/+26). In detail, bovine C/EBP site3 (**TT**GT**G**T**AAT**, -6/+3) motif is the same as the ovine C/EBP site (-65/-57). The rat C/EBP site (**TT**AT**G**C**AAT**) which was reported as a repressive C/EBP binding site (Tae et al., 1994) is identical to the mouse C/EBP site (-71/-63). Upstream of the C/EBP binding site in different species, there are several conserved hormonal control elements, such as E-box, USF and ChoRF (Koo et al., 2001; O'Callaghan et al., 2001; Travers et al., 2001). Despite variability of the absolute positions of C/EBP binding sites between rodent and ruminant, the relative positions of the C/EBP and other factors in the proximal region are similar (Figure 17).

Interestingly, there is no TATA box in bovine *ACC-α* PI (Mao et al., 2001), though a similar sequence motif is found in bovine (TA<u>A</u>AAA), ovine and mouse (TA<u>T</u>AAA). The third nucleotide of bovine is ‘A’, but that of ovine and mouse is ‘T’. This substitution means the motif cannot bend to allow RNA polymerase complex binding. Thus, bovine *ACC-α* PI does not contain a functional TATA-box (Keaveney et al., 1993), confirmed by the fact that the most 3′-TSS of bovine *ACC-α* PI lies upstream (overlapping with the C/EBP binding motif) of the TAAAAA sequence motif (Mao et al., 2001).

Moreover, one potential NF-Y binding site was found in bovine PI (-116/-102) and ovine PI (-175/-161). Due to the importance and the specificity of bovine proximal *ACC-α* PI promoter, the relevance and contributions of NF-Y and C/EBP factors were examined in this study.

A.



B.

Identity of Proximal PI	Ovine (-186/+29)	Murine (-200/+26)
Bovine (-127/+84)	93%	75%

C.

```
                                  -127 NFY site
b -127 CCTTGGCAGT GTCTGCAATT GGATAATAAG GAGGGAGGAG AATCTGAAAT
o -186 CCTTGGCAGT GTCTGCGATT GGATAATAAG GAGGGAGGAG AAGCTGAAAT
m -200 ...TGGAAGT GTTTGGGACT GGATAAGAAG TGGGGAGGGA GAGCC.AAAT

          C/EBP  site2
b - 77 GCCTTGTGAA AACTCAA... .......... ....GTGTCC ACGTGAAAAC
o -136 GCCTTGTGAA AACTCAA... .......... ....GTGTCC ATGTGAAAAC
m -154 GCCATGTGAA AACCCATAGC CTGTGGAACC CCCGGTGTCC ATGTGAAAAC

                                                C/EBP  site3
b - 44 ATTGTGTGGA GCCTCTGTGG CGAACTCTGT CTTTCTTGTT GTGTAAT...
o -103 ATCGTGCAGA GCCTCTGTGG CGAACGTTGT CTTTCTCGTT GTGTAAT...
m -104 ACTGTGGGCA GTCTCCAGGA CAAACACTGT CCA.....TT ATGCAATGGG

b +  4 ..CTTGCCTT TCATCACCAG GACCAGACTT AAAAAGCTGC CTTC.TGCGG
o - 56 ..CTTGCCTT TCATCATCAG GACCAGACTT ATAAAGCGGC CTTCCTGCAG
m - 59 ATCTTGCCTT TCGACACCAG GACTAGTCTT ATAAAGAGGC CTCCCTGAGG

b + 51 ACAGTTTCTG ACCTTTTGGT GTGAAGTTCC CTCAG
o -  8 ACAGTTTCTG ACCTTTTGGT GTGAAGCTCC CTCAG
m -  9 AGATTTTCTG ACCTTATGGT GTGAGGTTCC GAAAG
```

Figure 17 Multiple alignment of the proximal region of bovine, ovine and mouse PI

A) Putative binding sites in the proximal PI promoter of the bovine ACC-α gene. The black box represents the element EC region. The box filled gray represents the proximal PI promoter segment.
B) The identity of the proximal PI sequence of bovine PI (b, –127/+84), of ovine PI (o, –186/–29) and of murine PI (m, –200/+26).
C) The sequence comparison of the PIs of bovine, ovine and murine. The bold nucleotides represent the putative binding site as indicated. The underlined nucleotide is the transcriptional start site (+1) where relevant.

3.4.2 NF-Y activates proximal PI by binding to its proximal binding site in PI

3.4.2.1 Interaction of NF-Y with the its proximal binding site results in activation of PI

The CCAAT binding motif is usually located approximately 60-100 bp upstream of the transcription start site in many eukaryotic genes, just as was found for the putative proximal NF-Y binding motif in PI between -127 to -91. In order to determine whether NF-Y activates the proximal PI promoter, HC11 cells were cotransfected with the expression vectors of all NF-Y subunits and the proximal PI promoter (-127). Results revealed that NF-Y could significantly activate the proximal PI promoter (Figure 18). Thus, the NF-Y activational binding motif lies in the proximal region. To confirm the proximal NF-Y binding site of PI, the key binding site of NF-Y (CA**ATTGG**AT) was substituted with the sequence AAATACGGG resulting in the mutated construct, which was also cotransfected into HC11 cells. The results demonstrated that the mutation of the proximal NF-Y binding site significantly reduces the proximal PI activity in HC11 cells, and also significantly blocks the NF-Y mediated transactivation of proximal PI (Figure 18). Hence, NF-Y functions as an activator of proximal PI when it interacts with its binding motif in the proximal region.

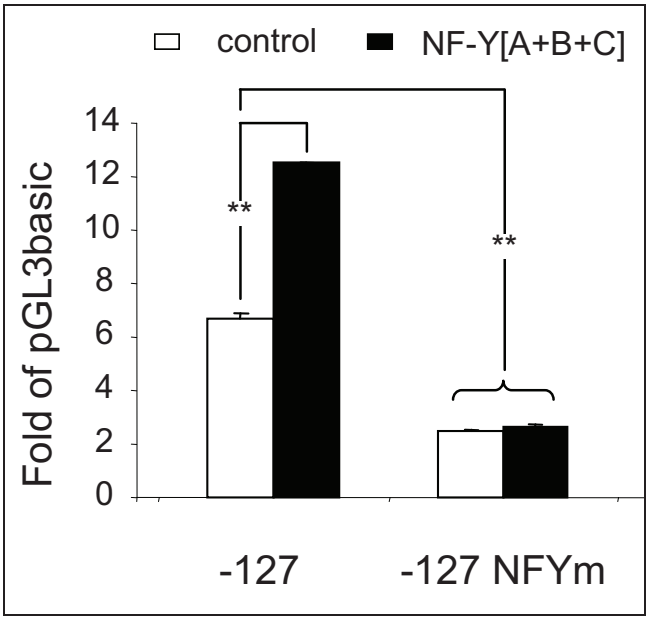


Figure 18 The NF-Y binding site in the proximal PI is required for its basal activity and NF-Y mediated activitation

600 ng of each NF-YA+B+C (each 200ng) were transiently cotransfected with 1.5 µg of -127 or -127NFYm. In the mutated reporter (-127NFYm), the NF-Y binding site was disrupted by fusion PCR mediated site-directed mutagenesis from CA**ATTGG**AT (wild type) into AAATACGGG. Error bars: SEM. Data shown are representative from three independent experiments assayed in triplicate. ** represents $p<0.01$.

3.4.2.2 NF-Y binds to proximal NF-Y binding motif

To further determine whether NF-Y binds to the reverted CCAAT element (-127/-91) in proximal PI region, EMSA experiments were performed with the oligonucleotides corresponding to sequences -127 and -91 (Figure 19). The sequence of NF-Y consensus binding motif (Cicchillitti et al., 2004) was also used as a probe. Nuclear extracts from HEK293 cells expressing three recombinant NF-Y subunits (NF-YA, NF-YB and NF-YC) were incubated either with or without adding anti-NF-YA to the probes. The results demonstrated that the consensus NF-Y motif competes efficiently for the complex formed with the proximal NF-Y motif. The complexes formed with both probes are supershifted by the anti-NF-YA antibody (Figure 19). Taken together, the NF-Y complex binds to its proximal motif and contributes to activate the PI promoter.

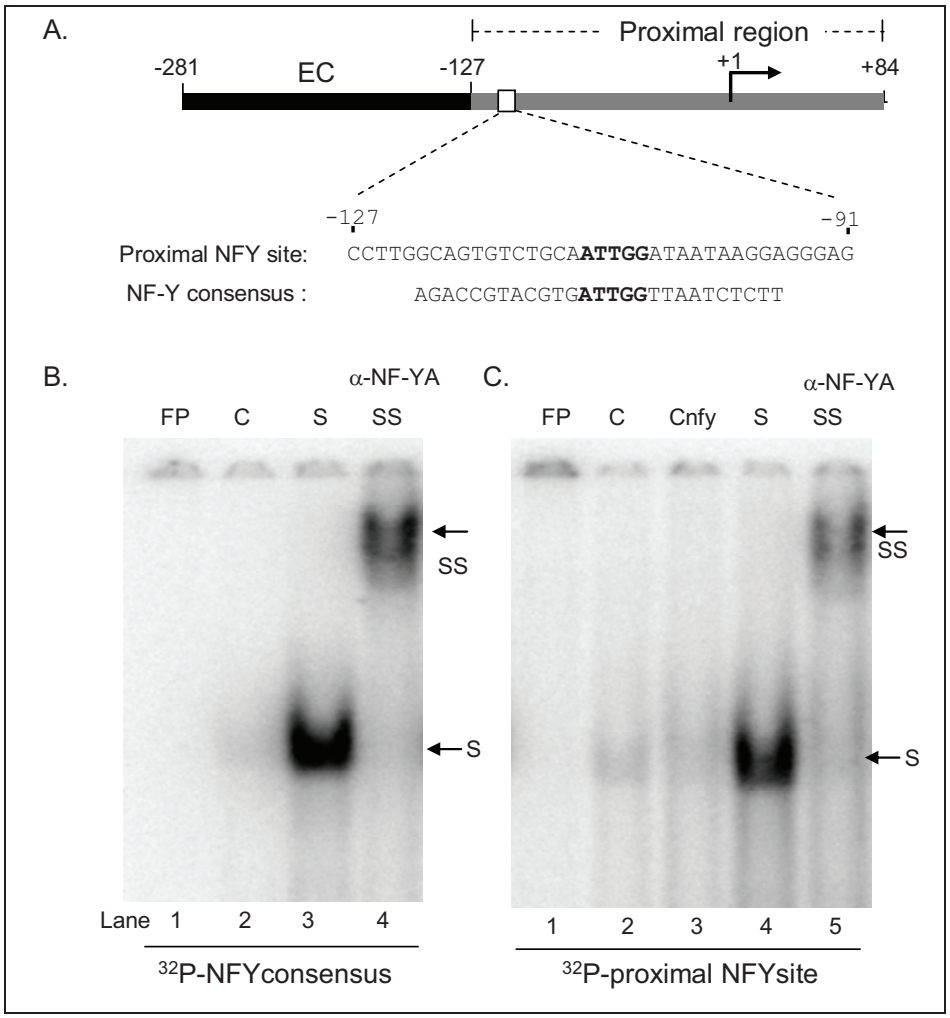


Figure 19 EMSA analysis of NF-Y binding site in the proximal PI promoter.

A) The NF-Y binding site of proximal PI and consensus. The bold letters represent the reverted CCAAT core site of NF-Y motif.
B) NF-Y site in *ACC*-α proximal PI were labelled and used for analyses. Nuclear extracts were prepared from HEK293 cells over-expressed with NF-Y[A +B +C]. FP: Free Probe; Cs: competition of itself cold probe; Cnfy: competition of NFY consensus probe; S: Shift; SS: Supershift with anti-NF-YA.

3.4.3 C/EBP factors activate the proximal PI promoter by interaction with the C/EBP site3 of proximal PI

3.4.3.1 C/EBP factors bind to the C/EBP site3, but not site2

Since the region -189/-166 in the PI promoter was identified as a C/EBP binding motif repressing PI activity, whilst C/EBPα, -β, -δ and -ε all up-regulated the PI promoter, I analyzed the proximal region (-127/+84) of PI and detected another two C/EBP binding motifs located at -74 bp and -6 bp. These are designated herein as C/EBP site2 and C/EBP site3 (Figure 20A). To identify nuclear factors that bind to this motif, again EMSA experiments were performed using nuclear extracts extracted from bovine and mouse cells or tissues. Incubation of a probe consisting of the C/EBP site3-wt with the nuclear extracts revealed three distinct protein-DNA complexes (‘S’ with three arrows, Figure 20C). Binding of the nuclear proteins to this probe was completely inhibited by an excess of unlabelled probe, while not affected by the addition of the mutated oligonucleotide C/EBP site3mu. Supershift assays further demonstrated the presence of the transcription factor C/EBP in the ‘S’ protein-DNA complexes. The entire DNA-protein complexes bands seen in the absence of antibody were supershifted when anti-C/EBPβ (Δ198) was included into the incubation. Comparable results were found by the addition of nuclear extracts from primary bovine mammary epithelial cells and mammary gland tissue. In contrast, incubation of a probe (C/EBP site2-wt) with nuclear extracts prepared from HC11 manifested no specific protein-DNA complexes (Figure 20C). Since the epitope recognized by the anti-C/EBPβ antibodies is conserved among C/EBP family members, these results indicate that members of the transcriptional factor C/EBP family can bind to the proximal C/EBP binding motif (C/EBP site3). However, it is unclear which ones.

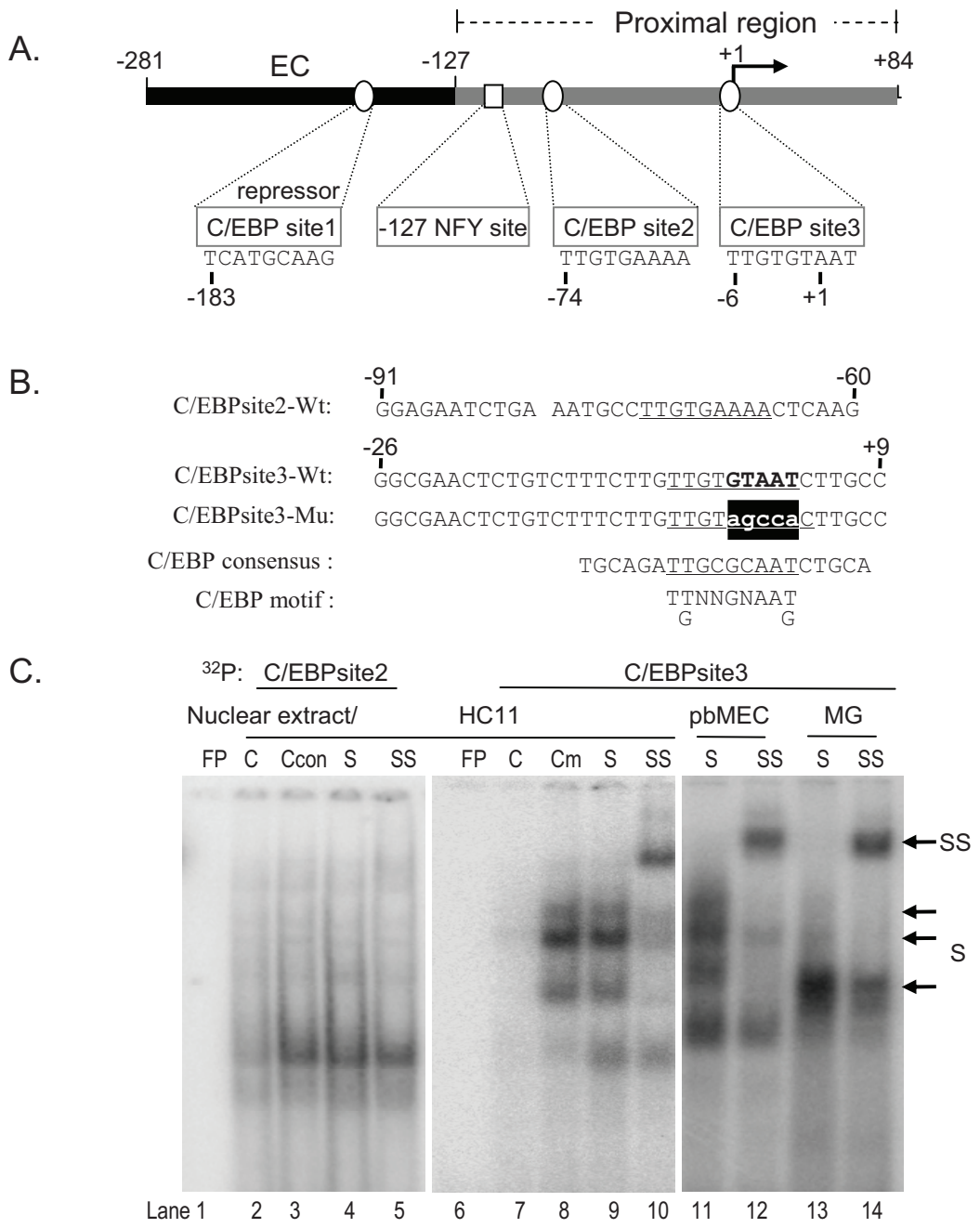


Figure 20 Identification of the C/EBP site in the proximal PI promoter

A) The putative C/EBP binding sites in the proximal PI and the repressive C/EBP binding motif (site1) are shown.
B) The sequences of the relevant probes derived from the proximal PI sequence. For mutation of site3, the original sequences (bold) were mutated into the white with black background. The C/EBP consensus binding sequence was as described Wu et al, 2003. C) C/EBP site2 and site3 were labelled and were used for EMSA. Lane1-5 are for site2, and Lane 6-14 are for site3. Wt: wild type; Mu: mutant type. Nuclear extracts were prepared as described in method from HC11, primary bovine epithelial cells (pbMEC) and mammary gland (MG) tissue. 'S' with arrows indicates the shift bands. 'SS' with an arrow represents the retarded bands supershifted by antibody in the experiment.

3.4.3.2 C/EBPα, -β and -ε may interact with the C/EBP site3

The C/EBP family members share a high degree of amino acid homology in the DNA binding and leucine zipper domains. In the family, four members (C/EBPα, -β, -δ and -ε) which have complete activation and repression domains, are regulated in different physiological conditions (Crosson et al., 1997; Matsuno et al., 1996; Ramji and Foka, 2002; Wu et al., 1998). In order to determine which member directly interacts with the C/EBP binding motif in the proximal PI and potentially regulates bovine *ACC-α* PI activity, HC11 nuclear extracts were isolated from control and DN-C/EBP vector-transfected cells. A labelled C/EBP site3-wt probe was incubated with each nuclear extract in the absence or presence of anti-C/EBPβ or anti-His-tag.

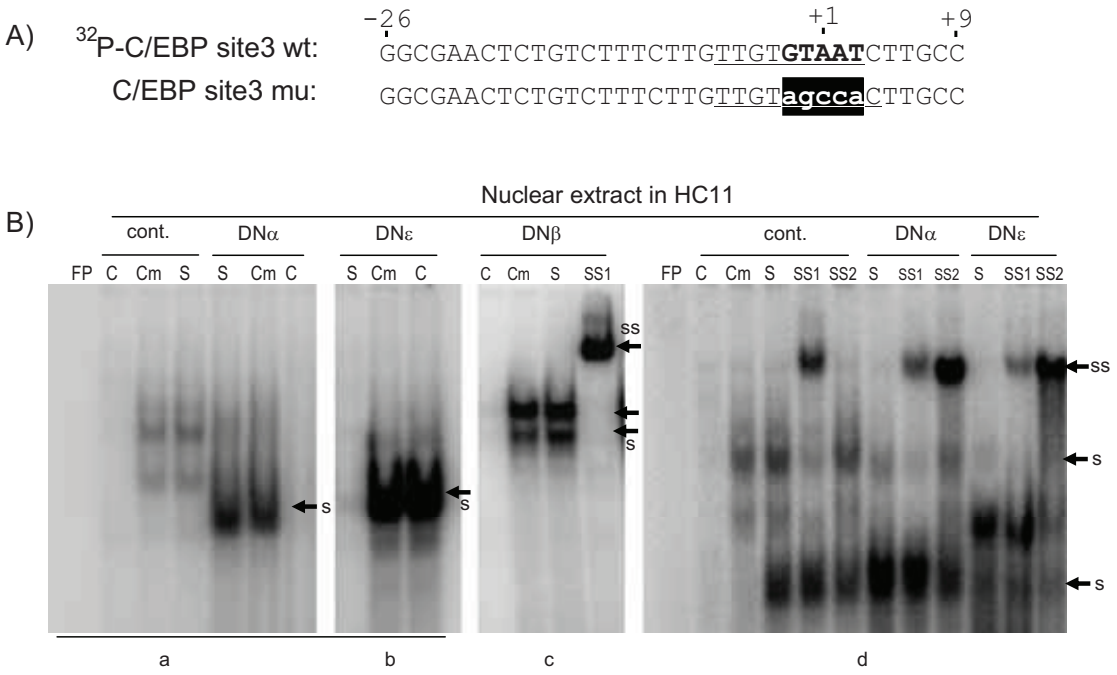


Figure 21 Identification of the members of the C/EBP family which directly bind to the proximal C/EBP binding site of PI

The labelled probe of C/EBP site3 was used. Nuclear extracts prepared in HC11 cells were of control (HC11) or cells expressing DN-C/EBPs (DN-α, DN- β DN- δ, DN- ε). Antibody of SS1 is anti-C/EBPβ and that of SS2 is anti-His6. FP is free probe; C is competition of itself (the same sequence used for hot probe); Cm is competition of mutation; S is shift. Gel `a´ and `b´ were from one intact gel.

Results revealed that nuclear extracts from transfected DN-C/EBPα, -ε (Figure 21B gel -a and -b) and β cells (Figure 21B gel -c) formed one additional and prominent complex with the labelled C/EBP site3-wt probe. The additional protein-DNA complexes are competed by a molar excess of cold C/EBP site3-wt competitor, but are unaffected by a molar excess of

C/EBP site3-Mu competitor (Figure 21B gel-a, -b, -c). Hence, C/EBPα, -β and -ε may directly bind to the C/EBP site3 in the proximal PI. In contrast, weak protein-DNA complexes were detected in control (Figure 21B gel -a) and DN-C/EBPδ transfected cells extracts (data not shown).

Antibodies were used to supershift the protein-DNA complexes in EMSA to further identify the specificity of direct binding of C/EBPα, -β and -ε. The results demonstrated that the retarded supershifted bands were visible by anti-C/EBPβ with nuclear extracts of control and DN-C/EBPα, -β and -ε transfected cells. However, the retarded complex could only be seen by anti-His-tag using the nuclear extracts prepared from DN-C/EBPα and -ε-transfected cells (Figure 21B gel `d´). For DN-C/EBPβ, all additional shifted bands are supershifted by anti-C/EBPβ antibodies (Figure 21B gel `c´). Taken together, C/EBPα, -β and -ε directly interact with the C/EBP site3 of proximal PI.

3.4.3.3 Low amounts of C/EBPα and -ε activate PI by dose dependent way

The results described above establish C/EBP factors as possible positive regulators of bovine *ACC-α* proximal PI activity. Transfection as little as 100 ng of C/EBPα and -ε expression vectors into HC11 cells evidently stimulates the activity of long PI (Figure 16B). The amount of expression vector in these previous experiments was relatively low compared to the amount of PI reporter construct (1.5 μg). To test whether low amounts of C/EBPα and -ε factors could function as transcriptional activators of the proximal PI, the effect of increasing amounts of C/EBPα and -ε expression vector on the proximal PI reporter construct was titrated. Increasing amounts (10, 40, 70, 100 ng) of C/EBPα expression vector were contransfected with a constant amount of the proximal promoter -127. The proximal promoter activity was increased from 15 to 42 -fold of pGL3basic (Figure 22A). A similar result was obtained for C/EBPε. The proximal promoter activity was increased in this setting from 15 to 37 -fold that of pGL3basic. The extent of activation by C/EBPε is slightly lower than that by C/EBPα. These data indicate that already, low amounts of C/EBPα and -ε transactivate the proximal bovine *ACC-α* PI promoter in a dose-dependent manner in HC11 cells.

In order to demonstrate which of the domains of C/EBP factors are relevant for PI activation, the C/EBPα factor was divided into two parts. ΔC-C/EBPα contains the first 263 amino acid residues of C/EBPα, carrying a Flag-tag and a strep-tag at its N-terminus and C-terminus respectively (Data not shown). ΔN-C/EBPα (named also DN-C/EBPα) contains 89 residues from amino acid 265 to amino acid 353, and a His-tag and a Strep-tag were fused to its

N-terminus and C-terminus respectively. To examine the functions of the fusion proteins in regulating the proximal PI, these vectors were individually transiently transfected into HC11 cells with the proximal PI reporter construct (-127). Overexpression of full length (FL) C/EBPα evidently increased the proximal PI activity. However, the proximal PI activities were not altered in ΔN- and ΔC-C/EBPα transfected cells (Figure 22B).

Taken together, these results show that C-terminal binding of C/EBPα to proximal PI is not sufficient for PI activation. The presence of the N-terminal domain is also required, highlighting the functional requirement of the N-terminus as a transcriptional activator.

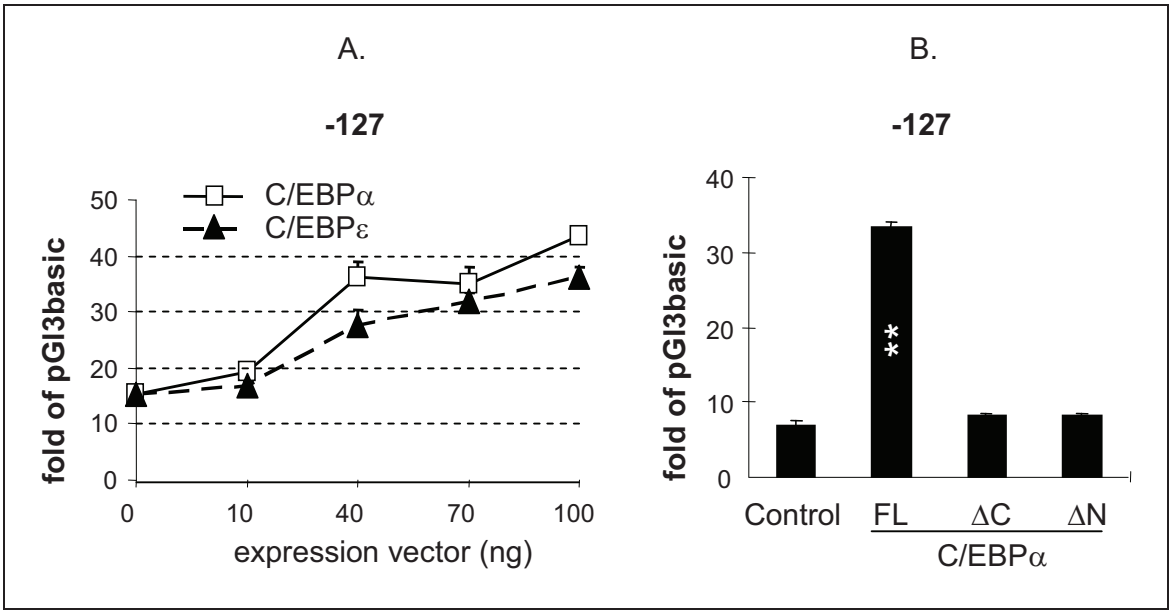


Figure 22 The functional role of C/EBPα and -ε on the proximal PI

A) Various amounts (10, 40, 70, 100 ng) of C/EBPα or -ε expression plasmid were transiently cotransfected into HC11 cells together with 1.5 μg of the proximal PI reporter construct (-127). The total amount of transfected DNA was kept constant, by filling up accordingly with the empty pFLAG.cmv. Error bars: SEM. Data was from three independent experiments.
B) 100 ng of full length (FL), N- terminus (ΔC) and C-terminus (ΔN) of C/EBPα expression vectors (constructions as described in Figure 43: ΔC=αP2, ΔN= DNα) were cotransfected with -127 reporter construct into HC11 cells. Activity indicated was from three assays of one experiment. Error bars: SEM. $^{**}p<0.01$.

3.4.3.4 High amounts of C/EBPβ and -δ activate proximal PI

Low amount of C/EBPβ and -δ did not activate the proximal PI promoter. To test the efficiency of larger amounts, different amounts of C/EBPβ and -δ were transiently transfected into HC11 cells with 1.5 μg of proximal PI reporter construct (-127). The activity of proximal PI was significantly increased up to 75 -fold that of pGL3basic by expressing 1000 ng of C/EBPβ vector (relatively high amount of C/EBPβ expression vector). A similar result was acquired by C/EBPδ, but the extent of activation (20 –fold of pGL3basic) is quite lower than that by C/EBPβ (Figure 23A).

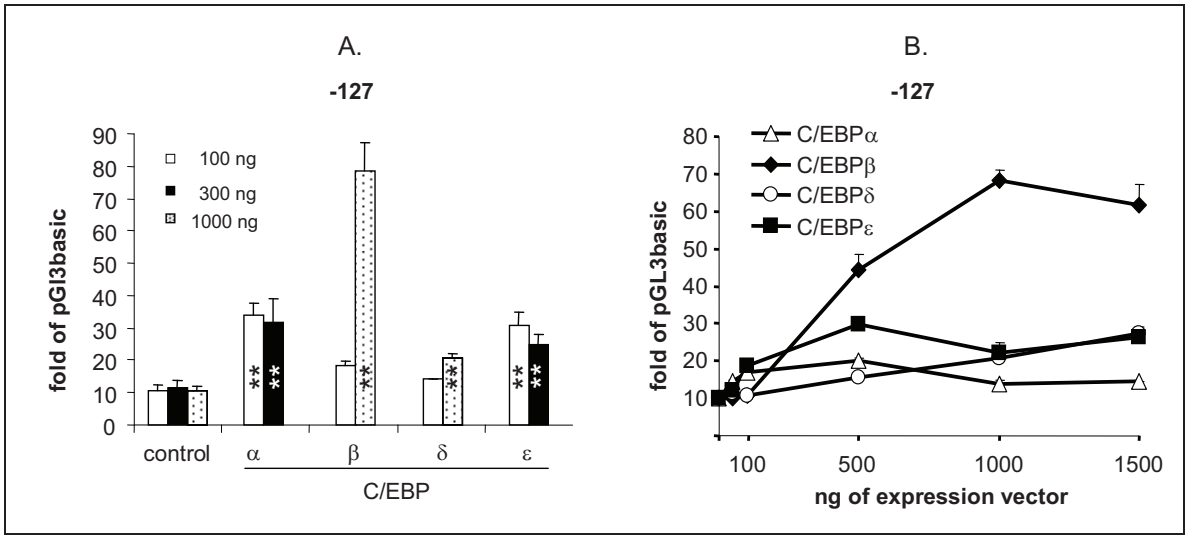


Figure 23 High amounts of C/EBPβ and -ε transactivate the proximal PI in a dose-dependent manner

A) Different amounts of C/EBP factors were transiently cotransfected into HC11 cells with 1.5 µg of proximal PI reporter (-127). The activity indicated was the fold change of mock in which the reporter was cotransfected with the empty plasmid pFLAG.cmv. Error bars: SEM. Data shown were the mean values of at least three independent experiments. **T-test against control: $p<0.01$.
B) Various amounts (50, 100, 500, 1000, 1500 ng) of C/EBPβ or -δ expression plasmid were transiently transfected into HC11 cells with 1.5 µg of proximal PI reporter construct (-127). The amount of expression vector was filled up to 1500 ng with pFLAG.cmv. Activity is the fold of pGL3basic. Error bars: SEM. Data were from three assays of one experiment.

To test the specificities of the activation by C/EBPβ and -δ, an ascending titration of C/EBPβ and -δ expression vectors (50, 100, 500, 1000, 1500 ng) were cotransfected into HC11 cells with 1.5 µg of proximal PI reporter construct. Below 100 ng, C/EBPβ and -δ expression vectors had no effect on the activity of proximal PI. Above that, increasing amounts of C/EBPβ and -δ activate proximal PI activity, but the activation extent by C/EBPδ is evidently lower than that by C/EBPβ (Figure 23B). The extent of activation peaks at 1000 ng of C/EBPβ and 1500 ng of C/EBPδ. In contrast, the activation extent seems to slightly decrease when the amount of the expression vector of C/EBPα and -ε increased from 100 ng to 300 ng (Figure 23A, B).

Taken together, these data indicate that high amounts of C/EBPβ and -δ can transactivate the proximal PI promoter.

3.4.3.5 Mutation of the C/EBP site blocks the activation by full length C/EBP proteins

To examine further the function of the C/EBP binding motif (site3) in the proximal PI region, a mutation was introduced into the core sequence of C/EBP site3 in the context of proximal PI

reporter (-127), resulting in the construct -127site3mu1 reporter (Figure 24). The responsiveness of this reporter to the binding of C/EBPα, -β and -ε was then determined in HC11 cells by transient transfection assays. Mutation of the proximal C/EBP binding motif abolished the basal promoter activity of the reporter construct (-127site3mu1). Furthermore, the C/EBPα, -β and -ε mediated transactivations were significantly reduced (Figure 24).

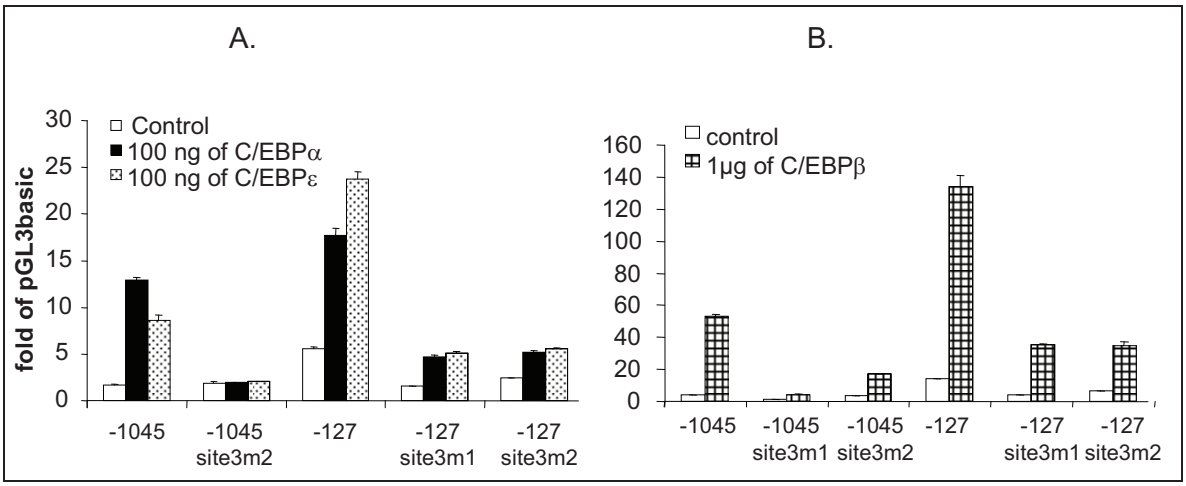


Figure 24 Mutational analysis of C/EBP binding site3 in proximal PI region by cotransfection with C/EBPα, -ε and -β

100 ng (A) and 1 μg (B) of expression C/EBP vectors was cotransfected with PI long (-1045, k609) and proximal (-127, k429) promoters. The C/EBP site3 in the proximal region was mutated from the wild type sequence TTGTGTAAT into TTGTagcca in m1 and gactagAAT in m2. The results show one representative from at least two independent experiments with assays in triplicate. Error bars: SEM.

Previously, our lab reported that *ACC-α* PI transcription starts at the first 'A' lying in the C/EBP site3. In site3mu1, the original sequence TTGTGT**A**AT (-6/+3) was mutated into TTGTagcca. Herein the TSS was blocked and the mutation should theoretically abolish the transcription start. In order to examine the role of the TSS in the regulation of PI when activated by C/EBP factors, the second mutation (site3mu2) was introduced keeping the TSS and converting the original sequence TTGTGTAA into gactagAAT. Results indicated that the second mutation also abolished the basal promoter activity of the reporter construct (-127site3mu2). Likewise, C/EBPα, -β and -ε mediated transactivations were evidently blocked (Figure 24).

Subsequently, the same mutations were analyzed in their effect on the activity of the long PI reporter construct (-1045). Both mutations blocked the transactivations mediated by C/EBPα, -β and -ε in mutated reporter constructs (-1045site3mu1, -1045site3mu2) transfected cells. Taken together, these data indicated that the C/EBP binding motif (site3) of the bovine *ACC-α* PI promoter is required not only for C/EBPα, -β and -ε-mediated transactivation, but also for basal promoter activity in HC11 cells.

3.4.3.6 DN-C/EBPs block its activation by full length C/EBPα and ε.

To further investigate the contributions of C/EBP factors to the basal activity of PI *in vivo*, DN-C/EBP proteins were transiently transfected into HC11 cells with the proximal PI reporter construct. Theoretically, DN-C/EBP proteins harboring only the DNA binding domain and leucine zipper domain will bind to their binding motif compete for originally bound transcription factor, and eventually abolish its transactivation function. The inhibitory activities of DN-C/EBPα and DN-C/EBPε were first titrated in transient transfection assays with the C/EBPα or C/EBPε expression vectors and the proximal PI reporter (Figure 25A). 2.7 μg of various DN-C/EBP expression vectors was cotransfected into HC11 cells with 0.9 μg of proximal PI reporter construct. Over-expression of DN-C/EBPβ and C/EBPζ (CHOP), but not -α, -δ and -ε significantly reduced the *ACC-α* PI activity by (~40 %) in HC11 cells. These data show that DN-C/EBPβ and CHOP efficiently function as repressors of the basal activity of *ACC-α* PI in HC11 cells (Figure 25B), whereas DN-C/EBPα or DN-C/EBPε sufficiently repress the activation by their respective full length proteins.

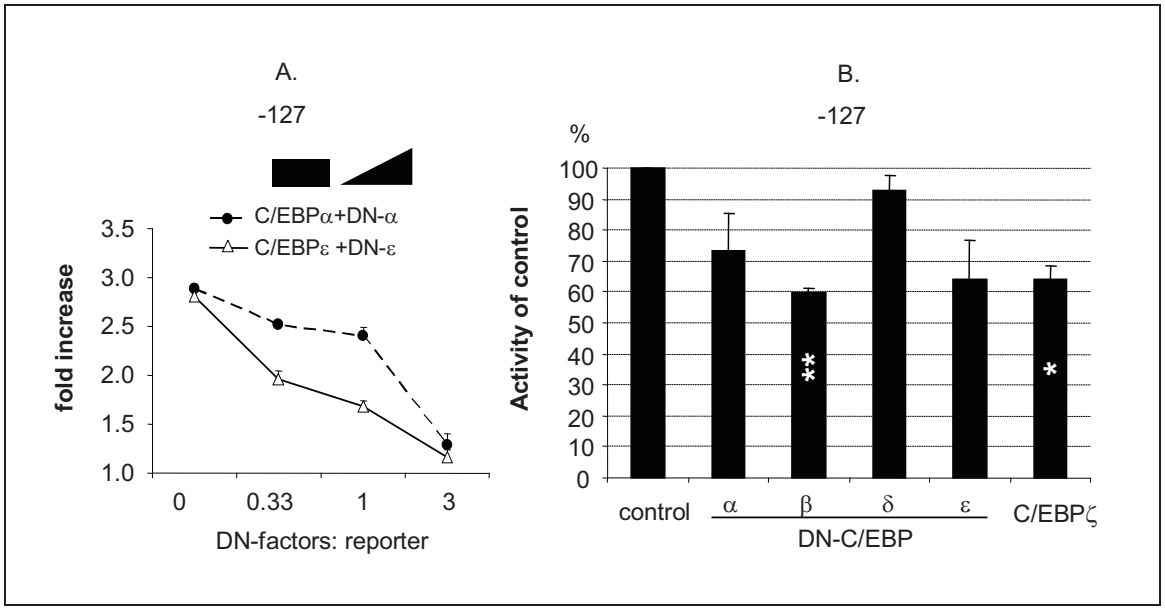


Figure 25 Inhibitional analysis for the basal activity of PI by DN-C/EBPs in HC11 cells

A) 0.9 μg of proximal PI reporter construct (-127) and a constant amount of C/EBPα or -ε (0.3 μg) was cotransfected into HC11 cells with no or increasing mounts of DN-C/EBPα or -ε expression vectors (0.33, 1 and 3 fold the amount of reporter construct -127).
B) 2.7 μg DN- expression vectors was contransfected into HC11 cells with 0.9 μg of proximal PI reporter construct. C/EBPζ vector expressing human CHOP protein. Data shown were from two independent experiments. Error bars: SEM. *:$p<0.05$, **:$p<0.01$

3.5 The molecular mechanism of PI repression

3.5.1 NF-Y synergistically represses C/EBPβ mediated activation of the proximal PI by specific protein-protein interaction

So far it has been identified and characterized that both NF-Y and C/EBPα, β and ε directly bind to respective binding sites in the proximal PI region and contribute to activate PI. The center position of the NF-Y binding site is at -110 bp, while C/EBP binding site lies at -2 bp. There are 109 nucleotides, including 10 α helixes, between these two binding sites. It is possible that one protein may interact with the other to regulate transcription initiation by DNA bending at transcription start site.

3.5.1.1 NF-Y specifically represses C/EBPβ mediated activation on proximal PI

To investigate the functional relationship between NF-Y and C/EBP factors in controlling the proximal PI activity, expression vectors of NF-Y [A+B+C] and/or respective C/EBP factors were cotransfected into HC11 cells with the proximal PI reporter construct. Consistent with the previous results, NF-Y (~1.5 -fold) and C/EBPβ (~5.3 -fold) separately and significantly transactivate the proximal PI activity (Figure 26A). Surprisingly, NF-Y[A+B+C] decreased the C/EBPβ mediated activation of proximal PI expression, from 45 –fold to 15 –fold of pGL3basic (Figure 26A).

To confirm the specificity of this observation, increasing amounts (30, 90, 300, 900, & 1500 ng) of NF-Y [A+B+C] expression vectors were cotransfected into HC11 together with constant amounts (1 μg) of C/EBPβ. Increasing amounts of NF-Y [A+B+C] decreased the extent of C/EBPβ mediated activation, from 100 % down to 37 % (Figure 26B). These data showed that NF-Y represses the C/EBPβ mediated activation in *ACC-α* PI in a dose-dependent manner.

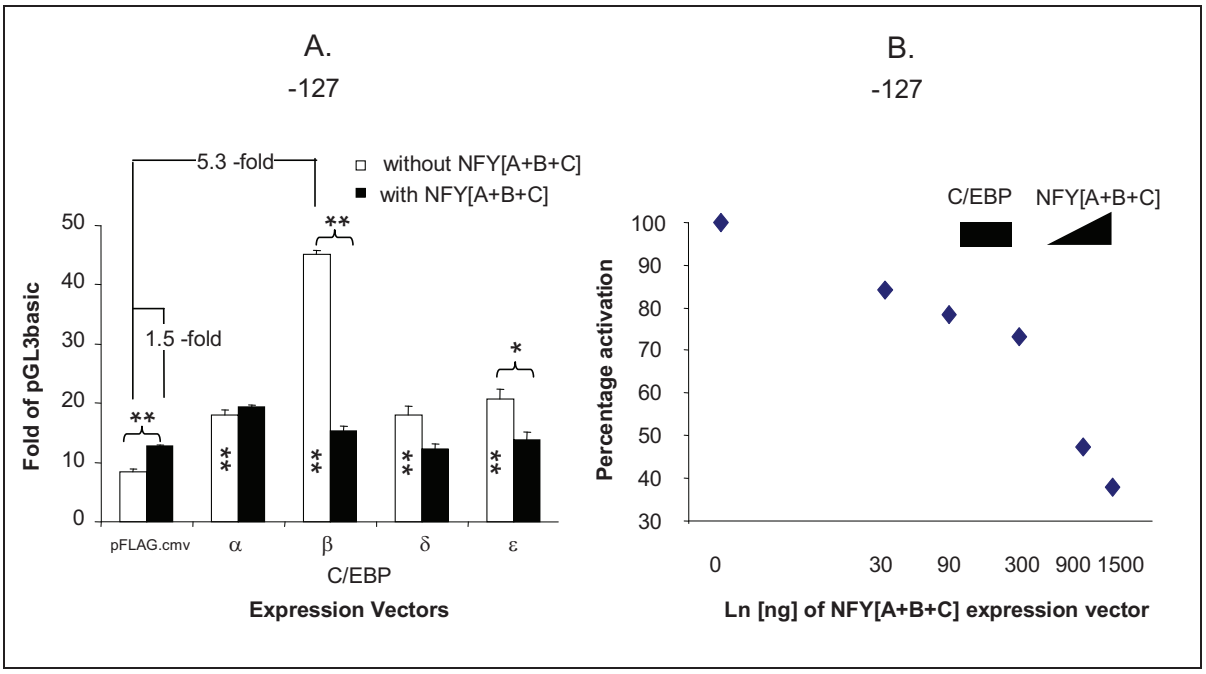


Figure 26 NF-Y and C/EBPβ synergism repressing the proximal PI

A) NF-Y significantly represses C/EBPβ-mediated activation. The ** on an empty column represents that the t-Test was performed against the control which was cotransfected with pFLAG.cmv, but without NF-Y[A+B+C]. Error bars: SEM. Data were from one experiment assayed in triplicate. **: p <0.01; *: p <0.05.
B) The activation of C/EBPβ was inhibited by NF-Y in a dose-dependent manner. X-axis is in natural logarithmic scale. Data shown were representative from two independent experiments assayed in triplicate.

Transfections were performed in HC11 cells. The total amount of DNA in each transfection was constant. 0.5 µg for each NF-Y subunit, C/EBPα and –ε, 1 µg for C/EBPβ and –δ were used to attain maximal activation. These amounts were determined previously by titration. The transfection efficiency was normalized with phRL-TK renilla; data were normalized with pGL3basic. B) Similar as A), the amount of NF-Y[A+B+C] was titrated from 30 ng till 1500 ng. NF-Y represses very significantly the C/EBPβ mediated PI activation.

3.5.1.2 All subunits of NF-Y are indispensable for the repression of C/EBPβ activated proximal PI

NF-Y regulates gene expression only if all three NF-Y factors (NF-YA, NF-YB, NF-YC) form a heterotrimer, known to constitute the active factor “NF-Y”. NF-Y can bind to its DNA binding motif by its NF-YA subunit, when NF-YA recognizes the NF-YB/NF-YC dimer. In order to identify if the inhibition is based on the entire NF-Y complex, NF-YA, -B, and/or -C were cotransfected either alone or together into HC11 cells with the C/EBPβ expression vector and proximal PI reporter construct. Moreover, to identify whether the inhibition is depended on the proximal NF-Y binding site of proximal PI, DNA binding function of NF-YA is devoid by substituting three amino acids (RGE→AAA) at its C-terminus as described (Mantovani et al., 1994), resulting NF-YAm29. Analyses showed that the binding activity of the nuclear extract from HEK293 cells expressing NF-YAm29, -YB and -YC subunits (non-binding NF-Y) was decreased by 28 % of the control (Figure 27B). In contrast, the

nuclear extract of cells expressing NF-YA, -YB, -YC subunits possessed strong binding activity (100 %) when detected using labelled NF-Y consensus probes.

Results indicated that the C/EBPβ mediated activation is repressed by NF-YA, -YB, -YC complex, and is also repressed by NF-YAm29, -YB, -YC complex (Figure 27A). But the C/EBPβ mediated activation can not be inhibited by any respective subunits of NF-Y including NF-YAm29. Hence, the inhibition of between NF-Y complex and C/EBPβ is independent from NF-Y binding to its proximal PI site.

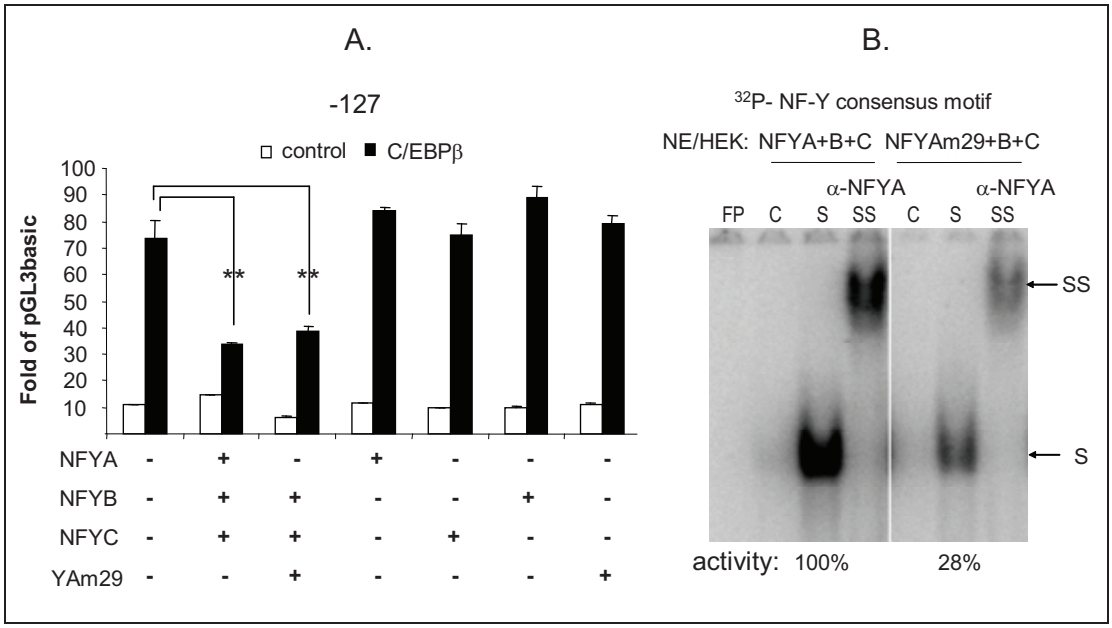


Figure 27 Comprehensive analyses of the interaction of NF-Y and C/EBPβ by cotransfection in HC11 cells

A) The inhibition of C/EBPβ activity is dependent of the intact NF-Y complex, but independent of the binding of NF-Y complex to its proximal binding site. 0.5 μg each different subunit expression vectors or combinations of NF-YA, YB, YC YAm29 were examined for inhibition of C/EBPβ activity (by 1μg of expression vector). Activity was normalized by pGL3basic. Data are representative of three independent experiments. Each assay is in triplicate. Error bars: SEM. ** represents $p<0.01$.
B) Binding activity of recombinant `non-binding NF-Y´ was blocked by a three amino acid AAA substitution of the original RGE in the binding domain of NF-YA, resulted in NFYAm29. The nuclear extracts (NE) were prepared from HEK293 cells expressing recombinant NFYA+YB+YC and NFYAm29+YB+YC. S with an arrow is the DNA-protein complex, and SS indicates the retarded supershifted band. The consensus NF-Y binding motif was labelled for probe in the experiment.

3.5.1.3 NF-Y and C/EBPβ bind to proximal PI by specific protein-protein interaction

In order to analyze if the protein-protein interaction model between NF-Y and C/EBPβ is relevant to PI promoter activity *in vivo*, HEK293 cells were cotransfected with NF-YAm29, YB, YC and C/EBPβ vectors together with the proximal reporter construct. Inclusion of the expression construct NF-YAm29, ensured that the mutational NF-Y complex could not

directly bind to their cognate DNA-binding motif on the -127 reporter construct.

Chromatin **I**mmuno**P**recipitation (ChIP) technique was used to determine whether C/EBPβ and NF-Y interacted directly and occupied the proximal PI promoter binding site. The NFY antibody precipitated 5.1 fold of the amount of the PI promoter molecules than that of control IgG (Figure 28C). The C/EBPβ specific antibodies precipitated 3.4 fold the mount of PI molecules than the control IgG. Hence, both 'non-binding NF-Y' and C/EBPβ effectively interact with the proximal PI region.

Because the binding domain of NF-Y was blocked using NF-YAm29, NF-Y interaction with the proximal PI promoter occurred via protein-protein interaction between NF-Y and C/EBPβ.

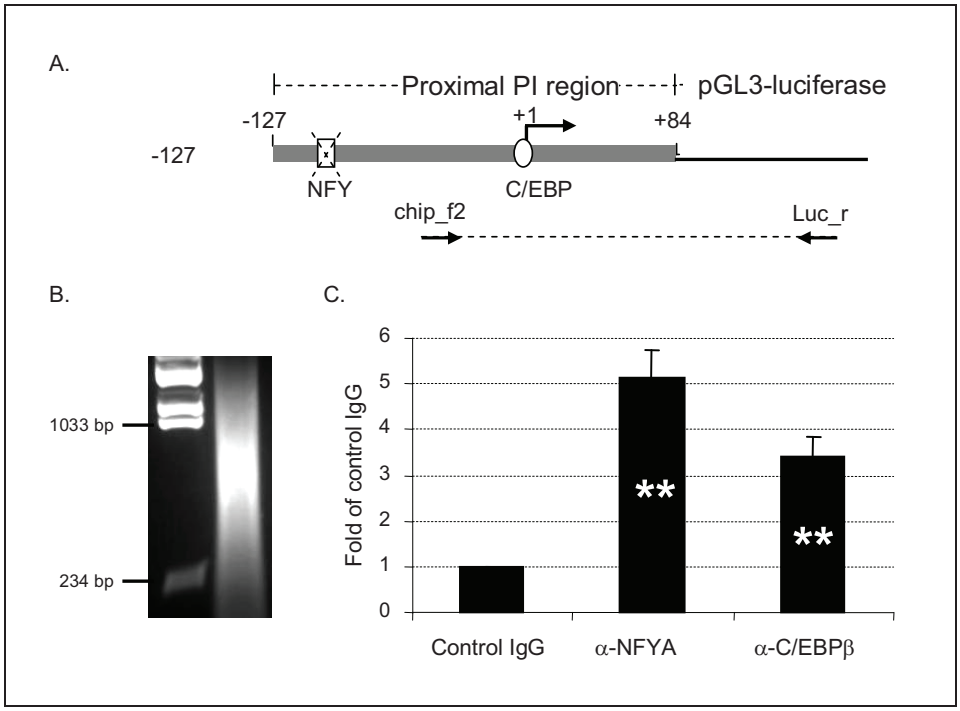


Figure 28 ChIP reveals that C/EBPβ binds to PI promoter and forms a complex with NF-Y *in vivo*.

A) Schematic representation of the proximal PI promoter on the reporter construct (-127) for ChIP. Arrows show the positions of the real-time PCR primers. B) Sonification of the transfected HEK293 cells. C) ChIP analysis using different antibodies. Data indicate the means of four qPCR quantifications from one precipitation. Error bars: SEM. ** represents $p<0.01$.

Four μg of proximal reporter construct (-127), 4 μg of C/EBPβ and 4 μg of NF-YC expression vector were cotransfected into HEK293 cells together with 8 μg of NF-YAm29, 8 μg of NF-YB expression vectors in a ~90-mm cell culture plate. Crosslinked protein-DNA complexes were incubated with anti-NF-YA or anti-C/EBPβ antibodies and isolated by immunoprecipitation with protein A-Sepharose beads. DNA was immunoprecipitated and the amounts of the proximal C/EBP site3 were measured by qPCR, using primer pairs (chip_ f2, Luc_r) derived from this region of the reporter construct.

3.5.2 Affinity comparison of the *cis*-elements in different region of PI

It has been proven in this study that NF-Y as well as C/EBP binding elements in the distal promoter both represses PI activity, opposing their function on the proximal promoter. It could

be possible that the binding affinity of these two factors for the distal and proximal sites is different, and thus eventually contributing different functions, either repressor or activator, to the wt promoter.

3.5.2.1 The NF-Y binding site in the proximal region has higher affinity than the repressive binding site in the distal region

To evaluate the relative binding affinity of the distal and proximal binding sites, competition EMSA was performed with a labelled NF-Y consensus probe. Double stranded oligonucleotides with the original sequence of either the distal or the proximal NF-Y binding sites were included as unlabelled competitor molecules. Nuclear extracts were prepared from HEK293 cells expressing recombinant bovine NF-YA, -YB and -YC subunits. The dissociation of the specific NF-Y-DNA complexes was determined by analyzing the complexes in the presence of different molar excesses of competitor (Figure 29).

The results manifested that an increasing molar excess of both competitors dissociate the NF-Y-DNA complexes formed by exogenous NF-Y consensus probes (Figure 29). However, 25 –fold molar excess of the NF-Y proximal site competitor efficiently reduce the NF-Y-consensus DNA complexes by 80 % (20 % NF-Y-DNA complexes residual), whereas as much as 200 -fold molar excess of the NF-Y distal site competitor was required to reduce the amount of the shifted NF-Y-DNA complexes (Figure 29). Moreover, the labelled NF-Y proximal site and distal site probe was efficiently extinguished by the proximal site competitors, but inefficiently by the distal site competitors (data not shown). These results demonstrated that the NF-Y binding affinity for the proximal binding site (activator) is significantly greater than that of the distal binding site (repressor).

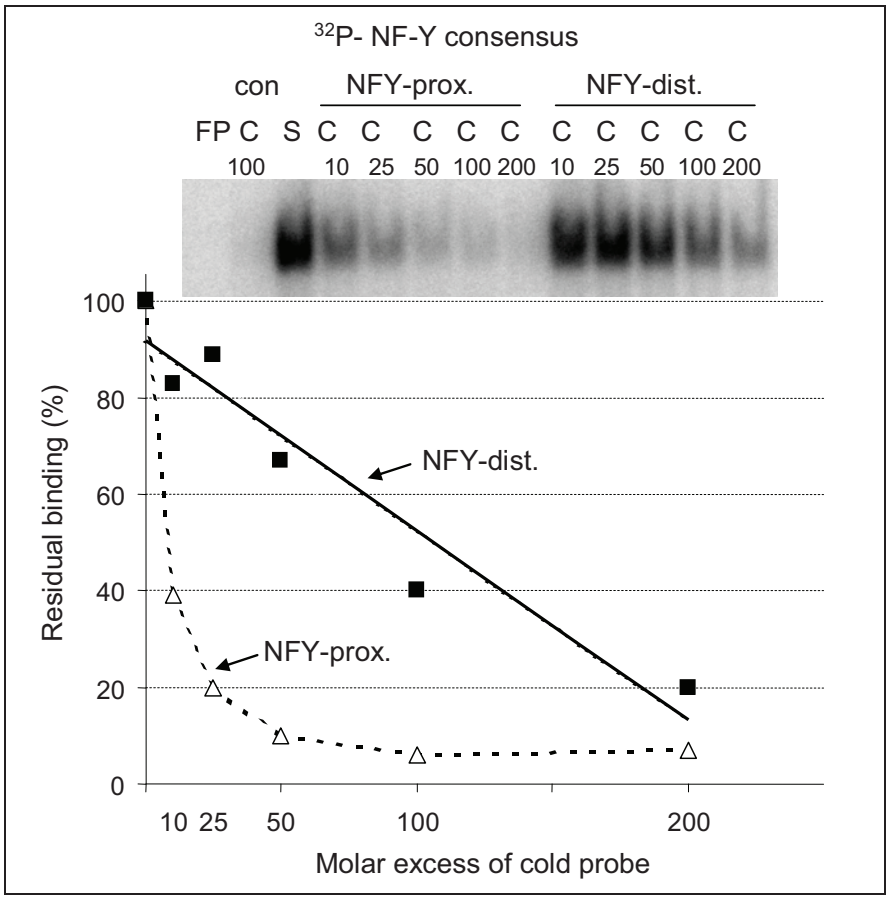


Figure 29 The affinity comparison of the NF-Y proximal and distal sites of PI

HEK293 cells expressing recombinant bovine NF-Y[A+B+C] and oligonucleotide probes of NF-Y distal and proximal sites (corresponding to sequences -1052 to -1009 and -127 to -91, respectively) were used in EMSA. The NF-Y consensus oligonucleotide (AGACCGTACGTG**ATTGG**TTAATCTCTT) probe was designed as described (Zhao et al., 2000). Amounts indicated are the molar excess of the corresponding competitor. The labelled probe is the NF-Y consensus binding motif. Densities of protein-DNA complexes were quantitated by phosphorimager analysis and the net value obtained for protein-DNA complexes in the absence of competitors was set to 100 %. The empty triangle represents the values obtained under competition with the NF-Y proximal binding site (-127 to -91), and the solid square represents the values generated by the NF-Y distal competitor (-1052 to -1009).

3.5.2.2 The C/EBP site in the proximal region has a higher affinity than the repressive site in distal region

The binding affinities of the distal and proximal C/EBP binding sites were evaluated in a similar experiment. The nuclear extracts were extracted from HC11 cells expressing recombinant bovine DN-C/EBPβ. In bovine DN-C/EBPβ, 154 amino acid residues of the N-terminus were deleted, but the DNA-binding domain and leucine zipper domain were kept. The C/EBP proximal binding site efficiently competes for the labelled consensus probe (Figure 30), as well as the labelled proximal or distal probe (data not shown). The distal C/EBP binding site, however, only ineffectively competes for the labelled probe.

Taken together, the proximal sites of both factors have much higher binding affinities than the

respective distal sites. Hence, small amounts of either factor are sufficient to saturate the proximal binding site and activate the promoter. Much higher concentrations, however, are necessary to saturate the distal binding sites. Once these are occupied, then the distal factors can feed back to repress the activation potential of the either NF-Y and/or C/EBP bound proximal attachment site.

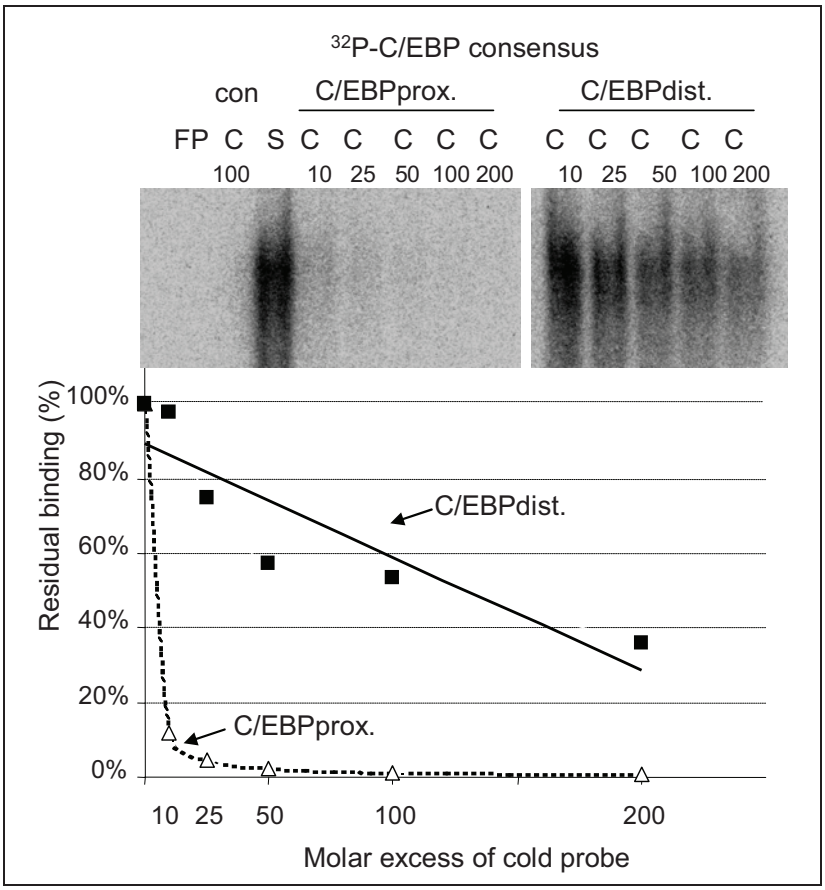


Figure 30 The affinity comparison of the C/EBP proximal and distal sites in PI

HC11 cells expressing recombinant bovine DN-C/EBPβ and oligonucleotide probes of C/EBP distal and proximal sites (corresponding to sequences -198 to -166 and -26 to +9, respectively) were used in EMSA. The sequence of the C/EBP consensus oligonucleotide (TGCAGATTGCGCAATCTGCA) probe was as described (Wu et al., 2003). Amounts indicate the molar excess of corresponding competitor. The labelled probe was C/EBP consensus binding motif. Densities of protein-DNA complexes were quantitated by phosphorimager analysis and the net value obtained for protein-DNA complexes in the absence of competitors is set to 100 %. The empty triangle represents the values by the proximal C/EBP binding site competitor (-26 to +9), and the solid square represents the values by the distal C/EBP competitor (-198 to -166).

3.6 The associations of the levels of NF-Y and C/EBP factors with PI activity

Data from this study indicate that *in vitro*, NF-Y and C/EBP factors play an essential role in the regulation of *ACC-α* PI activity in the model cells. The investigation of their changes during different physiological conditions will contribute to unraveling the molecular mechanisms controlling *ACC-α* PI activity.

3.6.1 The proportion of NF-Y over C/EBPβ correlates with the tissue specific level of PI activity

It was reported that the NF-YB and -YC heterodimer is ubiquitously expressed, being detectable in all tissues, whereas NF-YA is not ubiquitously expressed (Gurtner et al., 2003; Tomita and Kimura, 2008). Hence, the regulation of NF-YA expression controls levels of functional NF-Y complex in a tissue-specific manner (Farina et al., 1996). Since the current study revealed that NF-Y and C/EBPβ may control *ACC-α* PI activity by protein-protein interaction, I hypothesized that in the cow the interplay of NF-Y and C/EBPβ may also regulate *ACC-α* PI activity. Hence, the association between *ACC-α* PI activity and the mRNA levels of NF-Y (by measuring NF-YA) and C/EBPβ factors was examined in bovine tissues.

As has been examined, *ACC-α* PI activity is dramatically down-regulated by starvation (Figure 8A), but the mRNA levels of NF-YA or C/EBPβ have not been changed (see appendix J). Considering that fatty acid synthesis is active in the mammary glands, the C/EBPβ and NF-YA mRNA levels were investigated in these tissues from different groups, with different diets. Results indicated that C/EBPβ is significantly up-regulated by starch-enriched diets, but transcripts of *ACC-α* PI are insignificantly down-regulated. The mRNA levels of NF-YA are not changed.

However, *ACC-α* PI is maximally expressed in the mammary gland, while expression is moderate and minimal in the adipose tissue and liver respectively. Hence, *ACC-α* PI transcripts are expressed in a tissue specific manner (Table 8). The difference of the maximal PI activity and the minimal PI activity is ~400 –fold between mammary gland and liver. Surprisingly, similar levels of NF-YA mRNA copies were found in all tissues, for the most part, this remained true for C/EBP expression except for very low expression in the mammary gland. Further analyses indicated that the difference of NF-YA over C/EBPβ between liver and adipose is ~2 –fold, and that between liver and mammary gland is about ~10 –fold. Hence,

the levels of NF-YA over C/EBPβ also change in a tissue-specific way.

Table 8 mRNA copies of *ACC-α* PI, NF-YA and C/EBPβ in various tissues under different nutritional conditions

Copies/200 ng RNA	Liver		Adipose		Mammary Gland	
	starved[a]	fed[a]	fat-diet[b]	starch-diet[b]	starch-diet[b]	fat-diet[b]
ACC-α PI ($\times 10^4$)	0.10 ± 0.02	0.52 ±0.18	6.81 ±2.24	8.49 ±4.86	21.64 ±4.8	40.57 ±12.3
NF-YA ($\times 10^3$)	8.94 ±1.05	7.64 ±0.71	10.41 ±0.91	18.57 ±7.61	8.36 ±1.39	8.31 ±1.24
C/EBPβ($\times 10^6$)	43.71 ±2.49	37.06 ±7.98	24.50 ±3.53	30.83 ±10.99	6.12 ±0.81	3.78 ±0.60
NFYA:C/EBPβ	2.06 ±0.24	2.44 ±0.64	4.69 ±0.66	6.45 ±0.90	14.26 ±1.76	26.25 ±4.76
NEL [MJ]	18.0 ±1.1	110.2 ± 11.2	nd	nd	nd	nd

RNA from liver, fat and mammary gland tissues was reversely transcribed and measured by real-time PCR. NEL, Net Energy content for Lactation; nd, not determined. a: (n=6); b: (n=8). Each value represents the relative mRNA copies and is mean ± SEM.

Intriguingly, high PI expression levels in lactating udder appear to be correlated with a high proportion of NF-YA mRNA over C/EBP mRNA molecules (Table 8). The intermediate PI activity measured in adipose tissue also demonstrates an apparent link with an intermediate ratio of NF-YA over C/EBPβ mRNA molecules. This trend continues in the liver, where PI activity is low and NF-YA mRNA abundance compared to that of C/EBPβ is also low. Hence, the proportion of NF-YA over C/EBPβ correlates with the tissue-specific PI activity (Figure 31).

Statistically, this correlation is highly significant. Thus, tissue and nutrition related alteration in the RNA abundance of those factors previously shown *in vitro* to contribute to the molecular controls of PI activity, may also be true *in vivo*.

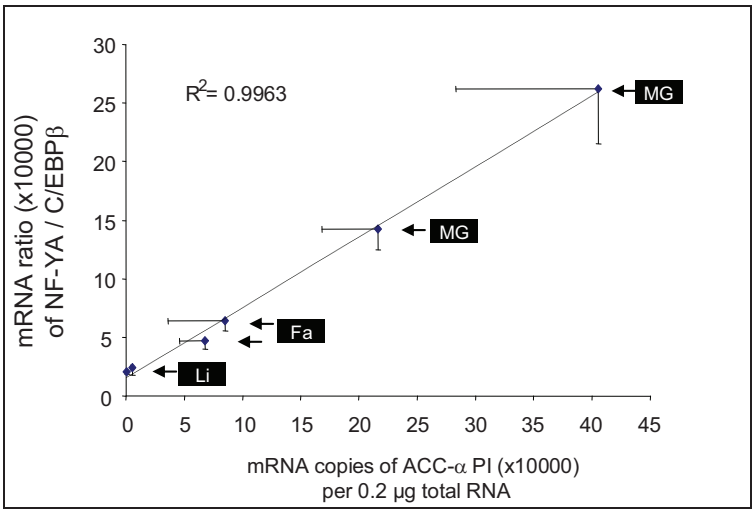


Figure 31 The correlation of *ACC-α* PI activity and the ratio of NF-YA/C/EBPβ

Data were from Table 8. Li, Fa and MG indicate liver, fat and mammary gland tissues respectively. Error bars are SEM. of 6 or 8 animals.

3.6.2 mRNA levels of NF-Y and C/EBP factors may not be regulated in the liver

To analyze the response of C/EBP factors to feeding, real-time PCR was performed to measure the mRNA levels of *ACC-α* derived from PI, C/EBPα, -β, -δ, –ε, –γ, –ζ and NF-YA in the liver. However, different diets did not influence these factors in according to PI activity. Surprisingly, C/EBPδ and -ε appear to increase in tendency ($p<0.1$) response to starvation, in opposition to *ACC-α* PI activity declining with starvation (appendix J). The mRNA level of beta-actin was not significantly altered by starvation. This measure was assessed for technical control.

3.6.3 C/EBPδ and –ε may be repressors, but C/EBPα and NF-YA may be activators in adipose tissues during the transition period (before and after calving)

To know the association of PI activity with the expressions of C/EBP factors in adipose tissue, the same samples in which I know that PI expression is physiologically and nutritionally regulated, were used to measure the mRNA levels of C/EBP factors by real-time PCR (Table 9). Results demonstrated that mRNA level of C/EBPα has a tendency to decrease after calving ($p<0.1$) in the starch-enriched group, and also insignificantly in the fat-enriched group. The ratio of before to after calving was 2.16±0.45 in starch-diet group, or 1.78±0.38 in fat-diet group analyzing on individuals (Table 9). Accordingly, mRNA expression of NF-YA decreased in tendency ($p<0.1$) after calving in the fat-enriched diets group. The ratio of before to after is 1.53±0.25. Hence, C/EBPα and NF-YA are positively correlated to PI activity before and after calving in adipose tissue by given diets.

Surprisingly, mRNA expressions of C/EBPδ and –ε increased after calving in both starch- and fat-enriched diet groups, and C/EBPδ decreased significantly in the fat-enriched group ($p<0.05$). The ratio of before calving to after calving of these genes is listed in Table 9. The trend shown by these two factors opposes that of PI activity, functioning as a repressor.

Taken together, C/EBPα and NF-YA may be the activators of PI expression in adipose tissue in the transition period (before and after calving), but C/EBPδ and -ε may function as repressors.

Table 9 mRNA expressions of relevant genes in adipose tissue before or after calving by different diets

			Copies (by diets)		*p*_calving		*p*_diets
			Starch-diets	Fat-diets	Starch-enri.	Fat-enri.	
ACC PI	Before Calving	x10^3	5.44±1.34	9.09±2.83	**<0.01**	**<0.05**	0.36
	After calving	x10^3	0.04±0.01	0.13±0.04			0.10
	Before: After		256±64	65±18			**<0.05**
C/EBPα	Before Calving	x10^5	1.43 ±0.25	1.38±0.33	<0.1	0.29	0.92
	After calving	x10^5	0.75 ±0.12	0.88±0.16			0.61
	Before: After		2.16±0.45	1.78±0.38			0.60
C/EBPβ	Before Calving	x10^6	1.69±0.34	1.93±0.28	0.44	0.83	0.66
	After calving	x10^6	1.32±0.22	1.79±0.41			0.42
	Before: After		1.51±0.40	1.34±0.22			0.76
C/EBPδ	Before Calving	x10^6	0.69±0.11	0.88±0.07	<0.1	**<0.05**	0.21
	After calving	x10^6	1.38±0.32	1.39 ±1.52			0.99
	Before: After		0.56±0.09	0.75±0.18			0.45
C/EBPε	Before Calving	x10^4	2.10±0.64	2.12±0.34	0.84	0.18	0.98
	After calving	x10^4	2.29±0.47	3.38±0.63			0.26
	Before: After		1.10±0.28	0.75±0.11			0.35
NF-YA	Before Calving	x10^3	2.36±0.91	1.40±0.13	0.44	<0.1	0.37
	After calving	x10^3	1.43±0.34	1.06±0.11			0.31
	Before: After		1.85±0.52	1.53±0.25			0.59
β-actin	Before Calving	x10^5	2.50±0.50	2.29±0.26	0.84	0.70	0.87
	After calving	x10^5	2.40±0.70	2.59±0.30			0.75
	Before: After		1.67±0.43	1.04±0.18			0.27

Adipose samples were used for analyses (n=8). Each value represents the mean ± SEM. The highest and the lowest PI expression values were deleted for statistic analysis of t-test, accordingly the expression of the relevant genes.

3.6.4 Bovine C/EBPβ-Thr^{269} (relative mouse Thr^{217}) is phosphorylated in refed liver

Considering the regulation of different transcription factors is tissue-specific, the mRNA levels of C/EBPβ remain constant during feeding changes in the liver (Figure 8A). Data *in vitro* have shown that C/EBPβ binds to and regulates the bovine *ACC-α* PI promoter (Figure 21B). Moreover, it is reported that Thr^{269} (relative to mouse Thr^{217}) was phosphorylated to activate the proliferation of stellate cells. Examination of the phosphorylation of bovine C/EBPβ-Thr^{269} was performed using an antibody that recognizes the conserved mouse phosphorylated Thr^{217} of C/EBPβ by western blot. Results showed that the Thr^{269}of C/EBPβ is greatly phosphorylated in the liver of refeeding cows (Figure 32). In contrast, the expression of LAP1, LAP2, LIP and alpha-actin prevails during diet changes. Hence, phosphorylation of bovine C/EBPβ Thr^{269} is increased by refeeding and may contribute to activate PI activity.

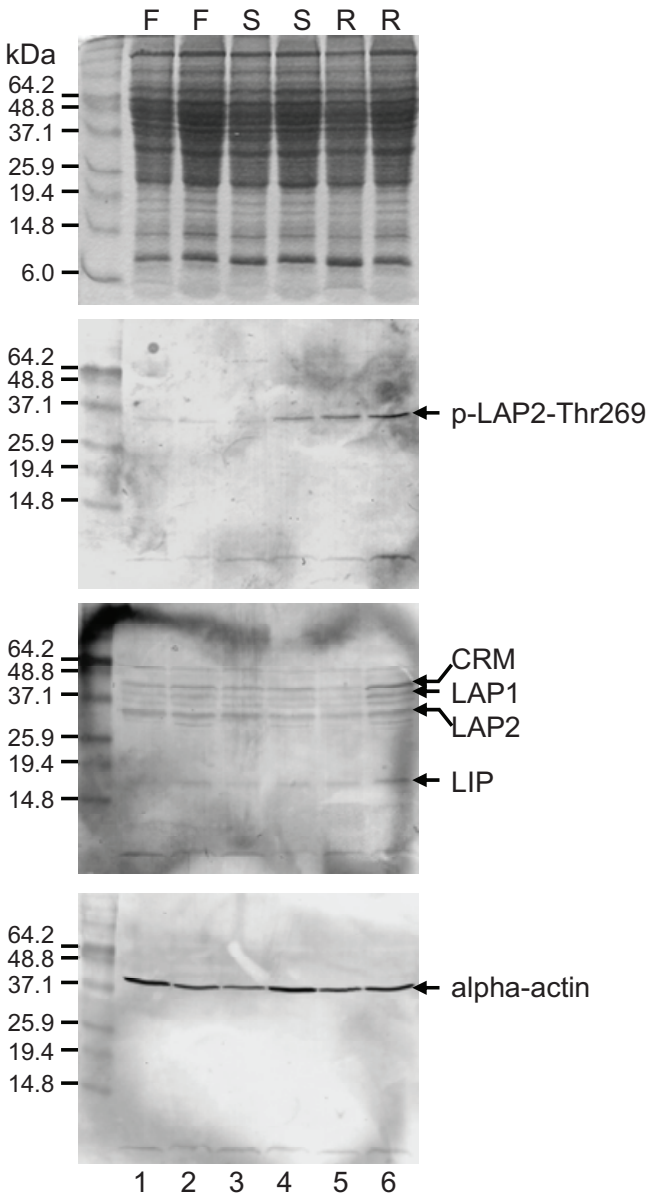


Figure 32 Refeeding robustly phosphorylates bovine C/EBPβ Thr^{269}

Liver samples were obtained from cows fed normally, and starved for 43 h, and then refed for 29 h. Tissues were ground in liquid N2, and lysed with lysis buffer (0.1 M Tris-Cl pH8.0, 0.15 M NaCl, 1 mM EDTA, 1Mm DTT, 1 mM PMSF, 0.5 % Sigma cocktail inhibitor, 1 % triton, 0.5 % NP-40, 20 mM NaF, 1 mM $NaVO_3$) at a concentration of 0.5 mg/ml. Cell lysates were sonicated for 2 min in an ice-cold water bath to shear genomic DNA. Samples loaded were from different animals. lane1:J2067; lane2 J2069; lane3: J2055; lane 4:J2057; lane5: J1550; lane6: J1553. Equal amounts of protein (50 µg) were loaded on 12 % SDS-PAGE for Coomassie brilliant blue staining and Western bloting. Phosphorylation of C/EBPβ Thr^{269} was detected by anti-mouse-C/EBP p-Thr^{217}; LAPs and LIP of C/EBPβ were probed by anti-C/EBPβ (sc-150); Anti-alpha-actin was used to probe for loading control. Detection antibody was conjugated with alkaline phosphatase. The ready membrane was developed with BCIP/NBT.

4 Discussion

4.1 ACC-α is the regulation target of lipid metabolism in cattle

ACC-α is the rate-limiting enzyme in long-chain fatty acid synthesis *de novo* (Kim, 1997). Malonyl-CoA, the product formed by ACC-α, is the two-carbon donor in the elongation of fatty acid in the cytosol. In mouse liver, conditional knockout of the ACC-α biotin binding site decreases both the level of malonyl-CoA and triglycerids, and the *de novo* synthesis of fatty acids (Mao et al., 2006). The results cited above support the role of ACC-α in *de novo* lipid biosynthesis.

ACC-α contributes to biological membranes, lipid signaling and the accumulation of large amounts of lipids in lipogenic tissues, such as liver, adipose tissues and mammary glands. The fatty acid synthesis is vital for making up of cells. Fatty acids are the main structural components of biological membranes, including the cellular plasma membrane and the intracellular membranes of organelles. The diverse functions of eukaryotic cells are dependent on the well compartmentalized organelles. Meanwhile, the derivative of fatty acids are involved in a vital part of the cell signalling (Wang, 2004), such as sphingolipid, diacylglycerol, phosphatidylinositol phosphates (PIPs) and the steroid hormones (Eyster, 2007). The fatty acids-formed triacylglycerols are a highly efficient form of energy storage in higher animals, whereas plants can afford to store energy in a less compact but more easily accessible form, carbohydrate.

Research on human and mouse manifests that the dysregulation of lipid metabolism may result in metabolic diseases such as obesity and diabetes. In cattle, the dysregulation of lipid metabolism in dry standing may result in metabolic disorders (e.g. ketosis). It is also reported that dysregulation of lipid metabolism accompanies cancer. The silencing of the *ACC-α*-encoding gene inhibits cell proliferation and induces caspase-mediated apoptosis of highly lipogenic prostate cancer cells (Brusselmans et al., 2005). Hence, regulation on ACC-α may contribute to the control of lipid metabolism.

ACC-α plays an essential role in cell activities. Thus, not surprisingly, ACC-α KO is lethal for mouse embryo (Abu-Elheiga et al., 2005). In contrast, ACC-β deficient mice can live normally, and it appears the lipid oxidation is not inhibited (Abu-Elheiga et al., 2003). Improved understanding of *ACC-α* regulation may provide an insight into the therapy of

metabolic disease and the enhancement of beef or milk quality. In the study, I focus on the regulation of PI in bovine *ACC-α*.

4.2 PI is the nutritionally regulated promoter

In cattle, four promoters (PIn, PI, PII, PIII) control ACC-α expression. PI, PII and PIII have been reported previously (Mao et al., 2001; Mao et al., 2002; Mao and Seyfert, 2002; Molenaar et al., 2003). PIn was isolated and identified as one of the *ACC-α* promoters in the current study.

4.2.1 PIn is not the principle promoter in lipogenic tissues

PIn is the most 5′ promoter of bovine *ACC-α*. The identified PIn features characteristics similar as its homologs of other species. It is GC-rich (Figure 37), a bidirectional promoter, and locates faraway upstream (41 kb) of the next promoter (PI). The isolation of PIn extends the knowledge about the regulation of the bovine *ACC-α*. A previously existing gap in the complete bovine genome DNA-sequence between the *TADA2L* gene and *ACC-α* is filled in by the sequence of PIn as characterized here. The transcripts generated from PIn in different species are similar. They contain exons [1n:4:5] and [1n:5] forming the 5′-UTR of the *ACC-α* mRNA in cattle (this work, Figure 35).

PIn is a tissue-specific promoter of *ACC-α* in brain. Functional analysis indicated that the bovine PIn has a very low activity in the murine mammary epithelial cells HC11. The activity of PIn is only ~3.3 –fold of pGL3basic (Figure 39). Hence, PIn barely drives the activity of this promoter-less vector. Expression profiles of PIn mRNA molecules demonstrate that PIn is highly expressed in the brain, but very low in liver, adipose tissues and mammary gland. These are the main lipogenic tissues (Figure 38). This highlights that bovine PIn is a brain-specific promoter, which is consistent with the homolog of sheep (Travers et al., 2005). Hence, bovine PIn may drive *ACC-α* transcription initiation in brain and regulate the synthesis of fatty acids in the brain neural cells, but it is not the main promoter for *ACC-α* expression in lipogenic tissues.

The key issue to characterize PIn for this study was to examine if (i) such a 5′-located PIn might be expressed in cattle as well, and (ii) if such a “PIn” might eventually override the regulation of PI. 5′-RACE showed that transcripts from PIn do not contain exon1 (Figure 35). Hence transcripts containing exon1 start from PI. Characterization of PI derived transcripts allows characterizing the activity of PI.

4.2.2 PI is the key promoter relevant for the nutritional regulation of fatty acid synthesis in lipogenic tissues

Previous work indicated that PI is the prominent promoter expressing *ACC-α* in the lipogenic tissues (Mao et al., 2001). In the present study, I found that PI activity in bovine liver is dramatically decreased (~20-fold) by prolongated starvation, and that refeeding activates PI expression ~3 -fold afterwards (Figure 8A). This finding is consistent with reports from the rat PI promoter, showing that starvation decreases PI activity in the liver, and that refeeding increases it again (Bianchi et al., 1990; Iverson et al., 1990; Kim and Tae, 1994). In adipose tissues, PI activity decreases during the transition period from pregnancy to lactation (Figure 8B). This finding is well supported by the fact that the rat PI activity is further diminished down to undetectable levels by the time of parturition in female white adipose tissue (Kim and Tae, 1994). Interestingly, the extent of decrease is slowed by fat-enriched diets, comparing to the standard carbohydrate diets (Figure 8B). No related results have been reported in other species. Hence, the observations of this study firmly establish that PI is the regulated promoter in the lipogenic tissues in the cattle.

Regarding PII and PIII, the former is a house keeping promoter, and it is not nutritionally or physiologically regulated in the liver (Figure 8A) and adipose tissues (Duske *et al.*, 2009, in press). The latter is a mammary gland specific promoter. Taken together, PI is the principally and nutritionally regulated promoter in the lipogenic tissues in the cattle. This study is to unravel the regulation mechanism of PI.

4.3 Characterization of a master control unit for PI promoter

4.3.1 NF-Y and C/EBP factors function as repressors via the distal PI promoter

Previous work reported that the bovine PI is basically repressed (Mao et al., 2001). The long promoter (-1045) has a very low activity, while the proximal promoter is very active in reporter gene analysis. Hence, PI is basically repressed by an upstream located element. The repressors and the repressive mechanism are unknown.

Primary investigation on bovine PI repressor was performed by Mao *et al.* 2001. But the detailed elements and repressors are unknown. In the study, serial deletions (Figure 10, 11), mutations (Figure 13, 15) and EMSA assays demonstrated that the upstream NF-Y (Figure 12B, D) and the downstream C/EBP (Figure 14B) comprise the bipartite repressor. Hence, NF-Y and C/EBP factors interacting with their distal binding sites are the bipartite repressor of PI promoter (Figure 33). Similar results were reported that rat PI repressor is bipartite

(Figure 33). The upstream element is CA28 repeat, and the downstream component is also a repressive C/EBP (Tae et al., 1994). O´Callaghan and colleagues further found the distal repressive region of rat PI reduces the extent of the response to insulin (O'Callaghan et al., 2001). Hence the elucidation of the repressive mechanism will contribute to understanding the regulation mechanism of fat metabolism in response to hormonal regulation, in cattle, rat and mouse. However, how do these repressors modulate PI activity *in vivo*?

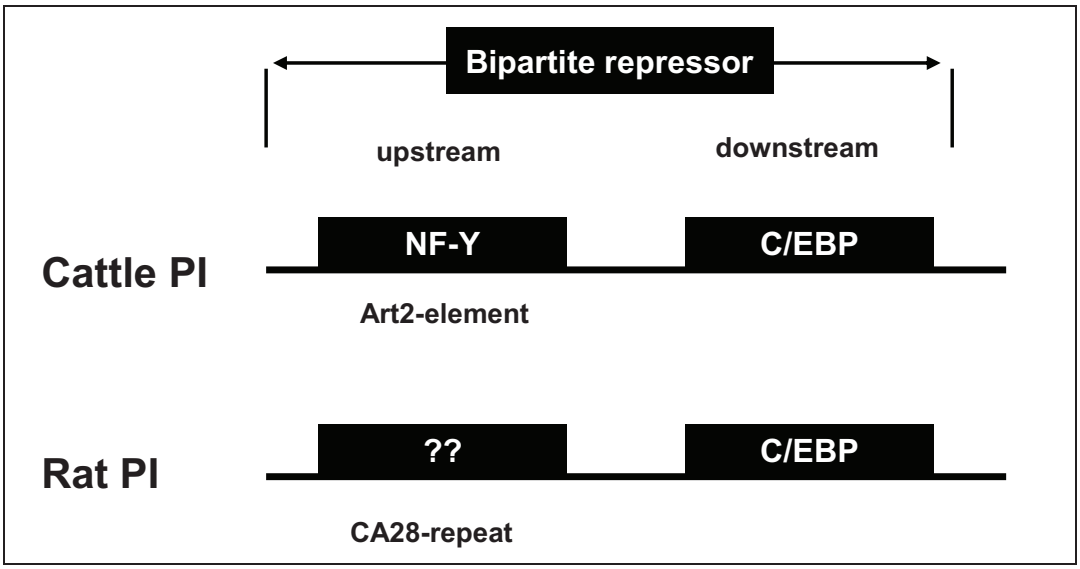


Figure 33 The bipartite repressor of *ACC-α* PI

4.3.2 NF-Y and C/EBP factors function as activators via the proximal PI promoter

Cotransfection analyses manifested that both NF-Y and C/EBP factors (–α, –β, –δ, –ε) activate rather than repress the long PI promoter (Figure 16). Hence, I hypothesized that the two family factors can also regulate PI promoter by interacting with the proximal region. The fact that both factors activate the proximal PI promoter further supported this hypothesis (Figure 18 and Figure 23). Subsequently, point mutation of the putative NF-Y binding site indicated that NF-Y binding to the proximal PI promoter drives expression (Figure 18). EMSA analysis further established that NF-Y factors bind to this site (Figure 19). Likewise, EMSA and point mutations showed that C/EBP factors are the key factors interacting with the binding site of proximal PI promoter, overlapping with transcription start site (Figure 20 and Figure 24). Sequence comparisons indicated that C/EBP involved in PI is conserved in ruminant and rodent, while NF-Y site is only found in bovine or ovine proximal PI (Figure 17). Taken together, the NF-Y and C/EBP factors interacting with their proximal binding site (-127/+84) are the drivers of PI promoter (Figure 34, green).

4.3.3 Both NF-Y and C/EBP factors are of key importance for PI regulation

NF-Y vastly regulates gene transcription by interacting with CCAAT box, and may do so either positively or negatively. Bucher found that the CCAAT box is one of the most ubiquitous enhancer elements, being present in approximately 30 % of eukaryotic promoters (Bucher, 1990). Typically, the CCAAT box is found as a single copy element in either forward or reverse orientation at the 5′- of the transcription start site (TSS). This was shown for the osteoclast differentiation factor gene (Kabe et al., 2005), the human homeobox gene (*TLX3*) (Borghini et al., 2006), the human collagen *COL11A1* gene (Matsuo et al., 2003) and the mouse adiponectin gene (Park et al., 2004). Despite the basal activation, NF-Y also mediates the transcriptional inhibition of genes, such as the mitotic cyclins and the cdc25C genes during p53-dependent G2 arrest induced by DNA damage (Manni et al., 2001), and the FGF-4 (fibroblast growth factor-4) gene (Bernadt et al., 2005). FGF-4 is regulated positively by its NF-Y binding site in embryonal carcinoma (EC) cells where the distal enhancer of the FGF-4 gene is active. However, the binding site functions as a negative *cis*-regulatory element when the FGF-4 enhancer elements are disrupted, or after the FGF-4 enhancer is inactivated during differentiation of EC cells.

However, in the present study, dual CCAAT boxes of NF-Y are found in bovine *ACC-α* PI promoter. The distal site extends from position -1040 to -1026 and the proximal site from position -116 to -102. The former site contributes to the repression of PI promoter, whereas the latter site is indispensable for NF-Y mediated activation and basal activity. Similarly, dual NF-Y binding sites have been reported in the human von willebrand factor (VWF) gene promoter (Peng and Jahroudi, 2002). In the VWF gene, the nonconsensus binding site (low affinity site) in exon1 contributes to repress VWF promoter activity, while the consensus NF-Y binding site (high affinity site) in the region from -30 to +1 functions as an activator. Peng et al further found that NF-Y interacts with histone deacetylases (HDACs) in a cell type-specific manner and recruits them to the VWF promoter to inhibit the promoter activity in non-endothelial cells through the repressive binding site (Peng and Jahroudi, 2003). Moreover, new evidence indicates that NF-Y can compact DNA (Guerra et al., 2007) and hence may repress the target gene promoter.

The NF-Y transcription factor is a heteromeric protein complex that consists of 3 subunits, NF-YA, NF-YB and NF-YC. Each subunit contains an evolutionary conserved HAP domain that is critical for subunit interaction and DNA binding (Mantovani, 1999). The function of NF-Y in regulation of gene expression is ubiquitous and depends on the physiological

condition. Solid evidence has been reported documenting that NF-Y may function as both, a repressor or an activator of gene expression (Bernadt et al., 2005; Ceribelli et al., 2008; Peng and Jahroudi, 2002). The functional role of NF-Y depends on the certain physiological condition (Bernadt et al., 2005) or the exact binding site in promoter (Peng and Jahroudi, 2002) and depends on other transcriptional factors which it may interact with. Hence, NF-Y is a bifunctional transcription factor and plays an important role in gene regulation.

Likewise, C/EBP is essential in gene regulation. Two C/EBP binding sites have also been found in the bovine PI promoter: a distal C/EBP binding site (-183/-175) and a proximal binding site (+6/-3). The proximal binding site contributes to basal promoter activity and activation. This was found in many C/EBP-dependent genes (Reddy et al., 2003; Zhang et al., 2003; Zhou and Snead, 2000). However, the distal binding site is relevant to the repression of bovine PI. This finding is consistent with the C/EBP binding motif of rat *ACC-α* PI (Tae et al., 1994). C/EBP cooperates with an unknown factor on upstream CA-28 repeat to repress rat *ACC-α* PI activity (Tae et al., 1994). The C/EBP repressive factor is further supported by fact that tumor necrosis factor-alpha (TNF-α) inhibits transcription of the α2 chain of type I collagen (α2(I) collagen) in cultured fibroblasts, by stimulating the synthesis and binding of repressive C/EBPs to a responsive element (Greenwel et al., 2000). It is known that there are several-members in the family of C/EBP factors. Each C/EBP member can be translated into an activator or a repressor isoform (Descombes and Schibler, 1991). Moreover, C/EBP factors regulate gene expression by interacting with other factors such as NF-Y and NF-κB. It has recently been reported that different C/EBP members interacting with HDAC1 in various physiology conditions control promoter activity (Wang et al., 2008a; Wang et al., 2008b). Hence, C/EBP factors can also positively or negatively regulate gene expression.

4.3.4 Both the driver sites have vastly different binding affinities from the repressor sites

It seems a paradox that the PI promoter has both driver binding site and repressor binding site for both, NF-Y and C/EBP factors. However, further investigation showed that the proximal binding sites have much higher affinities than those of the distal NF-Y and C/EBP binding sites (Figure 29, 30). Hence, the proximal binding sites with high affinities are responsible for activation, while the distal binding sites with low affinities are relevant to repression. The proximal *cis*-elements are indispensable for the basal activity of PI. These results are in agreement with the finding that the activity of promoter elements correlates well with the relative affinity of their factors (Mauxion et al., 1991). Interactions by low affinity sites and

the binding proteins may be functionally relevant *in vivo* because the binding affinity can be greatly enhanced by cooperative interactions with other transcriptional factors that recognize the adjacent sites (Carey and Smale, 2000). Hence, the difference of affinity is also an important factor in the complicate regulation mechanism of eukaryotic genes.

Therefore, a small amount of NF-Y and C/EBP factors are sufficient to saturate the proximal binding sites and activate the promoter. Much higher concentrations are necessary to saturate the distal binding sites. Once these are occupied, then the distal factors can feed-back repress the activation potential of the factors bound to the proximal attachment sites. Various affinity binding sites may cooperatively regulate *ACC-α* PI activity depending on different concentration of C/EBP factors and their isoforms.

4.3.5 NF-Y represses the activating capability of C/EBPβ

Using series of cotransfections, I demonstrated that C/EBPα, –β, –δ, –ε and NF-Y all activate PI activity (Figure 26A). The PI activity responds to different concentrations of C/EBP factors. PI activity is greatly activated by C/EBPα expressed in low concentrations (Figure 22). But C/EBPβ eventually is the strongest activator of the four members (–α, –β, –δ, –ε) of this factor family (Figure 23B). NF-Y significantly but moderately activates PI activity, as is consistent with the work of NF-Y (Xu et al., 2006). Combination of NF-Y and C/EBP factors indicated that NF-Y significantly represses the C/EBPβ-mediated activation (Figure 26A). In contrast, NF-Y represses the C/EBPε-mediated activation moderately by the extent. No inhibition of C/EBPα activity was found by NF-Y. Subsequently, the inhibition of C/EBPβ activity can be titrated by using increasing amount of NF-Y expression vectors (Figure 26B). However, separately the respective subunit of NF-Y (NF-YA, or NF-YB, or NF-YC) can not repress the C/EBPβ activity (Figure 27). I do not know whether the two subunits combination can repress the C/EBPβ-mediated activation, but the intact NF-Y complex can do.

4.3.6 NF-Y and C/EBPβ may physically interact via protein-protein interaction

The inhibition of C/EBPβ by NF-Y is independent of the proximal NF-Y binding site, because the `non-binding NF-Y´ still represses the C/EBPβ activity (Figure 27). The binding domain of NF-Y complex was mutated by 3-amino acid-substitution AAA for the original RGE in the binding domain of NF-YA (Mantovani et al., 1994). Hence, I deduced that the inhibition of C/EBPβ activity by NF-Y is based on protein-protein interaction.

The other potent evidences supporting the assumption that the interaction of NF-Y-C/EBPβ

synergistically regulates bovine *ACC-α* PI promoter are the pull-down (not shown) and `ChIP´ experiments (Figure 28). C/EBPβ can be detected in immunoprecipitated protein by both anti-NF-YA and anti-C/EBPβ, though the amount of pulled down C/EBPβ proteins by anti-C/EBPβ exceeds that being pulled-down by anti-NF-YA. This fact manifests the protein interaction between C/EBPβ and NF-Y complex (data not shown). In `ChIP´ analysis, the proximal PI promoter both can be pulled down by respective antibodies recognizing either the over-expressed `non-binding NF-Y´ or C/EBPβ (Figure 27). As discussed (Figure 27B), the `non-binding NF-Y´ can not bind to the NF-Y binding motif in the proximal PI promoter. The pulled down proximal PI fragment by anti-NF-YA indicates the interaction between the `non-binding NF-Y´ and C/EBPβ, which directly bind to the C/EBP binding site in proximal PI fragment.

The interaction between NF-Y and C/EBP factors is constituently and functionally biased in a factor-specific manner. Functional evidence was provided that the interaction between NF-Y and C/EBPβ in bovine represses PI activity (Figure 26A). This interaction could also occur between NF-Y and C/EBPε, rather than between NF-Y and C/EBPα and –δ on the grounds of my cotransfection analyses (Figure 26A). However, it was found in mouse that the protein-protein interaction between NF-Y and C/EBPα, but not C/EBPβ, positively regulates, rather than represses the C/EBPα activity in the amelogenin gene promoter (Xu et al., 2006). A similar result was reported for the human microsomal epoxide hydrolase gene (EPHX) regulating through the direct interaction of C/EBPα with NF-Y (Zhu et al., 2004). Interestingly, this interaction could be substituted by the interaction of NF-Y and C/EBPδ. When C/EBPα was knocked out *in vivo*, the interaction of C/EBPα-NF-Y was blocked, but a new interaction complex of C/EBPδ-NF-Y substituted for the previous one (Xu et al., 2007).

4.3.7 The mRNA proportion of NF-YA over C/EBPβ is greatly correlated in a tissue-specific manner with the *ACC-α* PI activity

Since above evidences indicate that NF-Y and C/EBP are crucial in regulating bovine *ACC-α* PI, I examined *in vivo* the physiological relevance of the proposed regulation circuit controlling PI activity. To this end, I measured the mRNA concentrations of PI activity and the relevant transcription factors in the liver, mammary glands and adipose tissues. The correlation between *ACC-α* PI activity and NF-Y, C/EBP factors is not evident considering the values from the individuals. However, the average proportion of NF-YA over average of C/EBPβ mRNA molecules correlates with *ACC-α* PI activity from udder, adipose to liver

tissue respectively (Figure 31). This correlation of the factors and the PI activity explains the followed conditions.

In the basal condition, both NF-Y and C/EBP factors are expressed at a relatively low level, and the proximal binding sites are saturated. Due to the low affinity of the distal sites, the repressor is barely occupied at low concentration of C/EBP, and the PI activity is high. Such a situation is seen in the mammary gland (Table 8). When the relative concentration of C/EBP factors is increased and with only a slight increase of NF-Y factors, the distal sites repressors feed back to repress PI activity, such as in adipose tissue (Table 8). However, a high level of C/EBP factors almost silence the expression of *ACC-α* PI, just as in the liver (Table 8). Taken together, this suggests that both factors are important for the regulation of *ACC-α* PI activity in a tissue-specific manner.

Detailed analyses indicated, in the liver, the PI activity is nutritionally regulated (Figure 8A). The PI activity is inhibited by starvation (Figure 8A) or energy reduction (Table 8). However, the NF-YA and C/EBP factors are barely regulated at the mRNA level by starvation in the liver of the cows, which were reduced down to 16 % energy (appendix J). Subsequently, I found that more C/EBP factors bind to the repressive C/EBP binding site after refeeding in the liver, but no evident changes were found between feeding and starvation, as well as for NF-Y complex (data not shown). These investigation manifested NF-Y and C/EBP factors are not regulated at the mRNA level, but may be at the protein level in the liver in response to nutritional regulation.

In adipose tissues, I found that calving greatly down-regulates the PI activity, but NF-YA and C/EBPα, β and ε repressors do not increase to inhibit the PI activity (Table 9). Though I found that C/EBPδ increases by fat-enriched diets after calving (Table 9), I can not explain why C/EBPδ activate, but not represses the proximal PI promoter in reporter gene analysis (Figure 23B).

Afterwards, I found, in the udder, the lowest C/EBPβ is expressed compared to the liver and adipose tissues (Table 8). C/EBPβ is significantly down-regulated at the mRNA level by fat-enriched diets ($p<0.05$). No significant changes were found on NF-YA expression. These investigations indicated that C/EBP factors may be regulable in the udder under nutrition controls.

Because the bifunctional nature of both factors depends on different cofactors (Caretti et al., 2003; Peng et al., 2007; Peng and Jahroudi, 2003; Wang et al., 2008a), it is unknown how C/EBP factors and NF-Y positively or negatively regulate *ACC-α* PI in a given condition.

Different isoforms (LAP1, LAP2 and LIP) may either activate or repress gene transcription resulting in a very comprehensive functional pattern of C/EBP factors. Hence, it is unclear at present how the different levels of NF-Y and C/EBP factors ultimately regulated PI activity. Taken together, the ratio of the concentration of NF-Y and C/EBP factors adjusts very different set points around which the activity of PI may be regulated by other factors of either acute or hormonal fatty acid synthesis regulation.

4.3.8 All C/EBP family members potentially regulate ruminant and rodent *ACC-α* PI

In the study, two C/EBP binding sites have been characterized in bovine *ACC-α* PI. C/EBPβ, -ε can directly bind to the distal site (Figure 14) and C/EBPα, -β and -ε can directly bind to the proximal binding site (Figure 21). Functionally, C/EBPα, -β, -δ and -ε all positively regulate bovine *ACC-α* PI (Figure 16, 23). Hence, these C/EBP factors are directly or indirectly relevant to bovine PI activity. Due to the identity of bovine and ovine C/EBP binding motif in proximal promoter region (Figure 17), and it is reported that C/EBPα and β both activate rat PI promoter (Tae et al., 1995), I hypothesize that all C/EBP family members will contribute to control *ACC-α* PI activity in ruminant and rodent.

4.3.8.1 C/EBPα is a tissues-specific regulable activator

It is well known that C/EBPα activates differentiation of adipocyte resulting in adipogenesis (Millward et al., 2007) but inhibits proliferation of liver cell (Cao et al., 1991). In the study, I found the *ACC-α* PI activity dramatically decreased in the transition period (before calving *vs* after calving, Figure 8). Accordingly, C/EBPα levels are down-regulated in tendency during the transition period (Table 9). In the liver, C/EBPα does not change in response to starvation (Appendix J). Similarly, Robinson *et al* reported that C/EBPα levels keep steady in the mouse mammary gland during starvation and refeeding (Robinson et al., 1998). Taken together, C/EBPα may be an adipose tissue-specific activator for bovine *ACC-α* PI promoter.

4.3.8.2 C/EBPβ may regulate PI at different levels

Several lines of evidence show that C/EBPβ is relevant for bovine ACC-α PI regulation. First, anti-C/EBPβ supershifted both the distal and the proximal binding elements formed shift bands in EMSA (Figure 14, 20). Second, recombinant DN-C/EBPβ can specifically bind to the two binding elements of bovine ACC-α PI promoter (Figure 14, Figure 21). Third,

increased C/EBPβ concentrations increased the bovine *ACC-α* PI activity (Figure 23, 24). Likewise, Tae *et al* showed that C/EBPβ activates the rat *ACC-α* PI activity (Tae et al., 1995), and adipogenesis can also be induced by ectopic expression of C/EBPβ (Ge et al., 2002; Yeh et al., 1995). Consistent with this conclusion, it is found that C/EBPβ knockout mice display decreasing of lipid accumulation and are protected from obesity (Millward et al., 2007; Rahman et al., 2007; Schroeder-Gloeckler et al., 2007). Hence, the present finding further provides novel evidence linking C/EBPβ expression to lipogenesis and energy balance, and important implications for the treatment of dysregulation of lipid metabolism.

I did not find altered mRNA concentrations (Appendix J), and protein expression (Figure 32) of C/EBPβ in the liver caused by feeding changes. It is well known that C/EBPβ may regulate the target genes by changing the ratio of various isoforms, especially of LAP and LIP (Raught et al., 1995). However, the expressions of these isoforms are unaltered in bovine liver (Figure 32). Subsequently, I examined the active C/EBP factors using the ^{32}P-labelled distal binding sites, and more active C/EBP binds to C/EBP binding site from refed liver (data not shown). The increased active C/EBPβ may be incurred by phosphorylation. Phosphorylation of C/EBPβ at Thr^{269} (orthologous to Thr^{217} in mouse C/EBPβ) is increased in the livers from refed-cows (Figure 32), but not from normally feeding and starved cows. Taken together, C/EBPβ may regulate PI at different levels.

4.3.9 The active NF-Y is regulated in a tissue-specific fashion at the protein level

NF-Y is a ubiquitous factor controlling gene regulation. However, NF-YA Expression fluctuates during the cell cycle and is down-regulated in postmitotic cells (Bolognese et al., 1999). In the study, I found that NF-YA levels decrease in tendency after calving in the adipose tissue, but the mRNA levels of NF-YA were relatively steady in the liver, adipose tissues and mammary glands (Table 8). This result is consistent with the finding by Bolognese and Farina, that NF-YA mRNA is relatively constant in growing and differentiated cells (Bolognese et al., 1999; Farina et al., 1999). However, I found high levels of NF-Y-DNA complex when examined the nuclear extracts from mammary gland using the EA-left side probe of NF-Y in EMSA experiment (Figure 12B `C2´). Small amounts of NF-Y complex were found in the livers of fed cows. NF-Y factors could not be detected in the liver of starved and refed cows. In contrast, high level of C/EBP factors was detected from all the three samples (data not shown). The functional NF-Y complex is controlled broadly in the protein level and post-translational level (Manni et al., 2008).

4.3.10 The master control unit controlling PI activity

In conclusion, a master control unit repressing PI activity is proposed as described in Figure 34 basing on the above results. The distal NF-Y (red) binding to element A represses the proximal C/EBPβ (green) activity by direct protein-protein interaction, while the distal C/EBP (red) binding to element C repress PI activity by interaction with the proximal NF-Y (green). Hence, the master control unit is composed of double protein-protein interactions between NF-Y and C/EBP.

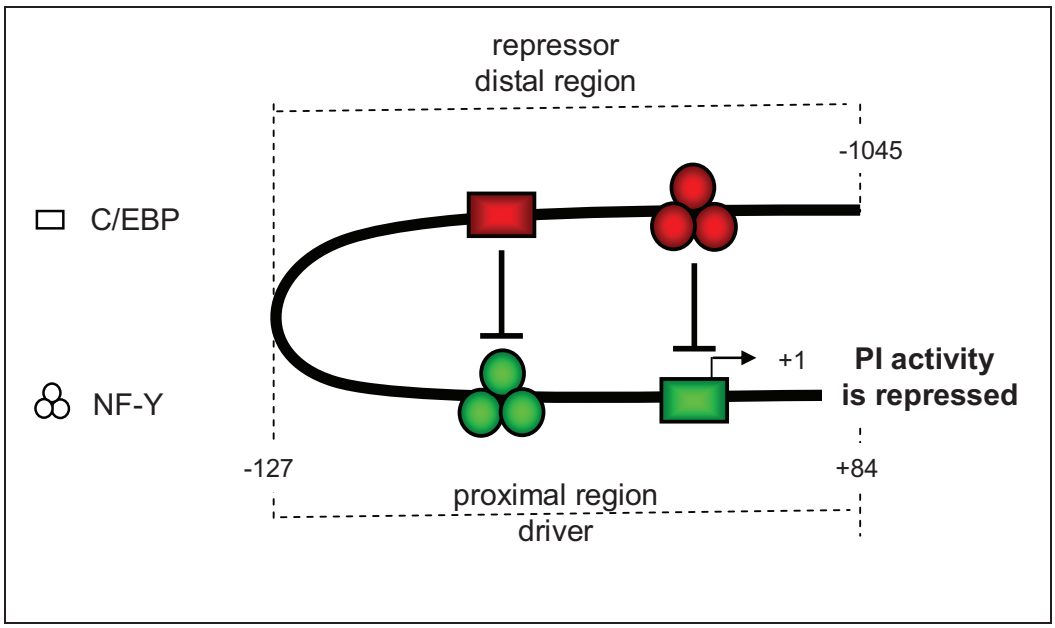


Figure 34 A master control unit in PI promoter of the bovine ACC-α gene

The map is not drawn to scale. +1 represents the transcription start site. Repressive factors are in red, and these drivering expression are in green.

The master control unit can be regulated by the mounts of the components (NF-Y and C/EBP) in a given tissue or in different physiology or nutritional conditions, or by competition of different family members, or by the posttranslational modification, and hence *ACC-α* PI displays tissue-specific activity. By recruiting coactivitors or corepressors, or post-translational modification, the master control unit can be modulated finely in response to nutritional or hormonal changes.

5 Summary

- PI of ACC-α is confirmed as a nutritionally regulated promoter.

- A repressive NF-Y binding site and a repressive C/EBP binding site (site1) have been identified in the distal PI promoter.

- A NF-Y binding site and a C/EBP binding site (site3) have been also identified in the proximal PI promoter, as driver binding sites.

- The affinities of the distal and proximal sites have been compared for NF-Y and C/EBP. The proximal sites have higher affinities for the respective binding factors than the distal site.

- C/EBP factors and NF-Y separately activate PI activity. However, NF-Y can specifically inhibit C/EBPβ activity on *ACC-α* PI by protein-protein interaction.

- The proportion of NF-YA over C/EBPβ mean values correlates with the tissue-specific PI activity. The mRNA levels of NF-YA and C/EBP factors are not regulated significantly in a certain tissue under nutritional changes.

- The phosphorylation of C/EBPβ Thr^{269} is up-regulated by refeeding in the liver, as indicates that a quick regulation by C/EBPβ phosporylaion may regulate PI activity.

- Bovine new promoter (PIn) and its derived *ACC-α* transcripts (5´-UTR) have been identified. The distance between PIn and PI is ~41 kb. The transcripts by PIn are composed of Exon [1n:4:5] and [1n:5], and are prominently expressed in the brain.

- To establish these results, expression constructs of bovine NF-YA, -YB, -YC, C/EBPα, -β, -δ and -ε, have been cloned and constructed for expression both in bacterial and mammalian cells. And the derived **D**ominant **N**egative factors: DN-C/EBPα, DN-C/EBPβ, DN-C/EBPδ and DN-C/EBPε have been constructed. The bovine *C/EBPδ* mRNA has been isolated and first time characterized.

- An indirect **A**vidin-**B**iotin **C**omplex based sandwich ELISA (ABC-ELISA) has been developed for the measurement of bovine ACC-α. The sensitivity is 1.6~3.2 ng /assay. To this end, the polyclonal antisera of rabbit and goat against a recombinantly expressed N-terminal segment of the bovine *ACC-α* have been developed by immunizations with purified recombinant protein expressed in *E.coli* BL-21. The two antibodies were purified by affinity chromatography on antigen carrying sepharose columns.

6 Acknowledgements

My deepest gratitude is to my supervisor, Prof. Dr. Hans-Martin Seyfert. Hans-Martin not only provides my family an ease and open research context, but also the possibility of a happy life in Dummerstorf. Hans-Martin guided me into the field of molecular biology from experiment designs, to results analyses and discussions, and to the manuscript and revision of this thesis. Without his patience, continuous encouragements, guidance of erudite knowledge on molecular biology and biochemistry, experiences on scientific writing, my dissertation could not possibly be finished.

I would like to acknowledge Mrs. Deike for sharing with her orderly managements of lab samples and data, and numerous technique experiences. I am also grateful to Mrs. Pletz, Mrs. Berndt, Mrs. Wego and Matina for abundant work supporting my graduate research. I am indebted to Kathrin Esch, Dr. Alexander Rebl and Dr. Juliane Günther for helps on experiments and my family living life. Again, I express my hearty thanks to all my lab members for help to start my family life in Dummerstorf.

I am obliged to Prof. Dr. Klaus Wimmers and PD Dr. Christa Kühn for providing a wide and active communication platform of seminar in the department, as makes my interests broad and deep. PD Dr. Jens Vanselow and Dr. Rainer Fürbaß always give me insightful comments and constructive suggestions. I am appreciative to them of considerable discussions. Many thanks are to PD. Dr. Tom Goldammer and Dr. Ronald M. Brunner especially for helps on fluorescence. I extend an immense gratitude to Dr. Brunner for his help on my laptop. It makes my thesis writing rapidly and smoothly. Many thanks are also to Dr. Fuchs for her numerous and response sequences of my clones. I am grateful to Mrs. Masermann, and to Mrs. Saß and Mrs. Künzel for my family visas and preparations of relevant documents. Mrs. Künzel's work makes me concentrate my energy on my thesis. I am appreciative to Dr. Wolfgang Tomek and Mrs. Krüger of their numerous helps in western blots. I extend my thanks to Lukas Wager for critical suggestions of my thesis manuscript.

I am also indebted to Bodo Brand, Sylvio Tetzlaff, Annett Eberlein, Sabine Schütze and Westphal Philipp. You always generously help me a lot of trifles and affairs. Thanks are also to Jinxian Xu, Srikanchai Tiranun, Nuchchanart Wirawan, Chomwisarutkun Kunsuda and Naraballobh Watcharapong for happy leisure time. I am grateful to Wei Yang and Yongjun Wei for the starts of my research and local life. At present, they share many experiences with me both on researches and future career.

I heartily appreciate my wife Shuzhen Liu, my son Weitao Shi and my extended family for numerous encouragements and understandings.

I owe my gratitude to DFG grant (Se 326/13-2), all those people who have made this dissertation possible, all those people who have cared for me and my family. I will cherish my graduate experience forever.

7 References

Reference List

Abraham,S., Matthes,K.J., and Chaikoff,I.L. (1960). Fatty Acid Synthesis from Acetate by Normal and Diabetic Rat Liver Homogenate Fractions. I. A COMPARISON OF COFACTOR REQUIREMENTS. *J. Biol. Chem.* **235,** 2551-2559.

Abu-Elheiga,L., Jayakumar,A., Baldini,A., Chirala,S.S., and Wakil,S.J. (1995). Human Acetyl-CoA Carboxylase: Characterization, Molecular Cloning, and Evidence for Two Isoforms. *Proceedings of the National Academy of Sciences* **92,** 4011-4015.

Abu-Elheiga,L., Matzuk,M.M., Kordari,P., Oh,W., Shaikenov,T., Gu,Z., and Wakil,S.J. (2005). Mutant mice lacking acetyl-CoA carboxylase 1 are embryonically lethal. *Proceedings of the National Academy of Sciences* **102,** 12011-12016.

Abu-Elheiga,L., Oh,W., Kordari,P., and Wakil,S.J. (2003). Acetyl-CoA carboxylase 2 mutant mice are protected against obesity and diabetes induced by high-fat/high-carbohydrate diets. *Proceedings of the National Academy of Sciences* **100,** 10207-10212.

Antonson,P. and Xanthopoulos,K.G. (1995). Molecular-Cloning, Sequence, and Expression Patterns of the Human Gene Encoding Ccaat/Enhancer-Binding-Protein-Alpha (C/Ebp-Alpha). *Biochemical and Biophysical Research Communications* **215,** 106-113.

Barber,M.C., Pooley,L., and Travers,M.T. (2001). Developmental regulation of alternatively spliced acetyl-CoA carboxylase-alpha mRNAs encoding isozymes with or without an eight amino acid domain upstream of the Ser-1200 phosphorylation motif in the mammary gland. *J Mol Endocrinol* **27,** 349-356.

Barber,M.C., Price,N.T., and Travers,M.T. (2005). Structure and regulation of acetyl-CoA carboxylase genes of metazoa. *Biochim Biophys Acta* **1733,** 1-28.

Barber,M.C., Vallance,A.J., Kennedy,H.T., and Travers,M.T. (2003). Induction of transcripts derived from promoter III of the acetyl-CoA carboxylase-alpha gene in mammary gland is associated with recruitment of SREBP-1 to a region of the proximal promoter defined by a DNase I hypersensitive site. *Biochem. J.* **375,** 489-501.

Beaty,N.B. and Lane,M.D. (1983a). Kinetics of Activation of Acetyl-Coa Carboxylase by Citrate - Relationship to the Rate of Polymerization of the Enzyme. *J. Biol. Chem.* **258,** 3043-3050.

Beaty,N.B. and Lane,M.D. (1983b). The Polymerization of Acetyl-Coa Carboxylase. *J. Biol. Chem.* **258,** 3051-3055.

Bernadt,C.T., Nowling,T., Wiebe,M.S., and Rizzino,A. (2005). NF-Y behaves as a bifunctional transcription factor that can stimulate or repress the FGF-4 promoter in an enhancer-dependent manner. *Gene Expr* **12,** 193-212.

Bianchi,A., Evans,J.L., Iverson,A.J., Nordlund,A.C., Watts,T.D., and Witters,L.A. (1990). Identification of an isozymic form of acetyl-CoA carboxylase. *J. Biol. Chem.* **265,** 1502-1509.

Bolognese,F., Wasner,M., Lange-zu Dohna,C., Gurtner,A., Ronchi,A., Muller,H., Manni,I., Mossner,J., Piaggio,G., Mantovani,R. et al. (1999). The cyclin B2 promoter depends on NF-Y, a trimer whose CCAAT-binding activity is cell-cycle regulated. *Oncogene* **18,** 1845-1853.

Boone,A.N., Chan,A., Kulpa,J.E., and Brownsey,R.W. (2000a). Bimodal activation of acetyl-CoA carboxylase by glutamate. *J. Biol. Chem.* **275,** 10819-10825.

Boone,A.N., Chan,A., Kulpa,J.E., and Brownsey,R.W. (2000b). Bimodal Activation of Acetyl-CoA Carboxylase by Glutamate. *J. Biol. Chem.* **275,** 10819-10825.

Borghini,S., Vargiolu,M., Di Duca,M., Ravazzolo,R., and Ceccherini,I. (2006). Nuclear Factor Y Drives Basal Transcription of the Human TLX3, a Gene Overexpressed in T-Cell Acute Lymphocytic Leukemia. *Mol Cancer Res* **4,** 635-643.

Brusselmans,K., De Schrijver,E., Verhoeven,G., and Swinnen,J.V. (2005). RNA Interference-Mediated Silencing of the Acetyl-CoA-Carboxylase-{alpha} Gene Induces Growth Inhibition and Apoptosis of Prostate Cancer Cells. *Cancer Res* **65,** 6719-6725.

Bucher,P. (1990). Weight Matrix Descriptions of 4 Eukaryotic Rna Polymerase-Ii Promoter Elements Derived from 502 Unrelated Promoter Sequences. *Journal of Molecular Biology* **212,** 563-578.

Buck,M. and Chojkier,M. (2007). A ribosomal S-6 kinase-mediated signal to C/EBP-beta is critical for the development of liver fibrosis. *PLoS ONE.* **2,** e1372.

Buck,M., Poli,V., van der Geer,P., Chojkier,M., and Hunter,T. (1999). Phosphorylation of rat serine 105 or mouse threonine 217 in C/EBP beta is required for hepatocyte proliferation induced by TGF alpha. *Molecular Cell* **4,** 1087-1092.

Burgess-Beusse,B.L., Timchenko,N.A., and Darlington,G.J. (1999). CCAAT/enhancer binding protein alpha (CIEBP alpha) is an important mediator of mouse C/EBP beta protein isoform production. *Hepatology* **29,** 597-601.

Cao,Z., Umek,R.M., and McKnight,S.L. (1991). Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes Dev.* **5,** 1538-1552.

Caretti,G., Salsi,V., Vecchi,C., Imbriano,C., and Mantovani,R. (2003). Dynamic recruitment of NF-Y and histone acetyltransferases on cell-cycle promoters. *J. Biol. Chem.* **278,** 30435-30440.

Carey,M. and Smale,S.T. (2000). *Transcriptional Regulation in Eukaryotes: Concepts, Strategies, and Techniques*. New York: Cold Spring Harbor Laboratory Press.

Carling,D., Zammit,V.A., and Hardie,D.G. (1987). A Common Bicyclic Protein-Kinase Cascade Inactivates the Regulatory Enzymes of Fatty-Acid and Cholesterol-Biosynthesis. *Febs Letters* **223,** 217-222.

Celada,A., McKercher,S.R., and Maki,R.A. (1996). Identification of the transcription factors NF-YA and NF-YB as factors A and B that bound to the promoter of the major histocompatibility complex class II gene I-A beta. *Biochem. J.* **317,** 771-777.

Ceribelli,M., Dolfini,D., Merico,D., Gatta,R., Vigano,A.M., Pavesi,G., and Mantovani,R. (2008). The histone-like NF-Y is a bifunctional transcription factor. *Mol. Cell. Biol.* **28,** 2047-2058.

Cesquini,M., Stoppa,G.R., Prada,P.O., Torsoni,A.S., Romanatto,T., Souza,A., Saad,M.J., Velloso,L.A., and Torsoni,M.A. (2008). Citrate diminishes hypothalamic acetyl-CoA carboxylase phosphorylation and modulates satiety signals and hepatic mechanisms involved in glucose homeostasis in rats. *Life Sciences* **82,** 1262-1271.

Choi,C.S., Savage,D.B., bu-Elheiga,L., Liu,Z.X., Kim,S., Kulkarni,A., Distefano,A., Hwang,Y.J., Reznick,R.M., Codella,R. et al. (2007). Continuous fat oxidation in acetyl CoA carboxylase 2 knockout mice increases total energy expenditure, reduces fat mass, and improves insulin sensitivity. *Proceedings of the National Academy of Sciences* **104,** 16480-16485.

Cicchillitti,L., Jimenez,S.A., Sala,A., and Saitta,B. (2004). B-Myb acts as a repressor of human COL1A1 collagen gene expression by interacting with Sp1 and CBF factors in scleroderma fibroblasts. *Biochem. J.* **378,** 609-616.

Crosson,S.M., Davies,G.F., and Roesler,W.J. (1997). Hepatic expression of CCAAT/enhancer binding protein alpha: hormonal and metabolic regulation in rats. *Diabetologia* **40,** 1117-1124.

Davies,S.P., Sim,A.T., and Hardie,D.G. (1990). Location and function of three sites phosphorylated on rat acetyl-CoA carboxylase by the AMP-activated protein kinase. *Eur J Biochem* **187,** 183-190.

Descombes,P., Chojkier,M., Lichtsteiner,S., Falvey,E., and Schibler,U. (1990). Lap, A Novel Member of the C/Ebp Gene Family, Encodes A Liver-Enriched Transcriptional Activator Protein. *Genes & Development* **4,** 1541-1551.

Descombes,P. and Schibler,U. (1991). A Liver-Enriched Transcriptional Activator Protein, Lap, and A Transcriptional Inhibitory Protein, Lip, Are Translated from the Same Messenger-Rna. *Cell.* **67,** 569-579.

Don,R.H., Cox,P.T., Wainwright,B.J., Baker,K., and Mattick,J.S. (1991). Touchdown Pcr to Circumvent Spurious Priming During Gene Amplification. *Nucl. Acids Res.* **19,** 4008.

Dorn,A., Bollekens,J., Staub,A., Benoist,C., and Mathis,D. (1987). A Multiplicity of Ccaat Box-Binding Proteins. *Cell.* **50,** 863-872.

Eyster,K.M. (2007). The membrane and lipids as integral participants in signal transduction: lipid signal transduction for the non-lipid biochemist. *Advan. Physiol. Edu.* **31,** 5-16.

Faergeman,N.J. and Knudsen,J. (1997). Role of long-chain fatty acyl-CoA esters in the regulation of metabolism and in cell signalling. *Biochem. J.* **323,** 1-12.

Farina,A., Gaetano,C., Crescenzi,M., Puccini,F., Manni,I., Sacchi,A., and Piaggio,G. (1996). The inhibition of cyclin B1 gene transcription in quiescent NIH3T3 cells is mediated by an E-box. *Oncogene* **13,** 1287-1296.

Farina,A., Manni,I., Fontemaggi,G., Tiainen,M., Cenciarelli,C., Bellorini,M., Mantovani,R., Sacchi,A., and Piaggio,G. (1999). Down-regulation of cyclin B1 gene transcription in terminally differentiated skeletal muscle cells is associated with loss of functional CCAAT-binding NF-Y complex. *Oncogene* **18,** 2818-2827.

Gallardo,D., Canovas,E., Lopez-Bejar,M., Ramirez,O., Pena,R., Quintanilla,R., and Amills,M. (2008). Alternative splicing at exon 28 of the acetyl-coenzyme A carboxylase alpha gene in adult pigs and embryos. *Animal Genetics* **39,** 205-206.

Ge,K., Guermah,M., Yuan,C.X., Ito,M., Wallberg,A.E., Spiegelman,B.M., and Roeder,R.G. (2002). Transcription coactivator TRAP220 is required for PPAR gamma 2-stimulated adipogenesis. *Nature* **417,** 563-567.

Ghosh,A.K., Lacson,R., Liu,P.X., Cichy,S.B., Danilkovich,A., Guo,S.D., and Unterman,T.G. (2001). A nucleoprotein complex containing CCAAT/enhancer-binding protein beta interacts with an insulin response sequence in the insulin-like growth factor-binding protein-1 gene and contributes to insulin-regulated gene expression. *J. Biol. Chem.* **276,** 8507-8515.

Greenwel,P., Tanaka,S., Penkov,D., Zhang,W., Olive,M., Moll,J., Vinson,C., Di Liberto,M., and Ramirez,F. (2000). Tumor Necrosis Factor Alpha Inhibits Type I Collagen Synthesis through Repressive CCAAT/Enhancer-Binding Proteins. *Mol. Cell. Biol.* **20,** 912-918.

Guerra,R.F., Imperadori,L., Mantovani,R., Dunlap,D.D., and Finzi,L. (2007). DNA compaction by the nuclear factor-Y. *Biophys J* **93,** 176-182.

Gurtner,A., Manni,I., Fuschi,P., Mantovani,R., Guadagni,F., Sacchi,A., and Piaggio,G. (2003). Requirement for down-regulation of the CCAAT-binding activity of the NF-Y transcription factor during skeletal muscle differentiation. *Mol. Biol. Cell* **14,** 2706-2715.

Ha,J., Daniel,S., Kong,I.S., Park,C.K., Tae,H.J., and Kim,K.H. (1994). Cloning of human acetyl-CoA carboxylase cDNA. *Eur J Biochem* **219,** 297-306.

Hardie,D.G. and Carling,D. (1997). The AMP-activated protein kinase - Fuel gauge of the mammalian cell? *European Journal of Biochemistry* **246,** 259-273.

Haystead,T.A., Campbell,D.G., and Hardie,D.G. (1988). Analysis of sites phosphorylated on acetyl-CoA carboxylase in response to insulin in isolated adipocytes. Comparison with sites phosphorylated by casein kinase-2 and the calmodulin-dependent multiprotein kinase. *Eur J Biochem* **175,** 347-354.

Haystead,T.A., Moore,F., Cohen,P., and Hardie,D.G. (1990). Roles of the AMP-activated and cyclic-AMP-dependent protein kinases in the adrenaline-induced inactivation of acetyl-CoA carboxylase in rat adipocytes. *Eur J Biochem* **187,** 199-205.

Heesom,K.J., Moule,S.K., and Denton,R.M. (1998). Purification and characterisation of an insulin-stimulated protein-serine kinase which phosphorylates acetyl-CoA carboxylase. *Febs Letters* **422,** 43-46.

Hsu,S.M., Raine,L., and Fanger,H. (1981). Use of Avidin-Biotin-Peroxidase Complex (Abc) in Immunoperoxidase Techniques - A Comparison Between Abc and Unlabeled Antibody (Pap) Procedures. *Journal of Histochemistry & Cytochemistry* **29,** 577-580.

Iverson,A.J., Bianchi,A., Nordlund,A.C., and Witters,L.A. (1990). Immunological Analysis of Acetyl-Coa Carboxylase Mass, Tissue Distribution and Subunit Composition. *Biochem. J.* **269,** 365-371.

Jurado,L.A., Song,S.L., Roesler,W.J., and Park,E.A. (2002). Conserved amino acids within CCAAT enhancer-binding proteins (C/EBP alpha and beta) regulate phosphoenolpyruvate carboxykinase (PEPCK) gene expression. *J. Biol. Chem.* **277,** 27606-27612.

Kabe,Y., Yamada,J., Uga,H., Yamaguchi,Y., Wada,T., and Handa,H. (2005). NF-Y is essential for the recruitment of RNA polymerase II and inducible transcription of several CCAAT box-containing genes. *Mol. Cell. Biol.* **25,** 512-522.

Katsurada,A., Iritani,N., Fukuda,H., Matsumura,Y., Nishimoto,N., Noguchi,T., and Tanaka,T. (1990). Effects of nutrients and hormones on transcriptional and post-transcriptional regulation of acetyl-CoA carboxylase in rat liver. *Eur J Biochem* **190,** 435-441.

Keaveney,M., Berkenstam,A., Feigenbutz,M., Vriend,G., and Stunnenberg,H.G. (1993). Residues in the Tata-Binding Protein Required to Mediate A Transcriptional Response to Retinoic Acid in Ec Cells. *Nature* **365,** 562-566.

Kim,K.H. (1997). Regulation of mammalian acetyl-coenzyme A carboxylase. *Annu Rev Nutr* **17:77-99.,** 77-99.

Kim,K.H. and Tae,H.J. (1994). Pattern and Regulation of Acetyl-CoA Carboxylase Gene Expression. *J. Nutr.* **124,** 1273S-1283.

Knudsen,J., Mandrup,S., Rasmussen,J.T., Andreasen,P.H., Poulsen,F., and Kristiansen,K. (1993). The Function of Acyl-Coa-Binding Protein (Acbp)/Diazepam Binding Inhibitor (Dbi). *Molecular and Cellular Biochemistry* **123,** 129-138.

Koo,S.H., Dutcher,A.K., and Towle,H.C. (2001). Glucose and Insulin Function through Two Distinct Transcription Factors to Stimulate Expression of Lipogenic Enzyme Genes in Liver. *J. Biol. Chem.* **276,** 9437-9445.

Lehninger,A.L. (1981). *Biochemistry the molecular basis of cell structure and function*. New York: Worth.

Li,S.J. and Cronan,J.E., Jr. (1992a). The gene encoding the biotin carboxylase subunit of Escherichia coli acetyl-CoA carboxylase. *J. Biol. Chem.* **267,** 855-863.

Li,S.J. and Cronan,J.E., Jr. (1992b). The genes encoding the two carboxyltransferase subunits of Escherichia coli acetyl-CoA carboxylase. *J. Biol. Chem.* **267,** 16841-16847.

Lin,F.T., Macdougald,O.A., Diehl,A.M., and Lane,M.D. (1993). A 30-Kda Alternative Translation Product of the Ccaat Enhancer-Binding Protein-Alpha Message - Transcriptional Activator Lacking Antimitotic Activity. *Proceedings of the National Academy of Sciences of the United States of America* **90,** 9606-9610.

Lopez-Casillas,F. and Kim,K.H. (1991). The 5' untranslated regions of acetyl-coenzyme A carboxylase mRNA provide specific translational control in vitro. *Eur J Biochem* **201,** 119-127.

Mabrouk,G.M., Helmy,I.M., Thampy,K.G., and Wakil,S.J. (1990). Acute hormonal control of acetyl-CoA carboxylase. The roles of insulin, glucagon, and epinephrine. *J. Biol. Chem.* **265,** 6330-6338.

Mahoney,C.W., Shuman,J., McKnight,S.L., Chen,H.C., and Huang,K.P. (1992). Phosphorylation of Ccaat-Enhancer Binding-Protein by Protein-Kinase-C Attenuates Site-Selective Dna-Binding. *J. Biol. Chem.* **267,** 19396-19403.

Manni,I., Caretti,G., Artuso,S., Gurtner,A., Emiliozzi,V., Sacchi,A., Mantovani,R., and Piaggio,G. (2008). Posttranslational Regulation of NF-YA Modulates NF-Y Transcriptional Activity. *Mol. Biol. Cell* E08-03.

Manni,I., Mazzaro,G., Gurtner,A., Mantovani,R., Haugwitz,U., Krause,K., Engeland,K., Sacchi,A., Soddu,S., and Piaggio,G. (2001). NF-Y Mediates the Transcriptional Inhibition of the cyclin B1, cyclin B2, and cdc25C Promoters upon Induced G2 Arrest. *J. Biol. Chem.* **276,** 5570-5576.

Mantovani,R. (1999). The molecular biology of the CCAAT-binding factor NF-Y. *Gene* **239,** 15-27.

Mantovani,R., Li,X.Y., Pessara,U., Hooft van Huisjduijnen,R., Benoist,C., and Mathis,D. (1994). Dominant negative analogs of NF-YA. *J. Biol. Chem.* **269,** 20340-20346.

Mao,J., Marcos,S., Davis,S.K., Burzlaff,J., and Seyfert,H.M. (2001). 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.* **358,** 127-135.

Mao,J., Molenaar,A.J., Wheeler,T.T., and Seyfert,H.M. (2002). STAT5 binding contributes to lactational stimulation of promoter III expressing the bovine acetyl-CoA carboxylase alpha-encoding gene in the mammary gland. *J Mol Endocrinol* **29,** 73-88.

Mao,J. and Seyfert,H.M. (2002). Promoter II of the bovine acetyl-coenzyme A carboxylase-alpha-encoding gene is widely expressed and strongly active in different cells. *Biochim Biophys Acta* **1576,** 324-329.

Mao,J., Chirala,S.S., and Wakil,S.J. (2003). Human acetyl-CoA carboxylase 1 gene: Presence of three promoters and heterogeneity at the 5'-untranslated mRNA region. *Proceedings of the National Academy of Sciences* **100,** 7515-7520.

Mao,J., DeMayo,F.J., Li,H., bu-Elheiga,L., Gu,Z., Shaikenov,T.E., Kordari,P., Chirala,S.S., Heird,W.C., and Wakil,S.J. (2006). Liver-specific deletion of acetyl-CoA carboxylase 1 reduces hepatic triglyceride accumulation without affecting glucose homeostasis. *Proceedings of the National Academy of Sciences* **103,** 8552-8557.

Martin,D.B. and Vagelos,P.R. (1962). The Mechanism of Tricarboxylic Acid Cycle Regulation of Fatty Acid Synthesis. *J. Biol. Chem.* **237,** 1787-1792.

Matsuno,F., Chowdhury,S., Gotoh,T., Iwase,K., Matsuzaki,H., Takatsuki,K., Mori,M., and Takiguchi,M. (1996). Induction of the C/EBP beta gene by dexamethasone and glucagon in primary-cultured rat hepatocytes. *Journal of Biochemistry* **119,** 524-532.

Matsuo,N., Yu-Hua,W., Sumiyoshi,H., Sakata-Takatani,K., Nagato,H., Sakai,K., Sakurai,M., and Yoshioka,H. (2003). The Transcription Factor CCAAT-binding Factor CBF/NF-Y Regulates the Proximal Promoter Activity in the Human {alpha}1(XI) Collagen Gene (COL11A1). *J. Biol. Chem.* **278,** 32763-32770.

Mauxion,F., Jamieson,C., Yoshida,M., Arai,K., and Sen,R. (1991). Comparison of Constitutive and Inducible Transcriptional Enhancement Mediated by Kappa-B-Related Sequences - Modulation of Activity in B-Cells by Human T-Cell Leukemia-Virus Type-I Tax Gene. *Proceedings of the National Academy of Sciences of the United States of America* **88,** 2141-2145.

Mcgarry,J.D., Leatherman,G.F., and Foster,D.W. (1978a). Carnitine Palmitoyltransferase I - Site of Inhibition of Hepatic Fatty-Acid Oxidation by Malonyl-Coa. *J. Biol. Chem.* **253,** 4128-4136.

Mcgarry,J.D., Takabayashi,Y., and Foster,D.W. (1978b). Role of Malonyl-Coa in Coordination of Fatty-Acid Synthesis and Oxidation in Isolated Rat Hepatocytes. *J. Biol. Chem.* **253,** 8294-8300.

McKnight,S.L. (2001). McBindall--a better name for CCAAT/enhancer binding proteins? *Cell.* **107,** 259-261.

Menendez-Hurtado,A., Santos,A., and Perez-Castillo,A. (2000). Characterization of the promoter region of the rat CCAAT/enhancer-binding protein alpha gene and regulation by thyroid hormone in rat immortalized brown adipocytes. *Endocrinology* **141,** 4164-4170.

Mick,G.J., Chun,K.Y., VanderBloomer,T.L., Fu,C.L., and McCormick,K.L. (1998). Inhibition of acetyl CoA carboxylase by GTP gamma S. *Biochimica et Biophysica Acta-Protein Structure and Molecular Enzymology* **1384,** 130-140.

Millward,C.A., Heaney,J.D., Sinasac,D.S., Chu,E.C., Bederman,I.R., Gilge,D.A., Previs,S.F., and Croniger,C.M. (2007). Mice With a Deletion in the Gene for CCAAT/Enhancer-Binding Protein {beta} Are Protected Against Diet-Induced Obesity. *Diabetes* **56,** 161-167.

Molenaar,A., Mao,J., Oden,K., and Seyfert,H.M. (2003). All Three Promoters of the Acetyl-Coenzyme A-Carboxylase {alpha}-encoding Gene Are Expressed in Mammary Epithelial Cells of Ruminants. *J. Histochem. Cytochem.* **51,** 1073-1081.

Munday,M.R., Campbell,D.G., Carling,D., and Hardie,D.G. (1988). Identification by amino acid sequencing of three major regulatory phosphorylation sites on rat acetyl-CoA carboxylase. *Eur J Biochem* **175,** 331-338.

Niehof,M., Kubicka,S., Zender,L., Manns,M.P., and Trautwein,C. (2001). Autoregulation enables different pathways to control CCAAT/enhancer binding protein beta (C/EBP beta) transcription. *Journal of Molecular Biology* **309,** 855-868.

Nikawa,J., Tanabe,T., Ogiwara,H., Shiba,T., and Numa,S. (1979). Inhibitory Effects of Long-Chain Acyl Coenzyme A Analogs on Rat-Liver Acetyl Coenzyme A Carboxylase. *Febs Letters* **102,** 223-226.

O'Callaghan,B.L., Koo,S.H., Wu,Y., Freake,H.C., and Towle,H.C. (2001). Glucose Regulation of the Acetyl-CoA Carboxylase Promoter PI in Rat Hepatocytes. *J. Biol. Chem.* **276,** 16033-16039.

Ogiwara,H., Tanabe,T., Nikawa,J., and Numa,S. (1978). Inhibition of Rat-Liver Acetyl-Coenzyme-A Carboxylase by Palmitoyl-Coenzyme-A - Formation of Equimolar Enzyme-Inhibitor Complex. *European Journal of Biochemistry* **89,** 33-41.

Pape,M.E. and Kim,K.H. (1989). Transcriptional Regulation of Acetyl Coenzyme-A Carboxylase Gene-Expression by Tumor Necrosis Factor in 30A-5 Preadipocytes. *Mol. Cell. Biol.* **9,** 974-982.

Park,S.k., Oh,S.Y., Lee,M.Y., Yoon,S., Kim,K.S., and Kim,J.w. (2004). CCAAT/Enhancer Binding Protein and Nuclear Factor-Y Regulate Adiponectin Gene Expression in Adipose Tissue. *Diabetes* **53,** 2757-2766.

Pei,D.Q. and Shih,C.H. (1991). An Attenuator Domain Is Sandwiched by 2 Distinct Transactivation Domains in the Transcription Factor-C/Ebp. *Mol. Cell. Biol.* **11,** 1480-1487.

Peng,Y., Stewart,D., Li,W., Hawkins,M., Kulak,S., Ballermann,B., and Jahroudi,N. (2007). Irradiation modulates association of NF-Y with histone-modifying cofactors PCAF and HDAC. *Oncogene* **26,** 7576-7583.

Peng,Y.W. and Jahroudi,N. (2002). The NFY transcription factor functions as a repressor and activator of the von Willebrand factor promoter. *Blood* **99,** 2408-2417.

Peng,Y. and Jahroudi,N. (2003). The NFY Transcription Factor Inhibits von Willebrand Factor Promoter Activation in Non-endothelial Cells through Recruitment of Histone Deacetylases. *J. Biol. Chem.* **278,** 8385-8394.

Pripbuus,C., Pegorier,J.P., Duee,P.H., Kohl,C., and Girard,J. (1990). Evidence That the Sensitivity of Carnitine Palmitoyltransferase-I to Inhibition by Malonyl-Coa Is An Important Site of Regulation of Hepatic Fatty-Acid Oxidation in the Fetal and Newborn Rabbit - Perinatal-Development and Effects of Pancreatic Hormones in Cultured Rabbit Hepatocytes. *Biochem. J.* **269,** 409-415.

Rahman,S.M., Schroeder-Gloeckler,J.M., Janssen,R.C., Jiang,H., Qadri,I., Maclean,K.N., and Friedman,J.E. (2007). CCAAT/enhancing binding protein beta deletion in mice attenuates inflammation, endoplasmic reticulum stress, and lipid accumulation in diet-induced nonalcoholic steatohepatitis. *Hepatology*

45, 1108-1117.

Ramji,D.P. and Foka,P. (2002). CCAAT/enhancer-binding proteins: structure, function and regulation. *Biochem. J.* **365,** 561-575.

Rasmussen,J.T., Rosendal,J., and Knudsen,J. (1993). Interaction of Acyl-Coa-Binding Protein (Acbp) on Processes for Which Acyl-Coa Is A Substrate, Product Or Inhibitor. *Biochem. J.* **292,** 907-913.

Raught,B., Liao,W.S.L., and Rosen,J.M. (1995). Developmentally and Hormonally Regulated Ccaat/Enhancer-Binding Protein Isoforms Influence Beta-Casein Gene-Expression. *Mol Endocrinol* **9,** 1223-1232.

Reddy,K.V., Serio,K.J., Hodulik,C.R., and Bigby,T.D. (2003). 5-lipoxygenase-activating protein gene expression - Key role of CCAAT/enhancer-binding proteins (C/EBP) in constitutive and tumor necrosis factor (TNF) alpha-induced expression in THP-1 cells. *J. Biol. Chem.* **278,** 13810-13818.

Robinson,G.W., Johnson,P.F., Hennighausen,L., and Sterneck,E. (1998). The C/EBP beta transcription factor regulates epithelial cell proliferation and differentiation in the mammary gland. *Genes & Development* **12,** 1907-1916.

Romier,C., Cocchiarella,F., Mantovani,R., and Moras,D. (2003). The NF-YB/NF-YC Structure Gives Insight into DNA Binding and Transcription Regulation by CCAAT Factor NF-Y. *J. Biol. Chem.* **278,** 1336-1345.

Ros,M., Lobato,M.F., Garcia-Ruiz,J.P., and Moreno,F.J. (1990). Integration of lipid metabolism in the mammary gland and adipose tissue by prolactin during lactation. *Mol Cell Biochem* **93,** 185-194.

Ryden,T.A. and Beemon,K. (1989). Avian retroviral long terminal repeats bind CCAAT/enhancer-binding protein. *Mol. Cell. Biol.* **9,** 1155-1164.

Sambrook,J., Fritsch,E.F., and Maniatis,T. (1989). *Molecular Cloing: A Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Schrem,H., Klempnauer,J., and Borlak,J. (2004). Liver-Enriched Transcription Factors in Liver Function and Development. Part II: the C/EBPs and D Site-Binding Protein in Cell Cycle Control, Carcinogenesis, Circadian Gene Regulation, Liver Regeneration, Apoptosis, and Liver-Specific Gene Regulation. *Pharmacol Rev* **56,** 291-330.

Schroeder-Gloeckler,J.M., Rahman,S.M., Janssen,R.C., Qiao,L., Shao,J., Roper,M., Fischer,S.J., Lowe,E., Orlicky,D.J., McManaman,J.L. et al. (2007). CCAAT/Enhancer-binding Protein beta Deletion Reduces Adiposity, Hepatic Steatosis, and Diabetes in Leprdb/db Mice. *J. Biol. Chem.* **282,** 15717-15729.

Tae,H.J., Luo,X., and Kim,K.H. (1994). Roles of CCAAT/enhancer-binding protein and its binding site on repression and derepression of acetyl-CoA carboxylase gene. *J. Biol. Chem.* **269,** 10475-10484.

Tae,H.J., Zhang,S., and Kim,K.H. (1995). cAMP Activation of CAAT Enhancer-binding Protein-beta Gene Expression and Promoter I of Acetyl-CoA Carboxylase. *J. Biol. Chem.* **270,** 21487-21494.

Tang,Q.Q. and Lane,M.D. (1999). Activation and centromeric localization of CCAAT/enhancer-binding proteins during the mitotic clonal expansion of adipocyte differentiation. *Genes Dev.* **13,** 2231-2241.

Thein,S.L., Lynch,J.R., Weatherall,D.J., and Wallace,R.B. (1986). Direct Detection of Hemoglobin-e with Synthetic Oligonucleotides. *Lancet* **1,** 93.

Tomita,T. and Kimura,S. (2008). Regulation of mouse Scgb3a1 gene expression by NF-Y and association of CpG methylation with its tissue-specific expression. *Bmc Molecular Biology* **9,** 5.

Trautwein,C., Caelles,C., Vandergeer,P., Hunter,T., Karin,M., and Chojkier,M. (1993a). Transactivation by Nf-Il6 Lap Is Enhanced by Phosphorylation of Its Activation Domain. *Nature* **364,** 544-547.

Trautwein,C., Vandergeer,P., Karin,M., Hunter,T., and Chojkier,M. (1993b). Transactivation of

Liver-Specific Genes by Lap Is Inhibited by Phosphorylation of Its Dna-Binding Domain. *Hepatology* **18,** A148.

Trautwein,C., Vandergeer,P., Karin,M., Hunter,T., and Chojkier,M. (1994). Protein Kinase-A and Kinase-C Site-Specific Phosphorylations of Lap (Nf-Il6) Modulate Its Binding-Affinity to Dna Recognition Elements. *Journal of Clinical Investigation* **93,** 2554-2561.

Travers,M.T., Cambot,M., Kennedy,H.T., Lenoir,G.M., Barber,M.C., and Joulin,V. (2005). Asymmetric expression of transcripts derived from the shared promoter between the divergently oriented ACACA and TADA2L genes. *Genomics* **85,** 71-84.

Travers,M.T., Vallance,A.J., Gourlay,H.T., Gill,C.A., Klein,I., Bottema,C.B., and Barber,M.C. (2001). Promoter I of the ovine acetyl-CoA carboxylase-alpha gene: an E-box motif at -114 in the proximal promoter binds upstream stimulatory factor (USF)-1 and USF-2 and acts as an insulin-response sequence in differentiating adipocytes. *Biochem. J.* **359,** 273-284.

Wang,G.L., Salisbury,E., Shi,X., Timchenko,L., Medrano,E.E., and Timchenko,N.A. (2008a). HDAC1 promotes liver proliferation in young mice via interactions with C/EBP beta. *J. Biol. Chem.* M803545200.

Wang,H., Peiris,T.H., Mowery,A., Le Lay,J., Gao,Y., and Greenbaum,L.E. (2008b). C/EBP{beta} IS A TRANSCRIPTIONAL REGULATOR OF PGC-1{alpha} IN THE REGENERATING LIVER. *Mol Endocrinol* me.

Wang,J. and Manley,J.L. (1997). Regulation of pre-mRNA splicing in metazoa. *Current Opinion in Genetics & Development* **7,** 205-211.

Wang,X. (2004). Lipid signaling. *Current Opinion in Plant Biology* **7,** 329-336.

Watkins,P.A., Tarlow,D.M., and Lane,M.D. (1977). Mechanism for Acute Control of Fatty-Acid Synthesis by Glucagon and 3'-5'-Cyclic Amp in Liver-Cell. *Proceedings of the National Academy of Sciences of the United States of America* **74,** 1497-1501.

Weinmann,A.S., Yan,P.S., Oberley,M.J., Huang,T.H.M., and Farnham,P.J. (2002). Isolating human transcription factor targets by coupling chromatin immunoprecipitation and CpG island microarray analysis. *Genes & Development* **16,** 235-244.

Weinmann,A.S., Bartley,S.M., Zhang,T., Zhang,M.Q., and Farnham,P.J. (2001). Use of Chromatin Immunoprecipitation To Clone Novel E2F Target Promoters. *Mol. Cell. Biol.* **21,** 6820-6832.

Witters,L.A., Friedman,S.A., Tipper,J.P., and Bacon,G.W. (1981). Regulation of Acetyl-Coa Carboxylase by Guanine-Nucleotides. *J. Biol. Chem.* **256,** 8573-8578.

Wu,J., Metz,C., Xu,X., Abe,R., Gibson,A.W., Edberg,J.C., Cooke,J., Xie,F., Cooper,G.S., and Kimberly,R.P. (2003). A Novel Polymorphic CAAT/Enhancer-Binding Protein {beta} Element in the FasL Gene Promoter Alters Fas Ligand Expression: A Candidate Background Gene in African American Systemic Lupus Erythematosus Patients. *J Immunol* **170,** 132-138.

Wu,Z.D., Xie,Y.H., Morrison,R.F., Bucher,N.L.R., and Farmer,S.R. (1998). PPAR gamma induces the insulin-dependent glucose transporter GLUT4 in the absence of C/EBP alpha during the conversion of 3T3 fibroblasts into adipocytes. *Journal of Clinical Investigation* **101,** 22-32.

Xu,Y., Zhou,Y.L., Gonzalez,F.J., and Snead,M.L. (2007). CCAAT/enhancer-binding protein delta (C/EBPdelta) maintains amelogenin expression in the absence of C/EBPalpha in vivo. *J Biol Chem* **282,** 29882-29889.

Xu,Y., Zhou,Y.L., Luo,W., Zhu,Q.S., Levy,D., MacDougald,O.A., and Snead,M.L. (2006). NF-Y and CCAAT/Enhancer-binding Protein {alpha} Synergistically Activate the Mouse Amelogenin Gene. *J. Biol. Chem.* **281,** 16090-16098.

Yamaoka,I., Taniguchi,Y., and Sasaki,Y. (1997). Rapid communication: nucleotide sequence of bovine C/EBP beta gene. *J. Anim Sci.* **75,** 587.

Yeh,L.A., Song,C.S., and Kim,K.H. (1981). Coenzyme-A Activation of Acetyl-Coa Carboxylase. *J. Biol. Chem.* **256,** 2289-2296.

Yeh,W.C., Cao,Z.D., Classon,M., and McKnight,S.L. (1995). Cascade Regulation of Terminal Adipocyte Differentiation by 3 Members of the C/Ebp Family of Leucine-Zipper Proteins. *Genes & Development* **9,** 168-181.

Yin,L., Zhang,Y., Charron,T., and Hillgartner,F.B. (2000). Thyroid hormone, glucagon, and medium-chain fatty acids regulate transcription initiated from promoter 1 and promoter 2 of the acetyl-CoA carboxylase-alpha gene in chick embryo hepatocytes. *Biochim Biophys Acta* **1517,** 91-99.

Yoon,J.W., Kita,Y., Frank,D.J., Majewski,R.R., Konicek,B.A., Nobrega,M.A., Jacob,H., Walterhouse,D., and Iannaccone,P. (2002). Gene Expression Profiling Leads to Identification of GLI1-binding Elements in Target Genes and a Role for Multiple Downstream Pathways in GLI1-induced Cell Transformation. *J. Biol. Chem.* **277,** 5548-5555.

Zhang,F., Lin,M., Abidi,P., Thiel,G., and Liu,J. (2003). Specific Interaction of Egr1 and c/EBP{beta} Leads to the Transcriptional Activation of the Human Low Density Lipoprotein Receptor Gene. *J. Biol. Chem.* **278,** 44246-44254.

Zhao,J.Q., Glasspool,R.M., Hoare,S.F., Bilsland,A., Szatmari,I., and Keith,W.N. (2000). Activation of telomerase rna gene promoter activity by NF-Y, Sp1, and the retinoblastoma protein and repression by Sp3. *Neoplasia* **2,** 531-539.

Zhou,Y.L. and Snead,M.L. (2000). Identification of CCAAT/Enhancer-binding Protein alpha as a Transactivator of the Mouse Amelogenin Gene. *J. Biol. Chem.* **275,** 12273-12280.

Zhu,Q.S., Qian,B., and Levy,D. (2004). CCAAT/Enhancer-binding Protein {alpha} (C/EBP{alpha}) Activates Transcription of the Human Microsomal Epoxide Hydrolase Gene (EPHX1) through the Interaction with DNA-bound NF-Y. *J. Biol. Chem.* **279,** 29902-29910.

8 Appendix

A. Isolation and identification of PIn of bovine *ACC-α*

8.1 Isolation of exon1n and PIn

8.1.1 Cloning of bovine ACC-α exon1n

Cloning exon1n (Ex1n) was achieved by a two-step procedure (Figure 35). Firstly, the 3´-terminus of Ex1n was isolated by RT-PCR; secondly, 5´-RACE was used to amplify the entire Ex1n sequence (Figure 35C).

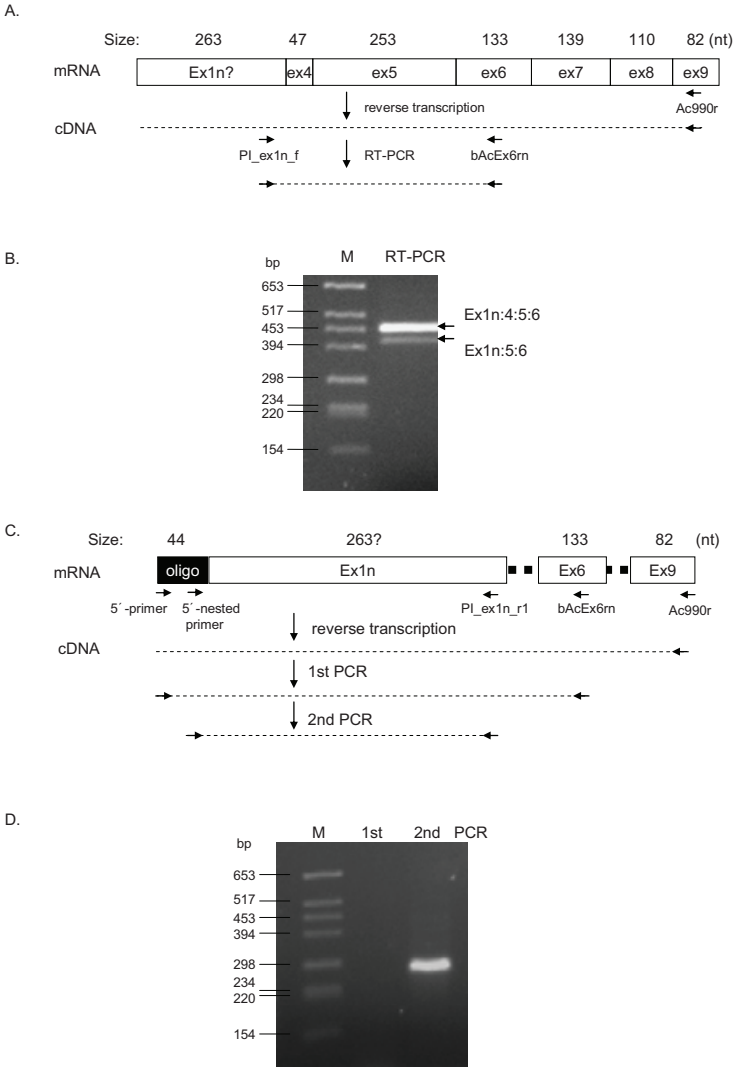


Figure 35 Identification and cloning of bovine *ACC-α* Ex1n by RT-PCR

Step 1: A) Schematic representation of the partial Ex1 cloning. The size of the exon in mRNA is listed above the respective exon. Relative primer positions are indicated by arrows. **B)** RT-PCR products were resolved on 2 % agarose gel electrophoresis.
Step 2: C) Schematic representation of the entire Ex1n cloning. The black frame represents the Gene Racer® RNA oligo (Invitrogen). The size of respective exon is listed above the according mRNA frame. Relative primer positions are indicated by arrows. **D)** PCR products of 5´-RACE were resolved on 2 % agarose gel electrophoresis.

Step 1: 2 µg of total RNA from brain was reversely transcribed into cDNA with primer Ac990r. The

cDNA was used as a template to amplify the 3´-terminus of Ex1n (Figure 35A). The primers were the reverse primer bAcEx6rn and the forward primer PI_ex1n_f, which is an Ex1n universe primer devised on the Ex1n sequences of ovine, mouse and human (Travers et al., 2005). Two bands (Figure 35B) were obtained, and subsequently cloned into pGEM-T easy vector for sequencing. The sequence alignments demonstrated the longer fragment contains a 53 bp unknown segment besides the known ex4, ex5 and partial ex6 of bovine *ACC-α*. The shorter fragment, except for the absence of ex4, contains the same exons as the longer. Further analysis indicated that the 53 bp unknown sequence is conserved in the Ex1n of ovine, mouse and human. Hence, part of the Ex1n sequence of bovine *ACC-α* was successfully achieved. The two transcripts [Ex1n:4:5:6] and [Ex1n:5:6] are derived from RNA-splicing.

Step 2: In order to clone the entire Ex1n, 5´-rapid amplification of cDNA ends (5´-RACE) was performed by nested PCR as described in the methods (Figure 35C). Primary PCR was carried out using the 5´-primer and the ex6 specific primer bACex6rn. Subsequently, the secondary PCR was performed using the 5´-nested primer and the Ex1n specific primer PI_ex1n_r1 from the partial Ex1n sequence. A dominant and specific PCR fragment was obtained (Figure 35D). This product was cloned into pGEM-T easy vector. The final clone was sequenced with universe SP6 and T7 primers. The 263 bp-Ex1n DNA sequence of bovine *ACC-α* is listed in the appendix H, and demonstrated differences from the known exons in the 5´-UTR of the ACC-α gene (Mao et al., 2001; Mao et al., 2002; Mao and Seyfert, 2002). The cDNA sequence of *ACC-α* has been deposited in EMBL Nucleotide Sequence Database with the accession number FN185962.

8.1.2 PIn is shared by *ACC-α* and *TADA2L*

In order to identify the PIn of bovine *ACC-α*, I blasted the entire Ex1n sequence against the bovine genomic sequence. Ex1n has 100 % homogeneity with the genomic contig (Sequence ID NW_001493653.2) in bovine chromosome 19 from position 50,244 to 50,506 bp (Figure 36A). However, gap presents 528 bp upstream of Ex1n. Hence, it is necessary to isolate the entire sequence of PIn.

To retrieve the sequence of the gap and to achieve the complete PIn, I analyzed our BAC478 clone which includes *ACC-α* PI and ex1 (Mao et al., 2001). *ACC-α* PI locates to the same genomic contig as mentioned above from position 6,261 to 9,179 bp as shown (Figure 36B). The sequence from BAC478 sequenced down from the vector with SP6 (346 bp) accords with 57, 639 to 57,984 bp of this contig. This region belongs to the intron2 of the TADA2L gene. Taken together, bovine *ACC-α* neighbors *TADA2L*. The finding is consistent with the previous report that *ACC-α* and *TADA2L* are located in vicinity in sheep, mouse and human. These two genes are transcribed in different orientations (Travers et al., 2005). Hence, BAC478 harbors the PIn promoter. The PIn of bovine is also an intergenic promoter between *ACC-α* and *TADA2L*. This conclusion was further confirmed by southern blot when probed with universe primer PI_ex1n_f (Figure 36C).

8.1.3 Cloning of the bovine *ACC-α* promoter PIn

Genome Walking was carried out to retrieve the genomic sequence of PIn from BAC478. Primary PCR was performed with the outer adaptor primer (AP1) and PI_ex1n_r1 using BAC478 libraries as the templates (materials and methods). The PCR product was used as a template of the Secondary PCR with the nested adaptor primer (AP2) and PI_ex1n_r2. The secondary PCR products were analyzed with 2 % agarose electrophoresis (Figure 36D). A band (~530 bp) was visible from *Stu*I libraries. Subsequently, the band was cloned into pGEM-T easy vector. Sequencing analysis indicated the partial PIn from *Stu* I libraries sequence was identical with the same genomic contig (Sequence ID NW_001493653.1) mentioned above.

Due to the low efficiency in amplification of a GC-rich promoter by genome walking, I did not get a longer sequence possibly bridging the gap. Hence, GC-rich PCR was performed with the forward primer PIn_f2 from the TADA2L gene and the reverse primer PI_ex1n_r1 from Ex1n of the ACC-α gene. Genomic DNA (genomic) and BAC478 DNA (BAC478) were used as templates separately (Figure 36D). The same size PCR product was generated from both templates. This PCR product was cloned into pGEM-T easy vector (k1336). Sequencing k1336 down from vector with SP6 showed

identity to the 5´- before the gap in the genomic contig. This region locates to the TADA2L gene. Whereas, sequencing with PIn_r2 indicated that a ~200 bp region showed identity to partial PIn sequence by genome walking of *Stu*I library. Moreover, the two sequences are overlapped. Taken together, the entire PIn sequence (1.2 kb) of the bovine ACC-α gene was achieved (Figure 37). This sequence has been deposited in EMBL Nucleotide Sequence Database with the accession number FN185963.

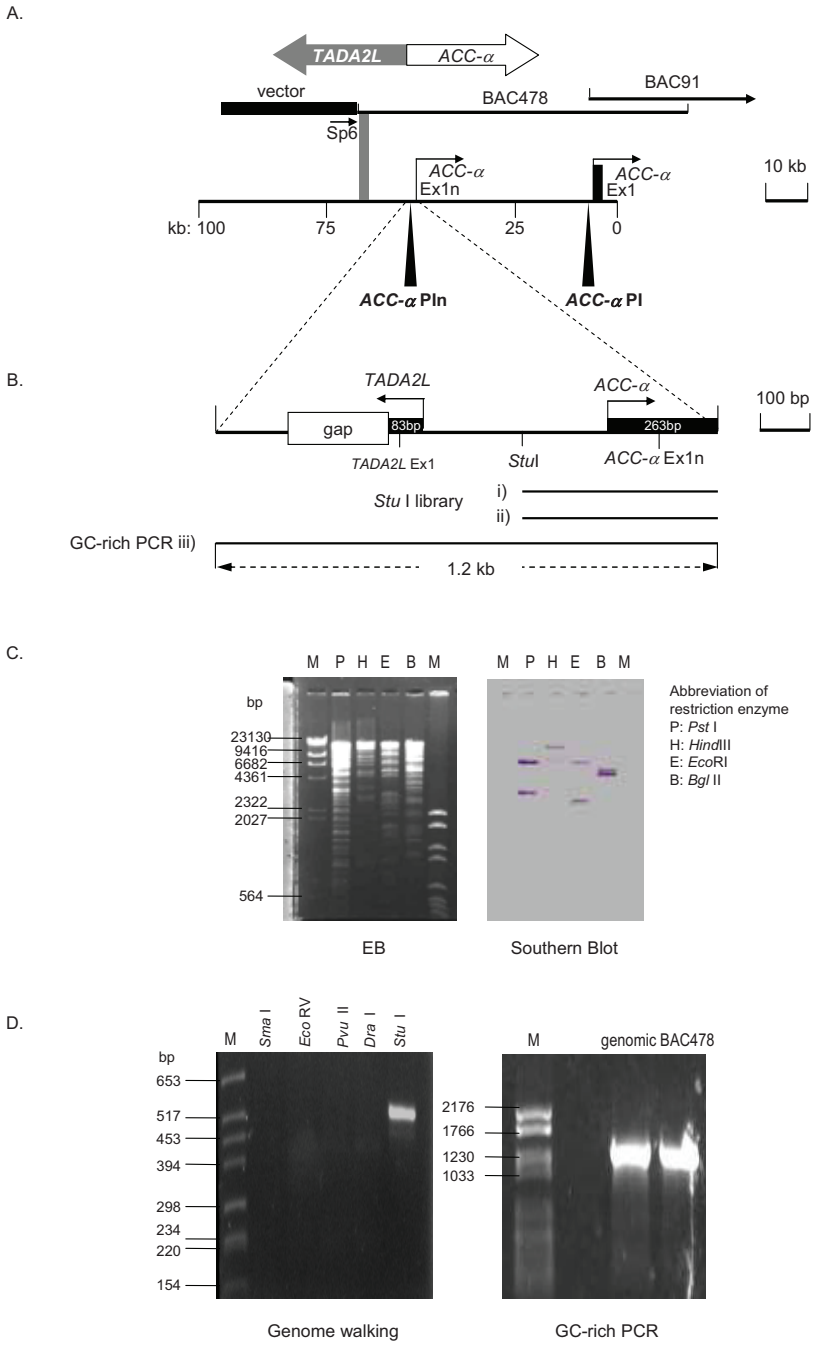


Figure 36 Isolation of PIn promoter of bovine *ACC-α*

A) The 100 kb genomic contig is from gene bank (Sequence ID NW_001493653.2). Arrows directed towards *TADA2L* and *ACC-α* represent the transcription orientation of the respective gene. The identity between 5´-flanking region of BAC478 and genomic contig was confirmed by sequencing with SP6 and alignment.
B) Genomic distributions of gap, ex1 of *TADA2L* and Ex1n of *ACC-α* are indicated.
C) Southern blot analysis of PIn on BAC478 DNA by EB staining (EB) and by Southern blot (SB) with Ex1n specific primer PI_ex1n_f.
D) Isolation of PIn based on Genome walking or GC-rich from BAC478 DNA.

8.1.4 Sequence analysis of PIn

```
-952    AGGTGCGTGA AGCAGAGGAC AGGGGCAGAC TTGGAAGAGA TTCGCAGACA AGATTCGAAG
             PIn_f2
-892    CCTCACTTGG TCCCTCATTA ATTATATGAT CTTGGGCGTC ATTTTACTGG AGACTCAGAC
                          HOXF/HOMF                CREB
-832    AACCTCAGCT CTATAGATAC GAAATGTCTA CTTGGAAGGC CTATCTGAAA ACAAGGGGTC
                                                 StuI                CREB(+)
-772    ACGTCAGTCC TTTGTTAACA ACGGGATTTC GCGCTCCGCC ACAGCAGCGA CAGGAATGCC
-712    GAGGCGGGGA CCCAGCGGAC CGTTCAGGGG GCGGGGCGCG GCCCCAGGGC TCAGTCCCGG
                                        SP1                          SP1(-)
-652    CCCGCGGCGC CCTCCCCCAC CGCGCGGCGT GGCCCGTGCC TCACGCCCGC CCGCCTGCCC
                       SP1(-)                              SP1(-)
-592    CTCTCGCCCC TGACGCCGCC GGGGCCCGCA GCCCGCCCGC AAACTGGCCG CCCTTCGCGG
                CREB  SP1                  SP1(-)
-532    TCCCCGCCTC CCACCTAGTC CCGCGAGTGG GGCTCACGGC CGGCGACAAG CTCCCGGAGC
            SP1(-)              SP1(-)       CREB
-472    ACCTCGTGCC TAACTCAGCG CGTTCCCCCT CCTCCGCGCC GAGGAAACGC CAGCCGTGCG
                                                   NF-AT (+)/NF-κB(-)
-412    CGCGCCCGAG CTTTGCGTGC GCGCTCCCTG CGGGCGGCGC AGGCGCGTGC GGGCCGCGCG
                                         SP1
-352    CCCGCCGCCT CCGCCCAGTC CCCGCCCCGG CCGTCGAAGC CCCGTCGCGG GTGGCGCGCC
                    SP1(-)
-292    TGCCGCCGCC GCCCCTGGCT CGGCCCCCGT CTGGGGCTCT CGCCGCGCGC CCGCCGCCGG
                 SP1(-)
-232    CCTCTTGCAG AGAGGCCTGA GAGGCTGGGA CTCTGAGCCG CCTGCCCGAG TTTTGAGTGG
                    StuI
-172    GACCAGTGAT TACTTTTTCC TGAGGGCGTG TTGCTGGTCG CCGCGGACCG GCCGGTGCCT
-112    GATGCCGCCG TTTTATCCGC TCCAGAGTGC GCTGAGCCGG AGCGGGCCAG TATGCCCCGC
                                                                +1(exon1n)
 -52    TTGTGTTTGT TTTCTGACAT TCCCACTCCC ACCCTTGTTA CCGCCTCGAA GGAGATCGAG

  +9    GCTAAGCGCC TGGGCCCAAG GACGGCGAGC GGGGGTAGCC TCTCGAAGCC GTATCTTGCA
 +69    GTGCCGCTGG TGGATTAATC AGACTGCCTG AATGGTTTAA GGGTACACGG GGTACGTTTG
+129    ATTCAGATGC TCCTGGAACG TCGAAGTTAT CATCTTTGGA AGGAACCATC CCCTCTTTGG
+189    GCTTCAGGGG CCCAGATTGA GGCGCAATGA TGAGAGGATG TGGTGGTCCA CTCTGATGTC
             PI_ex1n_f                                      PI_ex1n_r1
                          PI_ex1n_r2
+249    AATCTTGAGGG CTAG
```

Figure 37 The Nucleotide sequence of bovine *ACC-α* PIn including partial Ex1n with exact position of *cis*-elements

The 'A' base underlined with TSS (+1) was determined by 5´-RACE. +1 is the boundary of Ex1n (exon1n). The nucleotides underlined are the binding sites of the potential transcription factor. '-' indicated as reverse strand of the sequence. CREB: The cAMP response element binding protein.

The entire 952 bp PIn segment of the ACC-α gene was sequenced (Figure 37). No TATA box, no CCAAT box, but containing GC-rich boxes, characteristic of house-keeping promoters or tissue-specifically regulated promoters. A set of repeated *cis*-acting elements and transcription factors, including eleven Sp1 binding sites and four CREB binding sites were found in the PIn promoter by computer analysis against database. Sequence comparison indicated bovine PIn sequence (from position -611 to -1) showed 94 % identity to the ovine genomic analogue (Sequence ID AJ56446), and 86 % to that of human (NT_078100), but only 41 % to mouse ACC-α PIn (Sequence ID AJ619665). Regarding the ex1n sequences, they are highly conserved at 3´ terminal. In detail, bovine Ex1n showed 98 % identity to that of ovine (Travers et al., 2005), and 84 % to that of human (determined by human ACC1 sequence # NM_198834 and genomic sequence # NT_078100.1) and 68 % to that of murine (determined by the cDNA sequence # NM_133360.2). Taken together, ex1n is highly conserved in all species mentioned above, whereas PIn is highly conserved in only cattle, sheep and

human.

8.2 Expression profiles and functional analysis of PIn promoter

8.2.1 Bovine PIn is prominently active in the brain

In order to assess the tissue-specific activity of PIn, the abundance of *ACC-α* transcripts derived from PIn were used as criteria, to which the transcripts from PI were compared.

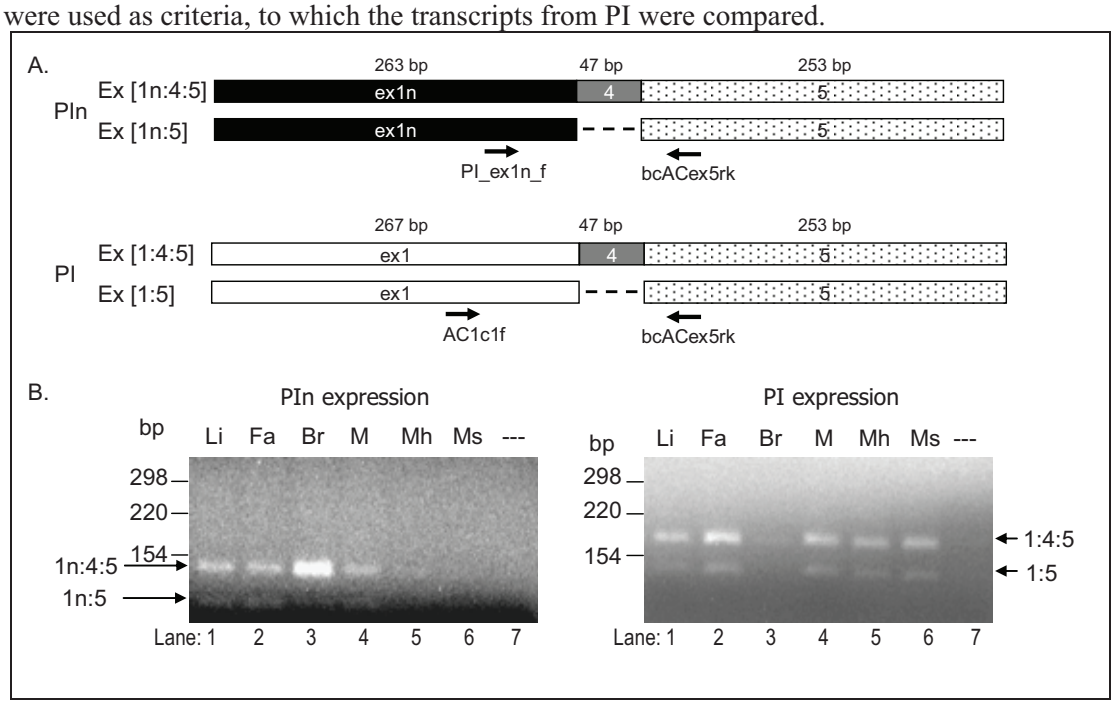


Figure 38 Relative transcriptional level of PIn and PI in bovine tissues

A) Represent of *ACC-α* transcripts derived by PIn or PI. The length of the exon was showed above on box.
B) Transcriptional profile of *ACC-α* transcripts derived from PIn or PI by RT-PCR.
Li: **li**ver, Fa: **fa**t, Br: **br**ain, M: **m**ammary gland, Mh: **h**ealthy **m**ammary gland. Ms: **s**ick **m**ammary gland. Lane1-4 of were for animal 18009. Lane5: animal 755, and Lane 6: animal 757. Lane7: PCR negative control.

To do so, I use Ac990_r as a common reverse primer for cDNA synthesis. Subsequently, PIn derived transcripts were amplified with PI_ex1n_f and the reverse primer bcACex5rk locating to exon5 of the known ACC-α transcript, and PI derived transcripts were amplified with Ac1c1f and the same reverse primer. Results indicated that PIn derived transcripts were abundant in the brain tissue, but not in the liver, fat and mammary gland (Figure 38B). In contrast, in the brain the PI transcripts was very low, but high in the liver, fat or mammary gland (Figure 38B), which is consistent with previous results (Mao et al., 2001). Interestingly, the transcript of Ex1n:4:5 is more prominent in brain, indicating a brain-specific promoter.

8.2.2 The basal activity of bovine *ACC-α* PIn is very low in HC11 cells

In oder to validate the promoter activity of PIn on *ACC-α* transcription, the PIn promoter and partial Ex1n from position -952 to position +255 was cloned into the *Sma*I site of pGL3basic. The correct insertion (PI-luc) was screened by restriction analysis. In order to examine the fine regulation of PIn, two deletions were constructed. An internal segment of 577 bp was deleted, between the *Stu*I sites at position -790 and -214 (Figure 39). The PIn promoter was truncated down to the proximal 214 bp, by deleting the distal 738 bp in the construct PIn-luc via *Kpn*I (the upstream site of insertion in pGL3basic) and the proximal *Stu*I site. Both subclones were confirmed by digestion and sequencing.

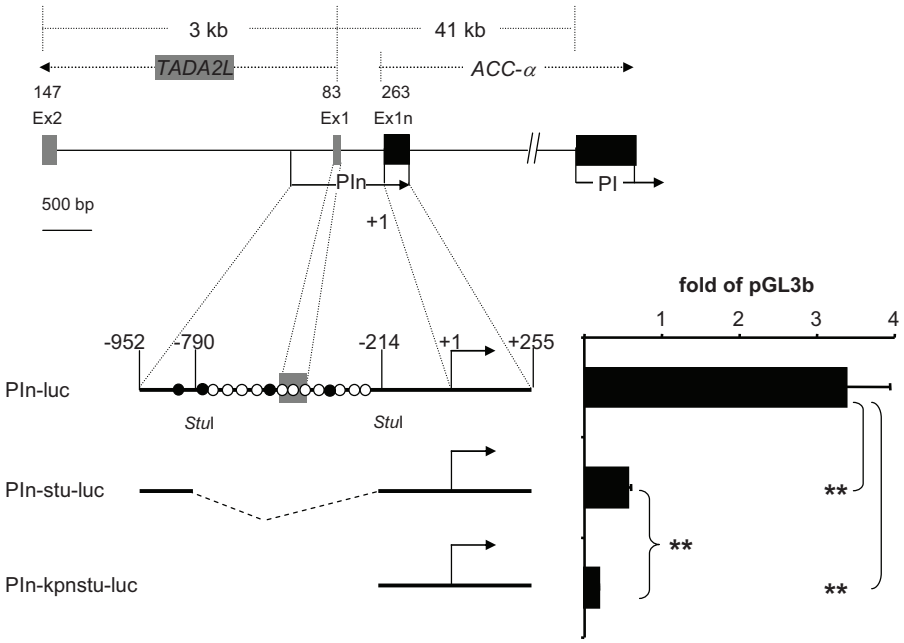


Figure 39 Mapping of cis relevant elements of PIn promoter in HC11 cells

The filled circles indicate the putative binding sites of CREBs. The empty circles in PIn-luc reporter indicate the putative binding sites of transcription factor SP1. Data shown were representative of three independent experiments assayed in triplicate. Error bar: SEM. ** $p<0.01$.

Subsequently, the wild type PIn reporter construct and the derived deletion series were transiently transfected into HC11 cells using cotransfected phRL-TK vector to normalize the transfection efficiency. Results indicated that the promoter activity of the wild type PIn is very low in HC11 cells (3.3 fold of pGL3b). The two deleted promoters dramatically decreased the promoter activity even further. Hence, the two regions of position -952 to -790 and -790 to -214 both contribute to increase the basal activity of PIn (Figure 39). Further analyses indicated that there are 11 potential SP1 and 3 potential CREB binding motifs in the region -790 to -214, and one putative CREB binding motifs in the region -952 to -790.

The data on PIn expression in the HC-11 mammary epithelial model cells show that PIn located far upstream does not encode many transcripts in these model cells.

B. Cloning of bovine transcriptional factors

Regarding Figure 7, I speculated that C/EBP and NF-Y factors may be relevant for PI regulation. Therefore, these factors are cloned as constructs for high yield expression in eukaryotes cells, to serve as valuable analytical tools.
C/EBP factors are highly conserved at the C-terminus. It is difficult to discriminate them among family members, bovine specific antibodies being unavailable. No discriminating antibodies are available for these family members of bovine. Thus, the factors were cloned to characterize their corresponding roles in specific gene regulation.

8.3 Cloning of four members (α, β, δ and ε) of bovine C/EBP family

Throughout this study, three tool vectors were used in the cloning, including pGEM-T easy (Promega), pASK-IBA43plus (IBA, Biotagnology) and pFLAG.CMV-1 (Sigma).
The pGEM-T easy vector is a normal clone vector to retrieve PCR product. The single 3´-T overhangs at the insertion site match to the 3´-A of the amplified fragments. A successful insertion interrupts the coding sequence of β-galactosidase in the pGEM-T easy vector. Recombinant clones can usually be identified by color screening on indicator plates.

pASK-IBA43plus vector is an expression vector in prokaryote such as *E.coli*. The expression cassette is under transcriptional control of the tetracycline promoter/operator. The recombinant protein has double tags. The His-tag is fused to N-terminus and the Strep-tag to C-terminus. The in-frame expression of recombinant protein can be confirmed by Western blot with antibody recognizing either/both tags.
To express the target genes in mammalian cells, the respective genes were subcloned from pASK-IBA43plus into pFLAG.CMV-1 expression vector, which is for the expression of N-terminal FLAG tagged protein in mammalian cells. The promoter-regulatory region of the human cytomegalovirus (CMV) drives transcription of FLAG-fusion constructs. Hence, the recombinant protein comprises double tags. The Flag-tag lies in N-terminus, and the Strep-tag to C-terminus (from pASK-IBA43plus). Generally, attach 5´- overhang to sequence specific oligos to introduce any restriction sites suitable for subsequent clonings. The construction map of C/EBP factors in the expression vectors is list in Figure 40.

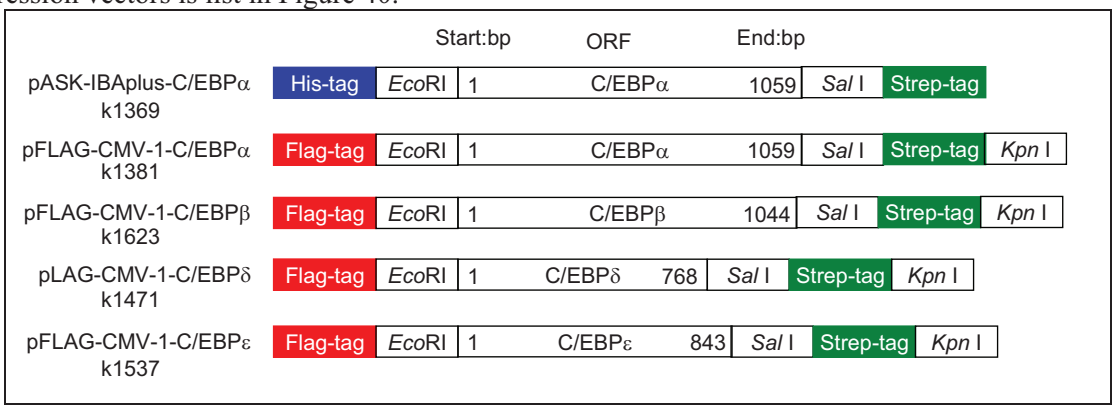


Figure 40 Schematic representations of the C/EBP expression vectors depicting the sequence of CDS and tags.

8.3.1 Cloning of bovine C/EBPα

A cDNA sequence of bovine C/EBPα was obtained from the GeneBank database (Sequence ID AY634627). This sequence was used to blast assembled genomes of *Bos taurus* at http://blast.ncbi.nlm.nih.gov/Blast.cgi. The sequence file (Sequence ID NW_001493603.2) was retrieved. Alignment indicated that the cDNA sequence of bovine C/EBPα completely matches its genomic sequence. Hence, bovine C/EBPα is intronless, like the homolog in human.

Because the GC% content in the C/EBPα CDS is 73%, GC-rich PCR was applied with the forward primer bg_c_Paf and the reverse primer bg_c_Par. Bovine genomic DNA served as a template. The PCR product was cloned into pGEM-T easy. The positive C/EBPα clone in pGEM-T easy (clone k1338) was confirmed by restriction analysis and sequencing.

Subsequently, the coding region of C/EBPα was generated by PCR. The forward and the reverse primers (CEBPa_f1, CEBPa_r2) were respectively introduced *Eco*RI and *Sal*I site. The amplified fragment was cloned into *Eco*RI/*Sal*I site of pASK-IBA43plus (clone k1369). The recombinant C/EBPα by pASK-BIBA43plus comprises the N-terminal His-tag and the C-terminal Strep-tag. The correct expression vector was validated by sequencing and Western blot for C-terminus Strep-tag.

Finally, the coding region was subcloned from pASK-IBA43plus to pFLAG.CMV-1 by PCR, for the expression in mammalian cells. The forward and reverse primers (CEBPa_f2, Strep_Kpn_r) were added the restriction sites of *Eco*RI and *Kpn*I respectively. The amplified fragment was cloned into *Eco*RI/*Kpn*I site of pFLAG.CMV-1, in which the *Sal*I site was previously disrupted. The recombinant C/EBPα will be also expressed with two tags: N-terminal Flag-tag and C-terminal Strep-tag. The correct construct was finally confirmed by sequencing and western blot with anti-flag antibody (Figure 43B).

8.3.2 Cloning of bovine C/EBPβ

Bovine C/EBPβ is also GC-rich (73 %) and intron-less (Yamaoka et al., 1997). Its sequence was retrieved from gene bank database (Sequence ID D82985).

To establish C/EBPβ expression vector, the gene was first amplified with the forward primer CE_Pbf1b and the reverse primer CEBPb_r5b by GC-rich PCR, using bovine genomic DNA as the template. The PCR product was cloned into pGEM-T easy vector. This clone (clone k1614) was confirmed by restriction analysis and sequencing.
Subsequently, the coding region of C/EBPβ was obtained by PCR. The forward and the reverse primers with added restriction sites were used (*Eco*RI: CEBPb_f7, *Sal*I: CEBPb_r5). The amplified fragment was cloned into *Eco*RI/*Sal*I site of the pFLAG.CMV-1 backbone, which was prepared by double digestion of K1381 with *Eco*RI and *Sal*I. The recombinant C/EBPβ was expressed with the same two tags as the recombinant C/EBPα in pFLAG.CMV-1. The correct construct (k1623) was finally confirmed by sequencing and western blot with anti-flag antibody (Figure 43B).

8.3.3 Cloning of bovine C/EBPδ

Regarding C/EBPδ, because there is no complete cDNA sequence, the full length cDNA of C/EBPδ was established by PCR-based rapid amplification of cDNA ends (RACE) and fusion PCR as indicated in Figure 41. GC-rich PCR was applied due to the high GC content (75%) of the coding region.
5´- and 3´- RACE experiments were conducted using total RNA derived from liver (Figure 41). Gene specific primers were designed on the CDS sequence of C/EBPδ from Gene Bank database (sequence ID NP_776692.1). The 1st PCR of 5´RACE was performed with the 5´-primer and the reverse primer CE_Pdr1, whereas the 1st PCR for 3´-RACE was conducted with the forward primer CE_Pdf1 and the 3´-primer. The 2nd PCR of 5´RACE was carried out with the 5´-nested primer and the reverse primer CE_Pdr2, while the 2nd PCR of 3´RACE was performed with the reverse primer CE_Pdf2 and the 3´-nested primer.
In order to achieve the intact cDNA fragment, fusion PCR was performed with the 2nd PCR products of 5´- and 3´- RACE. The final product was amplified by 5´- and 3´- nested primers and was cloned into pGEM-T easy vector for sequencing.

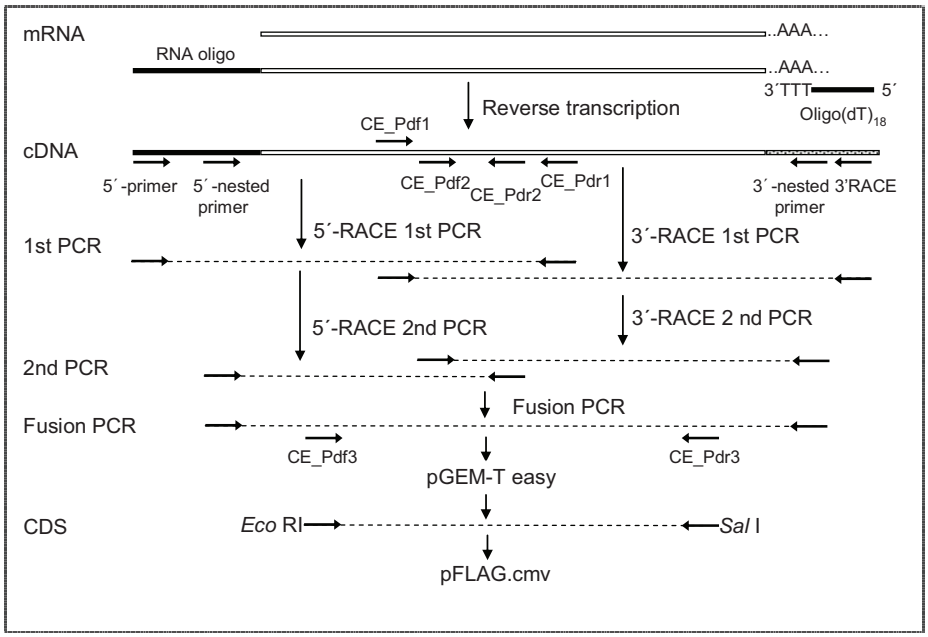


Figure 41 Cloning of C/EBPδ by 5´-, 3´- RACE and fusion PCR

1032 bp of entire cDNA sequence of C/EBPδ was successfully achieved. Alignments indicated that the cDNA sequence shows 98 % identity to the genome contig (Sequence ID NW_001493203). Hence, bovine C/EBPδ is also intron-less. Further sequence analyses demonstrated the bovine C/EBPδ cDNA is composed of 29 bp of entire 5´- untranslated region (UTR), 771 bp of an open reading frame, and 232 bp of entire 3´-UTR. This cDNA sequence has been deposited in EMBL Nucleotide Sequence Database (accession number AM980827, appendix G).

To construct the eukaryote expression vector of C/EBPδ, amplification was performed using forward and reverse primers with incorporated restriction sites (*Eco*RI: CE_Pdf3, *Sal*I: CE_Pdr3). The amplified product was cloned into *Eco*RI/*Sal*I site of the same pFLAG.CMV-1 backbone as above-mentioned for C/EBPb expression. Thus, recombinant bovine C/EBPδ also has the N-terminal FLAG-tag and the C-terminal Strep-tag. The final clone (clone k1471) was confirmed by sequencing as well as western blot with anti-FLAG antibody (Figure 43B).

8.3.4 Cloning of bovine C/EBPε

Expression vector of C/EBPε was constructed by fusion PCR as indicated in Figure 42.
First, the C/EBPε cDNA sequence (870 bp) was retrieved from Gene Bank database (Sequence ID XM_614092.2). It comprises 24 bp 5´- UTR and 846 bp of ORF. This sequence was used to blast bovine genome database. Alignment showed that it matches a genomic contig (Sequence ID NW_928095.1). Alignment also showed the genomic region of C/EBPε has an intron (Figure 42). Hence 5´- and 3´- RACEs were tried to clone the entire C/EBPε gene using total RNA derived from liver. Successful 3´-RACE fragment was obtained. However, 5´-RACE was failed to be acquired.
To clone the 5´-exon of the C/EBPε gene, GC-rich PCR was carried out with the forward primer CE_Pef5 and the reverse CE_Per5, and followed by the nested PCR using the forward primer CE_Pef3 (induced an *Eco*RI site) and the reverse primer CE_Per6 (introduced ~10 bp of 3´-exon sequence).

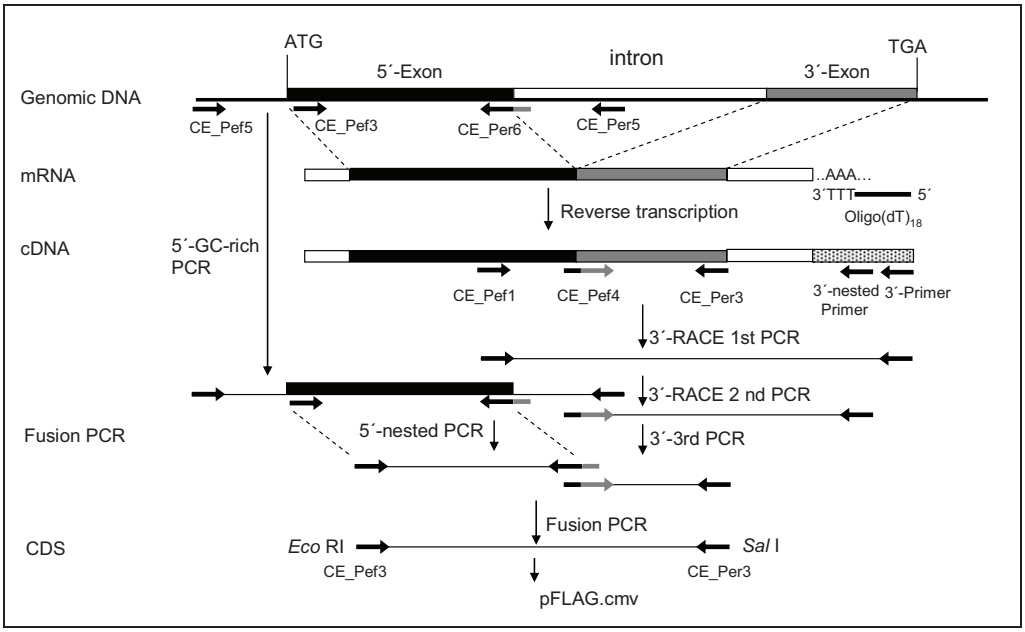


Figure 42 Cloning of C/EBPε by fusion PCR

Black box indicates the 5´- of C/EBPε expression region. Grey box represents the 3´- of C/EBPε expression region. ATG and TGA are the start and stop codon of C/EBPε. Primers CE_Per6 and CE_Pef4 include a part sequence of ex1 (black region) and a part sequence of ex2 (grey region). The other primers matter the according region as indicated.

For the 3´-RACE, primer pairs CE_Pef1 and the 3´-Primer were used in the primary PCR. The

secondary PCR was carried out with the forward primer CE_Pef4 and the 3´-nested primer. The obtained 3´-RACE product contains the entire 3´-exon and 3´-UTR of the C/EBPε gene. The fusion fragment of 3´-exon was amplified with the forward primer CE_Pef4 (introduced ~10 bp of 5´-exon sequence) and the reverse primer CE_Per3 (induced a *Sal* I site) in the 3rd PCR

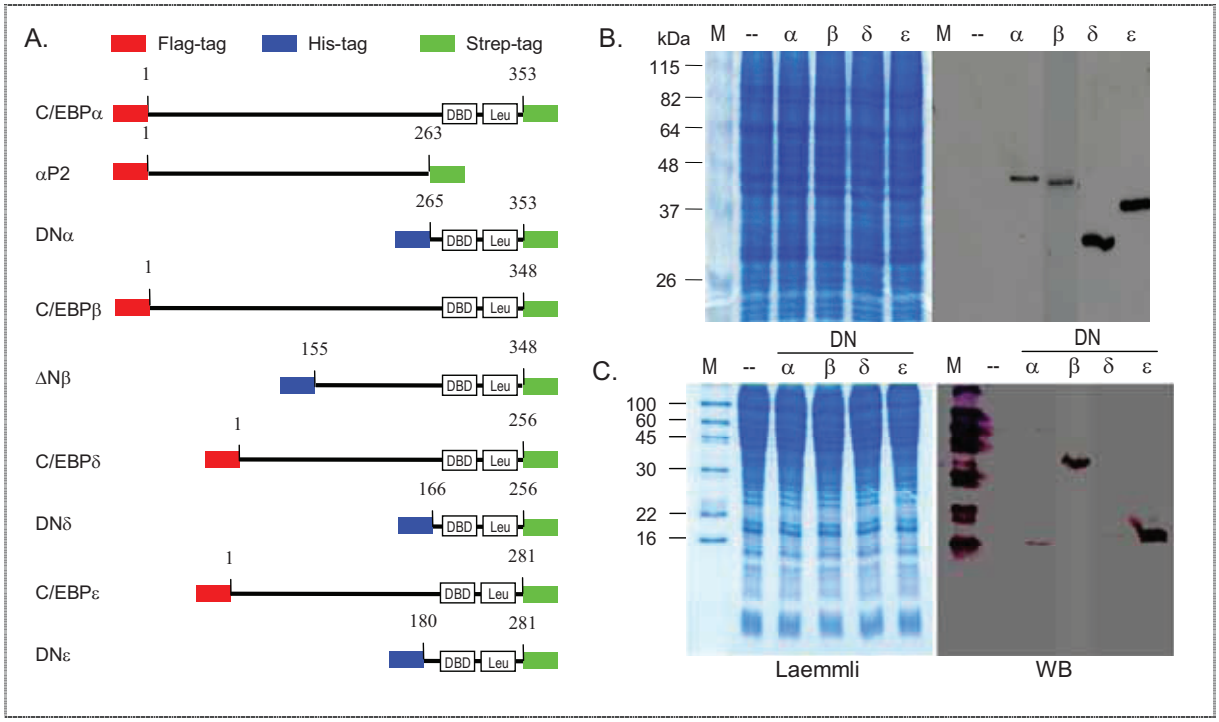


Figure 43 Expression of C/EBP and DN-C/EBP fusion proteins in HEK293 or HC11

A) Schematic representations of full length C/EBP and DN-C/EBP factors with flag, His or strep tags. αP2: ΔC-C/EBPα, in which the c-terminal of C/EBP will be deleted; DBD: DNA binding domain; Leu: leucine zipper domain. **B)** Western blot with anti-Flag-tag antibody (Sigma). M: protein ladder; α, β, δ and ε represent C/EBPα, -β, -δ and -ε. C) Western blot with Strep-tactin (IBA). M: protein ladder; DNα, -β, -δ and -ε represent DN-C/EBPα, -β, -δ and -ε.

For experiment B), 20µg of expression construct was transfected into HEK293 grown in a 10 cm plate. 48 h after transfection, cells were collected and washed with 1x cold PBS. Suspended the cells in 1x PBS containing 0.5 % (v:v) cocktail inhibitor (Sigma), and sonicated for 5 min to shear genomic DNA. 50 µg of protein was for Laemmli gel (left), 20 µg of protein for WB (right). Anti-flag recognize the FLAG-tag attached at N-terminus of C/EBP factors. The secondary antibody is anti-mouse IgG-HRP. Membrane was developed by ECL. For experiment C), 3 µg of expression construct was used to transfect HC11 in 6-Well-plate. 24 h after transfection, cells were split into 3 wells of 24-well-plate. Addtional 24 h later, cells were collected and washed with 1x cold PBS. Then were treated with in 1× lysis buffer for 30 min under RT with gentle shaking, and the protein of the lysate was determined by Bradford method. Lysate was condensed by cold acetone. 50 µg of lysate protein was loaded onto SDS-PAGE for Laemmli gel (left), and 100 µg was loaded for WB with Strep-tactin AP (right). The membrane was developed with BCIP/NBT.

In order to obtain the entire ORF segment of the C/EBPε gene, fusion PCR was performed using the 5´-exon and 3´-exon, and the product was amplified with the forward primer CE_Pef3 and the reverse primer CE_Per3. The fusion PCR product was cloned into *Eco*RI/*Sal*I site of the pFLAG.CMV-1 backbone as above-mentioned. Thus, recombinant bovine C/EBPε also has the N-terminal FLAG-tag and the C-terminal Strep-tag. The final clone (k1537) was confirmed by sequencing and western blot with anti-FLAG antibody (Figure 43B).
The results show that the C/EBP factors α, -β, -δ and -ε have been successfully cloned as expression constructs. The bovine respective factors are faithfully translated, since the N-terminal tag is expressed (Figure 43), and the C-terminally in-frame tag is recognized by antibody.

8.4 Cloning of the DN-C/EBP series

Dominant negative (DN)-C/EBP harbors the DNA binding domain and leucine zipper domain of C/EBP. DN-C/EBP may be used to detect the direct and specific binding activity of C/EBP factors, providing information about the functional importance of a C/EBP-DNA interaction. The dominant negative proteins will compete with the wild-type endogenous protein, thereby preventing the wild-type protein from activating reporter gene transcription. Inhibition of transcription with this type of dominant negative protein is often used to examine if the wild-type protein is a functional activator of the respective control region fused to a reporter gene.
The constructions of DN-C/EBPs were completed in two-step procedures. Firstly, the full length proteins were truncated by PCR or restriction digestion in pASK-IBA43plus. Secondly, the encoding fragment was cloned from pASK-IBA43plus into pcDNA3.1 by PCR. The schematic protein size was presented in Figure 43A.
In the first step, amplification was performed using the gene specific primer pairs: CEBPa_f3_IBA and CEBPa_r2 for DN-C/EBPα; the primer pairs CE_Pdf4_IBA and CE_Pdr3 for DN-C/EBPδ; the primer pairs CE_Pef7 and CE_Per3 for DN-C/EBPε. The templates were the according full length expression vectors in pFLAG.CMV-1. The amplified PCR products were cloned into *Eco*RI/*Sal*I site of pASK-IBA43plus.
The truncation of C/EBPβ was based on restriction digestion. The full length coding region of C/EBPβ was cloned from pFLAG.CMV-1(k1623) into pASK-IBA43plus by *Eco*RI and *Sal*I. The resulted clone was digested with *Eco*RI and *Sma*I, followed blunting by Klenow enzyme, and religated to result in DN-C/EBPβ in pASK-IBA43plus. All DN-C/EBP clones in pASK-IBA43plus were confirmed by sequencing and western blot by Strep-tactin (data not shown).

In the second step, the according DN-C/EBP fragments were cloned from pASK-IBA43plus into pcDNA3.1 (Invitrogen) by PCR. Two universal primers (IBA_Hind_Xho_f and IBA_Strep_BamHI_r) were used for amplification. The obtained respective DN-C/EBP fragments were cloned into *Hind*III/*Bam*HI site of pcDNA3.1+. The final DN-C/EBPs proteins have the His-tag at the N-terminus and the Strep-tag at the C-terminus (see Figure 43A). The final clones were verified by sequencing and Western blot with Strep-tactin (Figure 43C).

8.5 Cloning of the three subunits of NF-Y

All three subunits of the NF-Y family of transcription factors (NF-YA, -YB, -YC) are indispensable in NF-Y-dependent regulation. In order to know how bovine NF-Y complex regulate *ACC-α* PI activity, the three subunits were cloned. Liver RNA was used for RNA ligase-mediated 5´-RACE. cDNA was synthesized using GeneRacer® oligo-$(dT)_{18}$ as the primer. Subsequently, three PCR reactions were performed to obtain the entire coding region of NF-YA (see Figure 44A), or NF-YB (see Figure 44B), or NF-YC (see Figure 44C).

The 3rd PCR products were cloned into *Eco*RI/*Sal*I site of pASK-IBA43plus. The final constructs were confirmed by sequencing. The scheme of the NF-Y factors and their tags for expression is indicated in Figure 45A. To know if the insertions were in the proper frame in pASK-IBA43plus, western blots were performed with Strep-tactin to detect the C-terminal Strep-tag. Taken together, the cDNA fragments of NF-Y factors were successfully retrieved, and they properly cloned into the prokaryotic expression vector pASK-IBA43plus (Figure 45B). Hence, the recombinant NF-Y factors harbor His-tag in N-terminal and Strep-tag in the C-terminal.

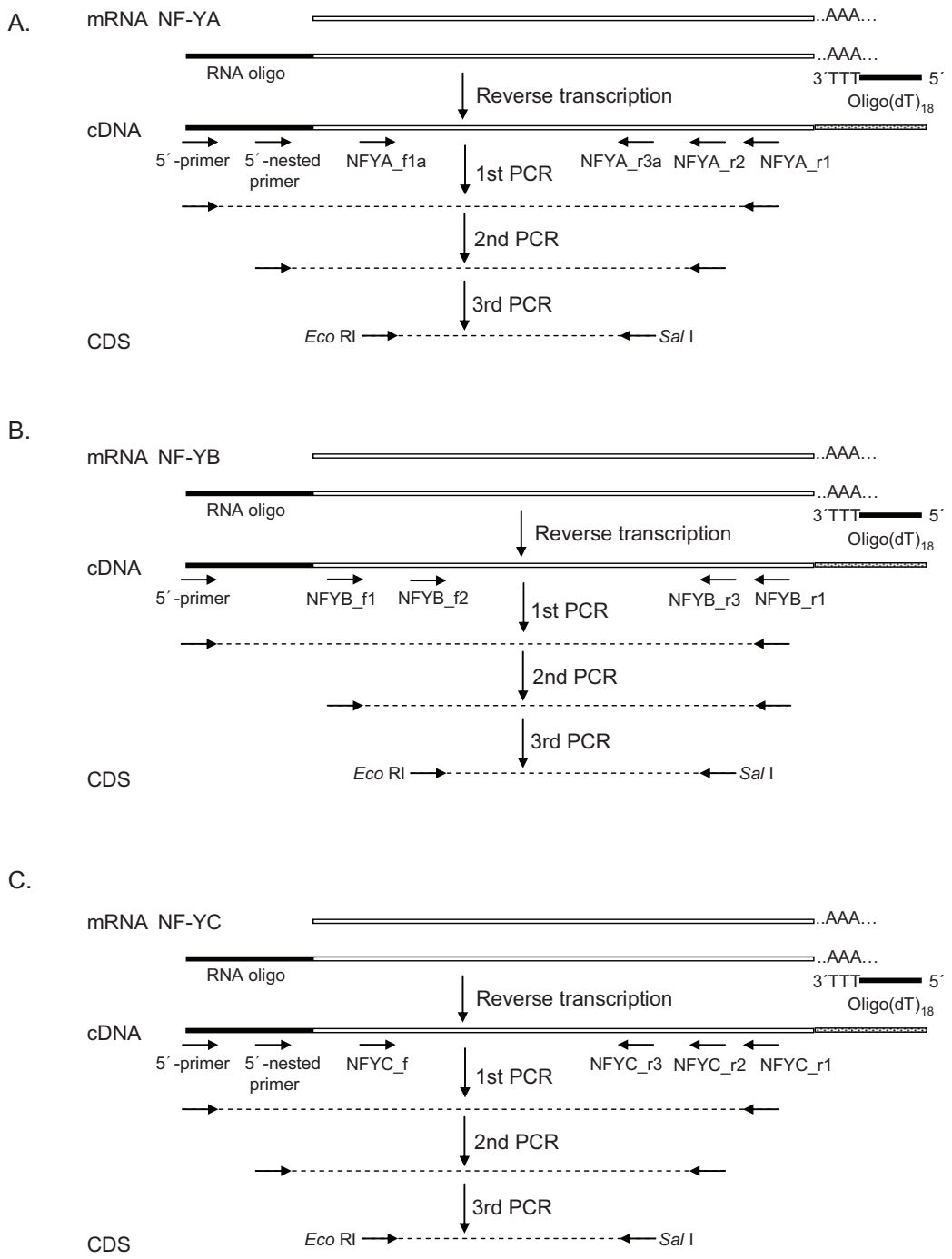


Figure 44 Schematics of cloning for NF-Y factors by 5´-RACE

A) NF-YA; B) NF-YB; C) NF-YC. The black boxes represent RNA oligo and oligo-(dT)18 from RACE kit (Invitrogen). Primers used are listed below the cDNA sequence with their names.

To express NF-Y factors in mammalian cells, the coding region from the start codon to the stop codon was amplified using primers (IBA_Hind_Xho_f and IBA_Strep_BamHI_r). The restriction sites *Hind*III and *Bam*HI were introduced into the forward primer and the reverse primer respectively. The procedures of subcloning are similar, but that for NF-YB has small changes. The amplified product of NF-YB was cloned into *Hind*III/*Bam*HI site of pcDNA3.1+. The others amplified products were blunted and cloned into *Hind*III/*Eco*RV site of pcDNA3.1+. To mutate the binding domain of NF-YA, fusion PCR was performed as materials and methods using four primers (P1: NFYA-f1a, P2: NFYA-r4m, P3: NFYA-f2m, P4: NFYA-r3a). The 2nd PCR product of NF-YA was a template. The

amplified product was blunted and subsequently cloned into *Hind*III/*Eco*RV site of pcDNA3.1+. All final clones were sequenced and confirmed by western blot (Figure 45C).

Results indicated that all factors were properly expressed in mammalian cells, because their C-terminal Strep-tags were in-frame expressed, or the specific antibody can recognize the recombinant proteins (Figure 45B,C). Three amino acids (RGE) in the DNA binding domain of NF-YA was successfully mutated into AAA, as was supported by sequencing and the western blot with anti-NF-YA (Figure 45C).

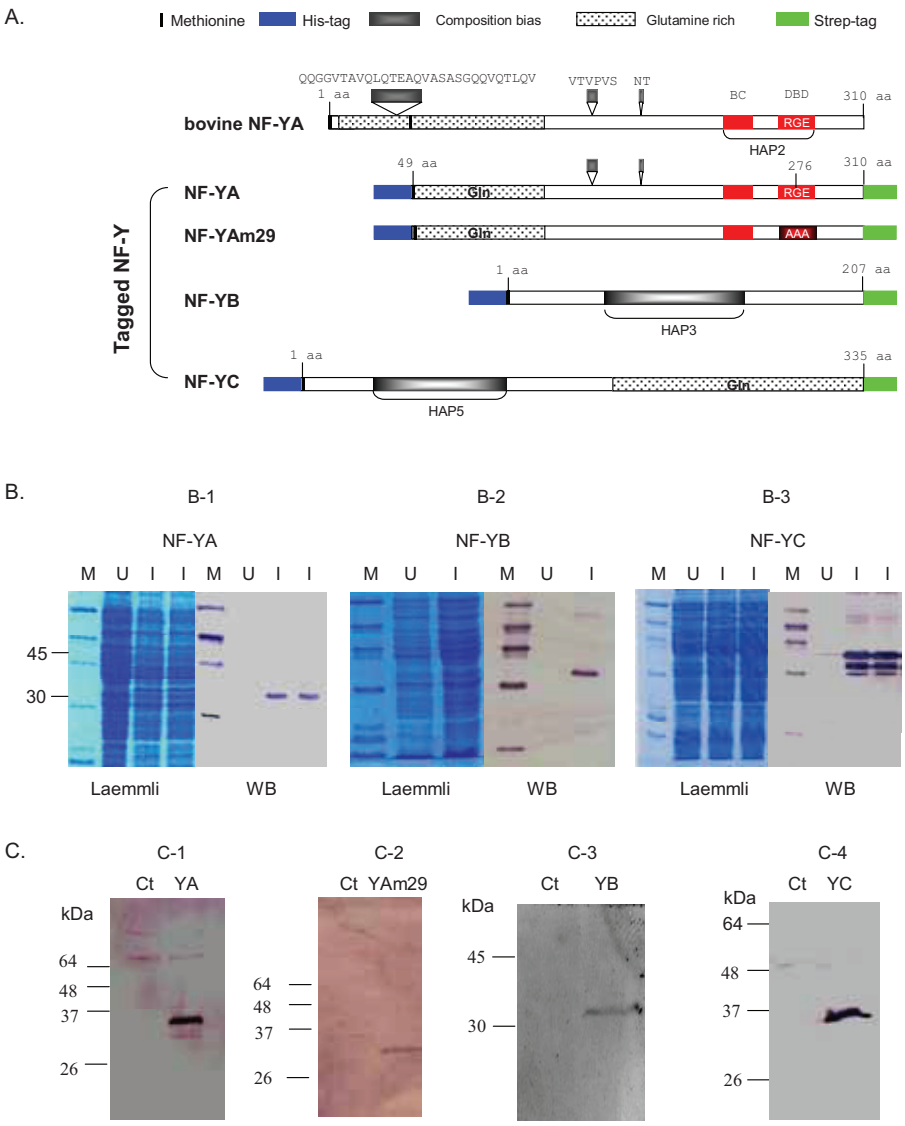


Figure 45 Expression of the recombinant NF-YA, YB or YC in *E. coli* XL1-blue and in HEK293 cells.

A Schematic representations of the tagged bovine NF-Y factors.

B The expressions of the recombinant NF-Y proteins in prokaryotes. -1): NF-YA; -2): NF-YB; -3): NF-YC; Laemmli gel (left) and Western blot (right) probed with Strep-tactin. U: uninduced, I: induced with anhydrotetracycline.

C. The expressions of the recombinant NF-Y proteins in mammalian cells. -1) NF-YA with anti-NF-YA; -2) NF-YAm29 with anti-NF-YA; -3) NF-YB with Strep-tactin; -4) NF-YC with Strep-tactin. Ct: the control cell lysate of HEK293. NF-YA: YA, YAm29, YB and YC indicated the cells were transfected with respective expression vectors.

C. Establish an ELISA

8.6 Establishment of an ELISA to measure the expression of bovine ACC-α

Measurements of ACC mass heavily rely on determination of its enzyme activity, or the mRNA concentration. The first method reflects ACC mass indirectly but not on the actual amount of enzyme. Since mRNA is regulated at different levels, the result by the second method would be affected by the decay and preparation of mRNA. Hence, it is necessary to develop ELISA, an efficient method, to directly measure the ACC mass. Moreover, the protein mass together with the enzymatic activity will give an insight into the post-translational regulation of ACC-α.

An **A**vidin-**B**iotin **C**omplex indirect sandwich ELISA (ABC-ELISA) was developed to measure the bovine ACC mass. Several procedures are required, including the preparation and purification of the recombinant ACC-α as an antigen, and the preparation and purification of specific anti-ACC-α from two different species. The recombinant technique was used to prepare the antigen for two main advantages. Firstly, specific antigen of ACC-α can be specifically and easily prepared. It is quite clear that both ACC-α and ACC-β have the conserved biotinylation sites in the domain of biotin carboxyl-carrier protein. Hence, the raised antibody based on the antigen of full length ACC-α cannot effectively differentiate between ACC-α and ACC-β. Secondly, the low molecular weight and the affinity tag (His-tag was used in the study) will facilitate the preparation and purification of the antigen. Because the molecular weight of the native ACC-α is ~265 kDa, the purification and analysis of such antigen of native ACC-α will be problematic and laborious. Hence, the cloning and expression of truncated ACC-α is necessary and important for the preparation of ACC-α antibodies.

8.6.1 Cloning and expression of truncated ACC-α

Cloning of the truncated bovine ACC-α was based on RT-PCR. The reverse transcription of liver total RNA was primed with a specific primer ACC1R:GA264. The DNA segment encoding the region of the 2nd~313th aa of the bovine ACC-α was amplified by nested PCR. The first primer pairs were bAcex5f and Ac1320_r. A secondary PCR was carried out using the forward primer (*Eco*RI: ACC_f) and 2 different reverse primers (*Sal*I: ACC_r1, *Hind*III: ACC_r2). Restriction sites were introduced by the primers. Both the PCR products were cloned into the pGEM–T easy vector for sequencing.

To construct the expression vectors of the truncated ACC-α, the coding region was retrieved from the two pGEM-T easy vectors by subcloning. Subsequently the obtained fragments were respectively cloned into *Eco*RI/*Sal*I site, or *Eco*RI/*Hind*III site of pASK-IBA43plus. The recombinant ACC-α expressed by clone K1214 only has the N-terminal His-tag, and that by clone K1215 has both tags: the N-terminal His-tag and the C-terminal Strep-tag.

Results indicated that both the recombinant *ACC-α* expression vectors with the Strep-tag and without the Strep-tag in C-terminus were highly expressed in *E.coli* BL-21. Figure 46A shows the expression of k1214 without the Strep-tag by Coomassie brilliant blue staining and Western blot with the anti-His-tag. The expression of k1215 with the Strep-tag was confirmed in Figure 46B.

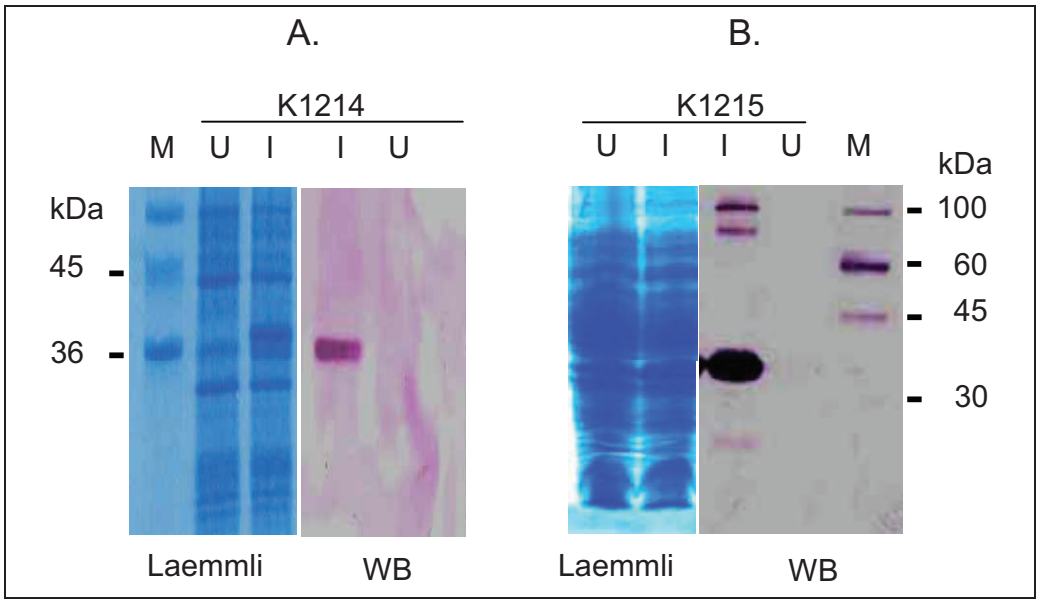


Figure 46 Performance of vectors expressing an N-terminal segment of the bovine ACC-α in *E.coli*

A) The expression of the His-tagged recombinant ACC-α by k1214 in *E.coli* BL-21.
B) The expression of the His and Strep-tagged recombinant ACC-α by k1215 in *E.coli* BL-21.
Laemmli gel (left), Western blot probed by Strep-tactin (right). U: uninduced, I: induced with anhydrotetracycline.

8.6.2 Two-step purification of antigen: His-tag affinity chromatography and gel purification

To raise specific antibodies for bovine ACC-α, the antigen was purified for immunization in the study. Furthermore, two specific antibodies were raised in the rabbit and goat to avoid the crosstalk signal interference for the indirect sandwich ELISA. The antigen was purified by two steps: affinity purification by His-tag and gel purification.

Firstly, insoluble expression of the recombinant ACC-α was confirmed and the induced cells were lyzed by sonication. The soluble and insoluble fractions were then separated by centrifugation. Results showed that the recombinant ACC-α was expressed as an inclusion body in the insoluble fraction (Figure 47A). The inclusion body of the recombinant ACC-α was soluble by sodium phosphate buffer (PBS) pH8.0 containing 8 M urea (Figure 47A).

The insoluble fraction of cell lysate from 1 L of cultured *E.coli* cells was dissolved in Equilibration buffer (PBS buffer pH8.0 with 8 M urea). Subsequently the solublized precipitate was applied to a Ni-NTA sepharose column. After washing (PBS buffer pH5.9 containing 8 M urea), the recombinant ACC-α was specifically eluted by PBS pH4.5 containing 8 M urea (Figure 47B).

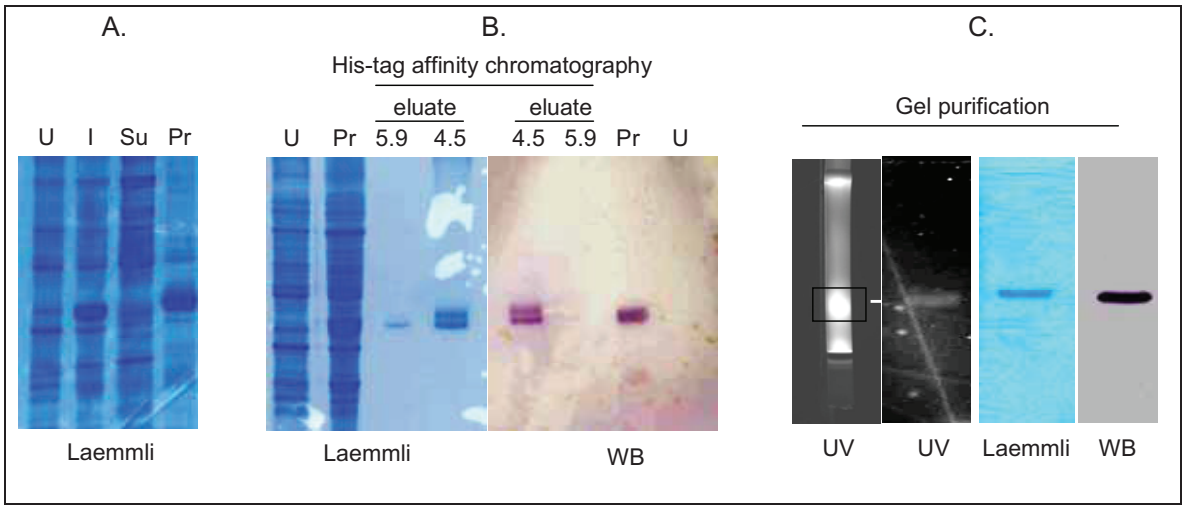


Figure 47 His-tag affinity chromatography and gel purification of the recombinant ACC-α

A) The solubility analysis of the recombinant ACC-α from k1214. Su: Soluble fraction of the induced cell lysate after centrifugation; Pr: the insoluble fraction dissolved in sodium phosphate buffer pH8.0 containing 8M urea.
B) Purification of the recombinant ACC-α by Ni-NTA sepharose. Laemmli gel (left), Western blot (right). 5.9: eluate by elution buffer pH5.9 containing 8M urea; 4.5: eluate by elution buffer pH4.5 containing 8M urea.
C) Gel purification for the recombinant ACC-α. UV, Slice1: partly labelled with Dansyl Chloride before excising from gel; UV, Slice2: The excised gel before injection was analyzed by SDS-PAGE, by staining; WB: Western blot probed with anti-His-tag.

To further purify the antigen and to visualize the desired protein bands of antigen in SDS-PAGE without staining, the purified proteins (by Ni-NTA sepharose) were solublized and partially dansylated for visibility under UV-light (Figure 47C, UV). The strongest band was excised from the gel column. Before injection, the antigen in the homogenized gel was confirmed by Coomassie brilliant blue staining and Western blot with anti-His-tag (Figure 47C, Laemmli, WB). Results showed that the technique to prepare the antigen was specific and efficient.

8.6.3 Preparation and purification of polyclonal antibodies against bovine ACC-α

The antigen was injected into animals as described in the methods and specific antisera were harvested. In order to obtain concentrated antibodies, antisera were purified by affinity chromatography whose resin is CNBr-activated sepharose coupled with antigen as described in methods. The affinity column was washed with strong detergent of 1 % triton, 8 M Urea and 2 % SDS in order to remove uncoupled antigen, because any trace amount of antigen in affinity purification will affect the accuracy of subsequent Western blots and especially ELISA.

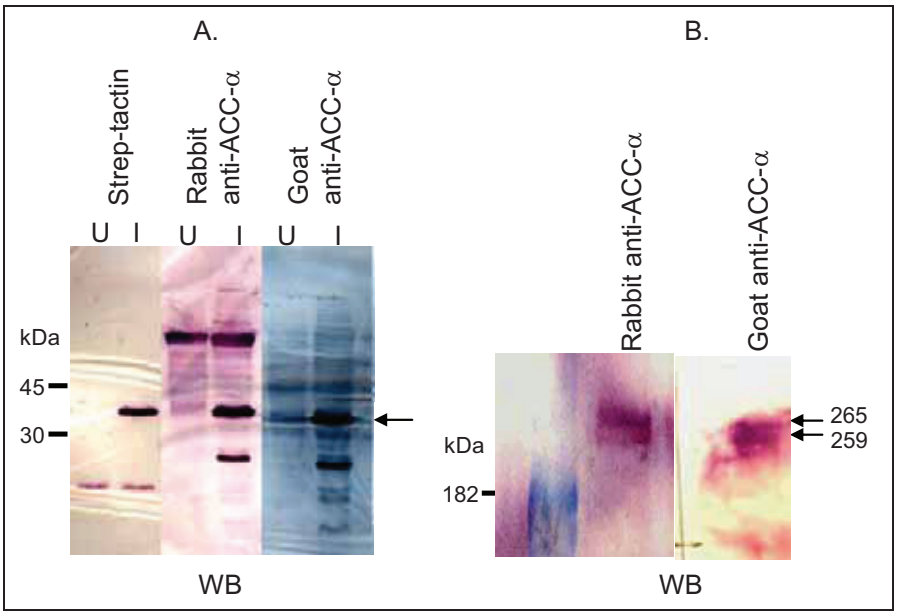


Figure 48 Evaluation of anti-bovine ACC-α antibodies origined from rabbit and goat by Western blot

A) The recombinant ACC-α expressed by K1215 as the detection subject. U: Uninduced crude protein; I: induced crude protein;
B) The whole cell lysate from bovine mammary gland as the detection subject.
Arrows indicate the expected the recombinant ACC-α and the endogenous bovine ACC-α bands.

Finally the purified antibodies from two origins were validated by Western blot. The whole cell lysates were from bovine mammary gland and a bacterial recombinant ACC-α with a strep-tag at the C-terminus. The rabbit and goat antibodies raised in the study all recognize the truncated recombinant ACC-α (Figure 48A). They also recognized the native bovine ACC-α (Figure 48B). In bovine mammary gland, two major bands may possibly be translated from various *ACC-α* transcripts containing different ATG. 265 kDa-isoform may be translated from *ACC-α* transcripts derived from promoter I and II, while the 259 kDa-isoform may be translated from mRNA molecules derived from promoter III, the mammary gland specific promoter.
Taken together, the purified antibodies against bovine ACC-α, which were raised from rabbit and goat, are suitable for ELISA assay.

8.6.4 The measurement sensitivity of ACC was greatly enhanced by ABC-ELISA

In order to examine the efficiency of the present system for ELISA, increasing concentrations (0.2, 1, 5 μg/ml) of rabbit anti-ACC-α and goat anti-ACC-α were coated onto the plates respectively. Constant amount of recombinant antigen (30 ng/well) were then added. After additional washing, secondary anti-ACC-α (2 μg/ml) from a different species was then added into the plates. Afterwards, species-specific detection antibody with HRP conjugate was added into the plate. Finally, the color reaction was performed with TMB for suitable time, and stopped with 2 M sulphuric acid. A_{450} was then measured. Blank well contains only the substrate and was stopped with the same stopping buffer. Results showed that the increasing of both coating antibodies resulted in linearly increased absorbability (Figure 49A), indicating the system conditions were feasible.

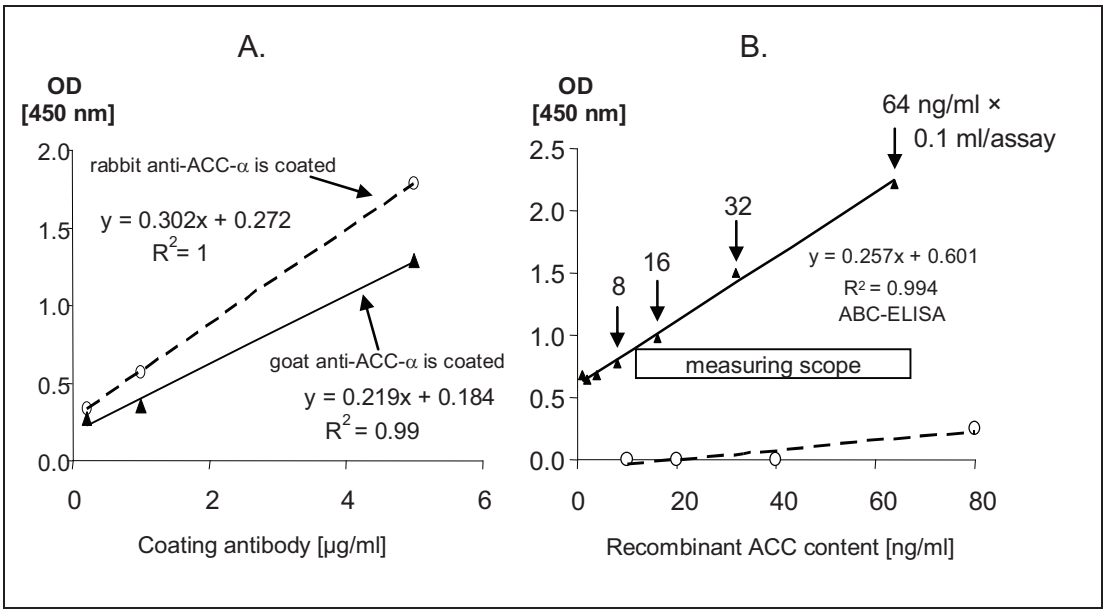


Figure 49 Establishment of standard curves for the measurement of bovine ACC-α

A) Test of antibodies by indirect sandwich ELISA. The solid line represents that goat anti-ACC-α was coated antibody. The dashed line represents the coated antibody as rabbit anti-ACC-α.
B) A standard curve allowing measuring ACC-α by ABC-ELISA. The solid line represents the standard curve by ABC-ELISA, and the variable concentrations of the recombinant ACC-α (standard) were assayed from 1 to 64 ng/ml (0.1 ng-6.4 ng/assay). The dashed line represents that by conventional ELISA, and the concentrations of the recombinant ACC-α were from 10-80 ng/ml (1-8 ng/assay).

For quantification of ACC-α, the **A**vidin-**B**iotin-**C**omplex indirect sandwich ELISA (ABC-ELISA) was performed for standard curve. 5 µg/ml of goat anti-ACC-α was coated onto the plate. Serial concentrations of recombinant purified ACC-α (1, 2, 4, 8, 16, 32, 64 ng/ml) were then added to the coated plate. The secondary anti-ACC-α antibody (rabbit anti-ACC-α) was then added (2 µg/ml). Subsequently, anti-rabbit IgG-biotin conjugate was added and was incubated with avidin-biotin mixture (Pierce). Results revealed that the slope of ABC-ELISA was 0.257 (R^2=0.994), the sensitivity being **16-32 ng/ml** (**1.6-3.2 ng/assay**, Figure 49B). In contrast, a similar experiment was performed by conventional ELISA (w/o ABC). The slope of the curve was very low (Figure 49B, dashed line). Hence, the ABC-ELISA evidently enhanced the sensitivity of measurement for ACC-α compared with the conventional ELISA (w/o ABC).

D. List of Primers

No.	Name	Sequence
72	5_Lucr	GCCTTATGCAGTTGCTCTCCA
92	BASENDf	GTACTAACATACGCTCTCCATC
101	bAcEx6rn	CAAATTCTGCTGGAGAGGCTACA
170	Acex7f	GTTCTCATTGCCAACAATGGCA
171	Ac990r	TTTCAAGAGAAGTTCTGGGAGCT
236	bAcex5f	CTCTGAGAGCTCATTTTGAAG
328	bAcex5rk	GAAGAAGGTTCATCCATTGCTTC
329	Ac5Aex6r	GCCAGACATGCTGGATCTTTG
330	Acex2f3'	CGCCGCCGCCGCCTTGAG
497	pAIBA3f	GTTATTTTACCACTCCCTATCAG
909	mActin_LCr	ACGTCACACTTCATGATGGAAT
944	PI_ec_1f	ATTCG***CATATG***CCACACGCTGCAAGGAAGAC
945	PI_ec_2f	ATTCG***CATATG***TACTGGAGGTGTCATGCAAGAG
946	PI_ec_3f	ATTCG***CATATG***AGCCAAGTTTTGGTGACTGCCA
947	PI_eb_r	ATTCG***CATATG***CCTCCCTGTCCATCACCAACT
948	ACCPf	ATCTGAATTCGATGAACCTTCTTCCTTGGCCA
949	ACCPr1	ATCT***GTCGAC***TGAGGCCTTGATCATTACTGGA
950	ACCPr2	ATCT***AAGCTT***ATGAGGCCTTGATCATTACTGGA
1000	PI_A_f4	GATGCTGAGGCTGAAACTCCAGTAC
1027	PI_ex1n_f	GCCCAGATTGAGGCGCAATGA
1048	PI_ex1n_r1	TTGACATCAGAGTGGACC
1049	PI_ex1n_r2	CTCATCATTGCGCCTCAATC
1062	BT_Fib_f1	AAACCGGACCATGACCATCC
1063	BT_Fib_r1	TGCCAGTTCATCCACACCAC
1064	BT_Fib_r2	TCTTCATGGACATCTTCTTCATTGAG
1101	PI_ec_CEBP_B_rm	CTGCCTC***ACTA***ATGACACCTCCAGTATCT
1102	PI_ec_CEBP_B_fm	GTGTCAT***TAGT***GAGGCAGCCAAGTTTTGG
1126	PIn_f2	AGGTGCGTGAAGCAGAGG
1137	bg_c_Paf	CATGCCGGGAGGACTTTAGCT
1138	bg_c_Par	CGGTGAGTTTGCGTTTCCAAG
1185	PI_ea_df	GGAAGGACTTTTGGCCACCTCATGCGAAG
1186	PI_ea_dr	GTGGCCAAAAGTCCTTCCAATGAACAC
1187	CEBPb_r5	AGTC***GTCGAC***GCAGTGGCCGGAGGAGG
1191	CEBPa_r2	AGTC***GTCGACC***GCGCAGTTGCCCATGGC
1192	CEBPa_f2	CAGT***GAATTC***AATGGAGTCGGCCGACTTCT
1193	CEBPa_r1	AGTC***GTCGAC***GCCGCCCGCACGGAGGTC
1194	CEBPa_f1	AGTC***GAATTC***ATGGAGTCGGCCGACTTCT
1221	pFLAG-f	GGGCGGTAGGCGTGTACG
1242	PI_CEBP2_r	CTTGAGTTTT
1243	PI_CEBP2_f	GGAGAATCTGAAATGCCTTGTGAAAACTCAAG
1244	PI_CEBP3_r	GGCAAGATTA
1245	PI_CEBP3_f	GGCGAACTCTGTCTTTCTTGTTGTGTAATCTTGCC
1286	PI_eA_m6r	GTCCTTCGTCTGAACACCCAGGTACCTATC
1287	PI_eA_m6f	GGTGTTCA***GAC***GAAGGACTGATGCTGAGGC
1293	NFYA-r1	TGGCGTCACCAGTCAACAC
1294	NFYA-r2	AGTGGGAAACTGTGAGGAAT
1295	NFYA-f1	CAGT***GAATTC***AATGGTCCAGGCTGTTCCTG
1296	NFYA-r3	AGTC***GTCGAC***TTAGGACACTCGGATGATCTG
1297	NFYA-f2m	GGAAG***GC***TG***C***TG***C***AGGCGGACGATTTTTCTCTCC
1298	NFYA-r4m	CCGCCT***G***CA***G***CA***GC***CTTCCGTGCCATGGCATGA
1299	NFYA-r3a	AGTC***GTCGAC***GGACACTCGGATGATCTGTG
1314	CE_Pef1	CCCAGGGCTGTGGCTGTGAA
1314	CE_Pef1	CCCAGGGCTGTGGCTGTGAA

1315	CE_Per1	GCGCACCGCGATGTTGTTC
1319	CE_Per3	AGTC***GTCGAC***GCTGCAGCCCCCCACG
1320	CE_Pdf1	AACCCGCTGCCTTCTACGA
1321	CE_Pdr1	GCATCTCCTGGTTGCGTC
1322	CE_Pdf2	GCCGACCTCTTCAACAGCAAC
1323	CE_Pdr2	CCCGCTTTGTGGTTGCTGT
1324	CE_Pdf3	CAGT***GAATTC***ATGAGCGCCGCGCTCTTCAGC
1325	CE_Pdr3	AGTC***GTCGAC***CCGCGCGTCCGCCGCC
1326	PI_ce3_rm	AGGCAAGTGGCTACAACAAGAAAGACAGAGTTCG
1327	PI_ce3_fm	CTTGTTGT***AGCCA***CTTGCCTTTCATCACCAGGA
1344	CE_Pef4	GCGTCCTCAAGGCCCCTT
1345	CE_Per4	AAGGGGCCTTGAGGACGC
1346	PI_CEBP3_f2m	GGCGAACTCTGTCTTTCTTGTTGT***AGCCA***CTTGCC
1369	CBF_Ef	AGACCGTACGTGATTGGTTAATCTCTT
1371	CBF_Er2	AAGAGATTAA
1372	FAST1_con_r	ATTTCCATGG
1373	FAST1_con_f	GCTGCCCTAAAA**TGTGTATT***CCATGGAAAT*
1374	hGLI_con_r	GAACCATCAT
1375	hGLI_con_f	TCGACTCCCGAA**GACCACCCA**CAATGATGGTTC
1395	PI_A_r6	TCAGCCTCAGC
1396	PI_A_f6	GACCAGTCCTGGGTGTTCATTGGAAGGACTGATGCTGAGGCTGA
1397	PI_A_f6mu1	GACCAGT**AAG**GGGTGTTCATTGGAAGGACTGATGCTGAGGCTGA
1398	PI_A_f6mu3	GACCAGTCCT**TTT**TGTTCATTGGAAGGACTGATGCTGAGGCTGA
1399	PI_A_f6mu4	GACCAGTCCTGGG**GAG**TCATTGGAAGGACTGATGCTGAGGCTGA
1400	PI_A_f6mu5	GACCAGTCCTGGGTGT**GAC**TTGGAAGGACTGATGCTGAGGCTGA
1401	PI_A_f6mu6	GACCAGTCCTGGGTGTTCA**GAC**GAAGGACTGATGCTGAGGCTGA
1402	PI_A_f6mu7	GACCAGTCCTGGGTGTTCATTG**TCC**GGACTGATGCTGAGGCTGA
1403	PI_A_f6mu8	GACCAGTCCTGGGTGTTCATTGGAA**TTG**CTGATGCTGAGGCTGA
1404	PI_A_f6mu9	GACCAGTCCTGGGTGTTCATTGGAAGGA**AGC**ATGCTGAGGCTGA
1408	NFYA-f1a	CAGT***GAATTC***ATGGTCCAGGCTGTTCCTG
1418	PI_ec_Cc_r2	TGGCTGCCT
1419	PI_ec_Cc_f2	GAGGTGTCATGCAAGAGGCAGCCA
1420	PI_ec_Cc_f2m	GAGGTGGAC***TAGT***CAAGGCAGCCA
1426	CE_Per6	AAGGGGCCTTGAGGACGCGCAGTGGCT
1427	CE_Pef3	CAGT***GAATTC***ATGTCCCACGGGACCTACT
1439	CE_Pef7	CAGT***GAATTC***TGCAGCCCGCTCCTCAAG
1450	STAT5/6_f	GTATTTCCCAGAAAAGGAAC
1451	STAT5/6_r	GTTCCTTTTC
1460	Pich_ba_r1	AAAAAAGACAAGAAGACCTGAT
1462	IBA_Hind_Xho_f	ATGCAAGCTTCTCGAGATGGCTAGCAGAGGATCGC
1463	IBA_Strep_BamHI_r	ATGCGGATCCTTATTATTTTTCGAACTGCGG
1486	CEBP_con_r	TGCAGATTG
1487	CEBP_con_f	TGCAGATTGCGCAATCTGCA
1488	CEBP_con_rm	TGCAGAGAC
1489	CEBP_con_fm	TGCAGAGACTAGTCTCTGCA
1490	CEBPa_f3_IBA	CAGT***GAATTC***GGCGGCGGCAAAGCCAAG
1492	CE_Pdf4_IBA	CAGT***GAATTC***GACAAGGCGGCAGGCAAG
1502	NFYB_f1	GAGGGTGAAATTGGGCAG
1503	NFYB_r1	TTAGTCAAGCATTGGGAAGC
1504	NFYB_f2	CAGT***GAATTC***ATGACAATGGATGGTGACAGT
1506	NFYC_r1	CAGGAGGCGTGGAGGTCG
1507	NFYC_r2	CTGAGGAGGGAGGCGGTG
1508	NFYC_f	CAGT***GAATTC***ATGTCCACAGAAGGAGGGTT
1509	NFYC_r3	AGTC***GTCGAC***GTCGCCGGTCACCTGGG
1550	NFYB_r3	AGTC***GTCGAC***TGAAAACTGAATTTGCTGAACAC
1557	pcDNA3,1_f	ACCCACTGCTTACTGGCTTA
1558	pcDNA3,1_r	GGGGGAGGGGCAAACAAC
1559	chip_ba_f2	TGAAAACTCAAGTGTCCACG

1570	PI_ce3_rm2	GGCAAGATTCTAGTCCAAGAAAGACAGAGTTCGCC
1571	PI_ce3_fm2	TTTCTTG***GACTAGAA***TCTTGCCTTTCATCACCAGG
1572	PI_ec_Cc_f2mu3	GAGGTGTCAT***TAT***GAGGCAGCCA
1574	PI_CEBP3_r3m	GGCAAGATTC
1575	PI_CEBP3_f3m	GGCGAACTCTGTCTTTCTTG***GACTAG***AATCTTGCC
1576	IBA_r	AGATCCGTGACGCAGTAGC
1592	CE_Pbf1b	CCGTGTTTCATGCAACGCC
1593	CEBPb_r5b	GCAGTGGCCGGAGGAGG
1595	CEBPb_f7	AGTC***GAATTC***AATGCAACGCCTGGTGGTCT
1599	bActin_LCf	GGAGAAGAGCTACGAGCTTC
1615	NFYA_f3	AGGCGGACGATTTTTCTCT
1633	PI_NFY5_Ef	CCTTGGCAGTGTCTGCA**ATTGG**ATAATAAGGAGGGAG
1634	PI_NFY5_Er	CTCCCTCC
1635	PI_NFY5_mf	GTCTG***A***AAT***AC***G***GG***AATAAGGAGGGAGGAGAATCT
1636	PI_NFY5_mr	CCTTATTCCCGTATTTCAGACACTGCCAAGGAAGG
1654	CEBPb_f8	AGTC***GAATTC***ATGCAACGCCTGGTGGTCT
1703	CEBPaLC_f	GCAAGAGCCGGGACAAGG
1704	CEBPaLC_r	TCGGGCAGCTGACGGAAG
1739	CEBPB_r9	CGACAGTTGCTCCACCTTCTTC
1740	CEBPb_f8	GACAAGCACAGCGACGAGTACA
1884	bCEBPg_ex1-2_f	GAGGGGCCAGGTACATGTGAA
1883	bCEBPg_ex2_r	TATGGATGACACTTATTC
1882	bCHOP_ex2-3_f	ACCTGAAAGCAGAGCCTGATC
1881	bCHOP_ex3-4_r	GATTCTTCCTCCTTGTTTCCAG
GA264	ACC1R	ACTCGCTGCCACCTGACGA
GA324	Ac1cx1f	GTCTGTCCATCTGTGAAGTATC
GA387	Ac1320 r	CTGTCGGAAGAGGTTAGGGAAG

E. List of Figures

Figure 1 Acetyl-CoA is the key precursor in biosynthesis of lipids. 2
Figure 2 Functional domains of bovine ACC-α. 4
Figure 3 The distribution of exons and bovine *ACC-α* promoters 5
Figure 4 PIn and PI comparisons of different species 6
Figure 5 Candidate region for the repressive elements of PI 10
Figure 6 Schematic representation of the C/EBP family members. 11
Figure 7 Putative attachment sites for transcription factors in bovine *ACC-α* PI 51
Figure 8 Expression patterns of bovine *ACC-α* in the liver and adipose tissues during different nutritional conditions by real-time PCR 52
Figure 9 Structure of PI and its deletion series in reporter constructs 54
Figure 10 Mapping of the repressive elements of PI promoter by deletion in HC-11 and HepG2 cells 55
Figure 11 The fine mapping of element A repressing the PI activity 57
Figure 12 Comprehensive EMSA analyses of the factors binding to element EA 58
Figure 13 Derepression of PI promoter activity by mutation of the NF-Y binding site in the left side of element EA 59
Figure 14 Determination of the C/EBP factors binding to the repressive element EC 61
Figure 15 Derepression of PI promoter activity by mutation of the repressive C/EBP binding site in element C 63
Figure 16 Cotransfection analyses of NF-Y and C/EBP factors on the PI promoter 64
Figure 17 Multiple alignment of the proximal region of bovine, ovine and mouse PI 66
Figure 18 The NF-Y binding site in the proximal PI is required for its basal activity and NF-Y mediated activitation 67
Figure 19 EMSA analysis of NF-Y binding site in the proximal PI promoter. 68
Figure 20 Identification of the C/EBP site in the proximal PI promoter 70
Figure 21 Identification of the members of the C/EBP family which directly bind to the proximal C/EBP binding site of PI 71
Figure 22 The functional role of C/EBPα and -ε on the proximal PI 73
Figure 23 High amounts of C/EBPβ and -ε transactivate the proximal PI in a dose-dependent manner 74
Figure 24 Mutational analysis of C/EBP binding site3 in proximal PI region by cotransfection with C/EBPα, ε and β 75
Figure 25 Inhibitional analysis for the basal activity of PI by DN-C/EBPs in HC11 cells 76
Figure 26 NF-Y and C/EBPβ synergism repressing the proximal PI 78
Figure 27 Comprehensive analyses of the interaction of NF-Y and C/EBPβ by cotransfection in HC11 cells 79
Figure 28 ChIP reveals that C/EBPβ binds to PI promoter and forms a complex with NF-Y *in vivo*. 80

Figure 29 The affinity comparison of the NF-Y proximal and distal sites of PI 82
Figure 30 The affinity comparison of the C/EBP proximal and distal sites in PI 83
Figure 31 The correlation of *ACC-α* PI activity and the ratio of NF-YA/C/EBPβ 85
Figure 32 Refeeding robustly phosphorylates bovine C/EBPβ Thr^{269} 88
Figure 33 The bipartite repressor of *ACC-α* PI 92
Figure 34 A master control unit in PI promoter of the bovine ACC-α gene 100
Figure 35 Identification and cloning of bovine *ACC-α* Ex1n by RT-PCR I
Figure 36 Isolation of PIn promoter of bovine *ACC-α* III
Figure 37 The Nucleotide sequence of bovine *ACC-α* PIn including partial Ex1n with exact position of *cis*-elements IV
Figure 38 Relative transcriptional level of PIn and PI in bovine tissues V
Figure 39 Mapping of cis relevant elements of PIn promoter in HC11 cells VI
Figure 40 Schematic representations of the C/EBP expression vectors depicting the sequence of CDS and tags. VII
Figure 41 Cloning of C/EBPδ by 5´-, 3´- RACE and fusion PCR VIII
Figure 42 Cloning of C/EBPε by fusion PCR IX
Figure 43 Expression of C/EBP and DN-C/EBP fusion proteins in HEK293 or HC11 X
Figure 44 Schematics of cloning for NF-Y factors by 5´-RACE XII
Figure 45 Expression of the recombinant NF-YA, YB or YC in *E. coli* XL1-blue and in HEK293 cells. XIII
Figure 46 Performance of vectors expressing an N-terminal segment of the bovine ACC-α in *E.coli* XV
Figure 47 His-tag affinity chromatography and gel purification of the recombinant ACC-α XVI
Figure 48 Evaluation of anti-bovine ACC-α antibodies origined from rabbit and goat by Western blot XVII
Figure 49 Establishment of standard curves for the measurement of bovine ACC-α XVIII

F. List of tables

Table 1 Reagents 17
Table 2 Antibodies 18
Table 3 Plasmid vectors 18
Table 4 DNA molecular weight ladder 24
Table 5 Sequencing primers used in this study 27
Table 6 Growth area of culture plates 34
Table 7 Labeling of hot probe 46
Table 8 mRNA copies of *ACC-α* PI, NF-YA and C/EBPβ in various tissues under different nutritional conditions 85
Table 9 mRNA expressions of relevant genes in adipose tissue before or after calving by different diets 87

G. cDNA sequence of bovine C/EBPδ

ACCGCGCTCGCCGCCGGCCCGACGCCGCCATGAGCACCGCGCTCCTCAGCCTGGACGGCCCAGCGCGCGGCGCGC
CCTGGACGGCGGAACCCGCTGCCTTCTACGAGCCCGGCCGCGCGGGGAAACCGGGTCGGGGAGCCGAGCCCGCCG
CGTCCGCCATGTACGACGACGAGAGCGCCATCGACTTCAGCGCCTACACCGACTCCATGGCCGCCGTGCCCACCC
TGGAGCTGTGCCACGATGAGCTCTTCGCCGGCCTCTTCAACAGCAACCACAAAGCGGGCGCCCTGGAGCCACTGC
CCGGGGGCCCCGCGCGCCTCGGGGGCCCTGGCCCGGCGCCGCGACCCCCCAAGCGCGAGCCCGACTGGGGCGACG
GCGACGCGCCCGGGCCCCTGCTGCCCGCGCAGGTGGCCGCGTGCGCGCAGACGGTGGTGAGCCTCGCGGCCGCTG
CGCAACCCACACCGCCCGCGTCGCCAGAGCCGCCGCGCCGGAGCCCCGCGCCCCCAGCGCCCGGGCCCGCGCGAG
ACAAGGCGGCAGGCAAGCGGGGCCCGGACCGCGGCAGCCCCGAGTACCGGCAGCGACGCGAGCGCAACGACATCG
CTGTGCGCAAGAGCCGCGACAAGGCCAAGCGACGCAACCAGGAGATGCAGCAGAAGCTGGTGGAGCTTTCGGCCG
AGAACGAGAAGCTGCAGCAGCGCGTGGAGCAGCTCACGCGGGACCTGGCCGGACTGCGGCGGTTCTTCAAGCAGC
TGCCCGGCGCGCCCTTCCTGCCCGGCGCGGGGGCGGCGGACGCGCGGTGACGCGGGGGCCGCGCGGGCTGCGGCC
GCACTGCCTGGAGCCGCCGGGCCGGACTCCCGCGCTTGGCCGGGACTCGGACAGCTGCCGCGTGGACCCTAAGTT
ACTGTGACGGCAGCTTCTCCTACATCCTACGCCTAGGATGCAGCTAAGGTACTTTCGTAAAAGAGACTTTTCCGA
CAAGCCTTTGTACTGTAGATACGAGGGAAAGACTGAGAAAAAAAAAAAAAAAAAAAA

The underlined `ATG´ is the start codon. The `TGA´ is the stop codon.
Accesssion number: AM980827

H. Exon1n Sequence of bovine ACC-α

AGATCGAGGCTAAGCGCCTGGGCCCAAGGACGGCGAGCGGGGGTGGCCTCTCGAAGCCGTATCTTGCAGTGCCGC
TGGTGGATTAATCAGACTGCCTGAATGGTTTAAGGGTACACGGGGTACGTTTGATTCAGATGCTCCTGGAACGTC
GAAATTATCATCTTTGGAAAGAACCATCCCCTCTTTGGGCTTCAGGGGCCCAGATTGAGGCGCAATGATGAGAGG
ATGTGGTGGTCCACTCTGATGTCAATCTTGAGGGCTAG

Included in the submitted gene bank sequence (accession number FN185962).

I. PIn sequence

AGGTGCGTGAAGCAGAGGACAGGGGCAGACTTGGAAGAGATTCGCAGACAAGATTCGAAGCCTCACTTGGTCCCT
CATTAATTATATGATCTTGGGCGTCATTTTACTGGAGACTCAGACAACCTCAGCTCTATAGATACGAAATGTCTA
CTTGGAAGGCCTATCTGAAAACAAGGGGTCACGTCAGTCCTTTGTTAACAACGGGATTTCGCGCTCCGCCACAGC
AGCGACAGGAATGCCGAGGCGGGGACCCAGCGGACCGTTCAGGGGGCGGGGCGCGGCCCCAGGGCTCAGTCCCGG
CCCGCGGCGCCCTCCCCCACCGCGCGGCGTGGCCCGTGCCTCACGCCCGCCCGCCTGCCCCTCTCGCCCCTGACG
CCGCCGGGGCCCGCAGCCCGCCCGCAAACTGGCCGCCCTTCGCGGTCCCCGCCTCCCACCTAGTCCCGCGAGTGG
GGCTCACGGCCGGCGACAAGCTCCCGGAGCACCTCGTGCCTAACTCAGCGCGTTCCCCCTCCTCCGCGCCGAGGA
AACGCCAGCCGTGCGCGCGCCCGAGCTTTGCGTGCGCGCTCCCTGCGGGCGGCGCAGGCGCGTGCGGGCCGCGCG
CCCGCCGCCTCCGCCCAGTCCCCGCCCCGGCCGTCGAAGCCCCGTCGCGGGTGGCGCGCCTGCCGCCGCCGCCCC
TGGCTCGGCCCCCGTCTGGGGCTCTCGCCGCGCGCCCGCCGCCGGCCTCTTGCAGAGAGGCCTGAGAGGCTGGGA
CTCTGAGCCGCCTGCCCGAGTTTTGAGTGGGACCAGTGATTACTTTTTCCTGAGGGCGTGTTGCTGGTCGCCGCG
GACCGGCCGGTGCCTGATGCCGCCGTTTTATCCGCTCCAGAGTGCGCTGAGCCGGAGCGGGCCAGTATGCCCCGC
TTGTGTTTGTTTTCTGACATTCCCACTCCCACCCTTGTTACCGCCTCGAAGG

Accession number FN185963

J. mRNA copies of relevant genes in the livers from fed and starved cows

	copies	Fed	Starved[a]	Fed/starved	P_fed vs starved
ACC-α PI	x10^3	5.22±2.22	1.00±0.15	5.24	0.06
C/EBPα	x10^5	6.89±1.11	8.84±0.94	0.78	0.23
C/EBPβ	x10^7	2.22±0.44	2.62±1.49	0.85	0.41
C/EBPδ	x10^6	5.27±0.56	6.34±0.12	0.83	0.09
C/EBPε	x10^4	1.85±0.40	3.39±0.56	0.55	0.05
C/EBPγ				1.07	0.89
C/EBPζ				0.90	0.46
NF-YA	x10^3	7.64±0.65	8.93±0.98	0.86	0.33
β-actin	x10^6	1.85±0.10	2.46±0.37	0.75	0.17

Total RNA was prepared from tissues and reverse transcribed with CE_Per1 (S1315) and oligo-dT (S792). cDNA was measured with CE_Pef1 (S1314) and CE_Per4 (S1345). Beta-actin was measured with bActin_LCf (S1599) and mActin_LCr (S909) to normalize different samples. Copy number was determined with serial concentration of standard expression vector k1357 linearized with *Eco*RI. 'a' represents the cows fed with 16 % energy of normally diets, n=6.

Abbreviation list

°C	centigrade or Grade Celsius
µg	microgram
µl	micro liter
µM	micro molar
aa	amino acid
Ab	antibody
ABC	avidin-biotin complex
ACC	acetyl-coA carboxylase
ACC	acetyl-coA carboxylase encoding gene
Acc NO.	accession number
Acetyl-CoA	acetyl-coenzyme A
ADP	adenosine diphosphate
Ag	antigen
Amp	Ampicillin
AMP-PK	AMP-dependent protein kinase
AMV	avian myeoblastosis virus
AP	alkaline phosphatase
AP1	activator protein1, or adaptor primer1
AP2	activator protein2, or adaptor primer2
ATP	adenosine triphosphate
BAC	bacterial artificial chromosome
BAT	brown adipose tissue
BC	biotin carboxylase
BCCP	biotin carboxyl carrier protein
BCIP	5-bromo-4chloro-3-indolyl-phosphate (disodium salt)
β-Gal	β-galactosidase
BiFC	bimolecular fluorescence complementary
bp	base pair
BSA	bovine serum albumin
C/EBP	CCAAT/enhance-binding protein
$CaCl_2$	Calcium chloride

cAMP	adenosine-3',5'-cyclophosphate
cAMP-PK	cAMP-dependent protein kinase
cDNA	complementary DNA
ChIP	chromatin immuno-precipitation
CHOP	C/EBP homologous protein
ChoRE	carbohydrate response element
Ci	Curie
CIAP	calf intestine alkaline phosphatase
CMV	cytomeglovirus
CoA	coenzyme A
cpm	counts per minute
CPT-1	carnitine palmitoyl transferase-I
CRE	cAMP-respose element
CREB	cAMP-respose element binding protein
CsCl	cesium chloride
CT	carboxyl transferase
Da	Dalton
DN	Dominant negative
DNA	deoxyribonucleic acid
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxy-cytidine-5'-triphosphate
DEPC	diethylprocarbonate
Dex	dexamethasone
dGTP	2'-deoxyguanosine-5'-triphosphate
DIG	digoxigenin
DMEM	dulbecco's modified Eagle' medium
DMSO	dimethyl sulfoxide
DNase	deoxyribonulase
dNTPs	deoxyribonucleoside triphosphate
ds	double-stranded (DANN)
DTT	dithiothreitol
dTTP	thymidine-5'-triphosphate
E.coli	*Escherichia coli*
EA	element A
EB	element B
EC	element C
ECL	enhanced chemiluminescent
EDTA	ethylenediaminetetraacetic acid (disodium salt)
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
EtBr	ethidium bromide
Ex	exon
Ex1n	exon1 new
FA	formaldehyde
FAS	fatty acid synthase
FCS	fetal calf serum
FP	free probe
g	gram
G	guanosine

G418	geneticin
GFP	green florescent protein
GH	growth hormone
GLI	Glioma associated oncogene
GSP	gene-specific primer
h	hour
H_2O	water
HC11	murine mammary epithelial cell
HEK293	human embryonic kidney
HEPES	4-(-2hydroxyethyl)-1-piperazineethane sulfonic acid
HepG2	Human hepatocellular liver carcinoma cell
HRP	horseradish peroxidase
IBMX	3-isobutyl-1-methylxanthine
IP	Immuno-precipitation
IPTG	isopropyl-β-D-thiogalctopyranoside
kb	kilo base pairs
kDa	kilo Dalton
KO	Knock out
L	liter
LAP	Liver activator protein
LIP	Liver inhibitory protein
LB	Luria-Bertani Medium
M	molar
MEC	mammary epithelial cells
Mg	magnesium
MG	mammary gland
mg	milligram
MgSO4	magnesium sulfate
min	minute
ml	milliliter
mM	milimolar
MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	messenger RNA
mu	mutant
NaCl	sodium chloride
NaOAC	sodium acetate
NBT	nitroblue tetrazolium
NE	Nuclear extracts
NF1	nuclear factor1
NF-κB	nuclear factor-κB
NF-Y/CBF	nuclear factor-Y
ng	nanogram
nm	nanometer
nt	nucleotide
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase Chain reaction
PEG	polyethylene glycol
Pi	phosphate
PI	promoter I

PII	promoter II
PIII	Promoter III
PIn	Promoter I new
pmol	picomole
PMSF	phenylmethylsulfonyl fluoride
poly [d(I-C)]	poly-deoly-insosinic-deoxy-cytidylic acid
PPAR	peroxisome proliferator-activated receptor
PUFA	polyunsaturated fatty acid
RACE	rapid amplification of cDNA ends
RL	*Renilla* luciferase gene
RNA	ribonucleic acid
Rnase	ribonuclease
rpm	revolution per minute
RT-PCR	reverse transcription-polymerase chain reaction
s	second
SEM	The standard error of the mean
SDS	sodium dodecyl sulfate
SRE	sterol regulatory element
SREBP-1	sterol regulatory element binding protein-1
SSC	saline-sodium citrate
STAT	signal transducers and activators of transcription
T	thymidine
T3	tri-iodothyronine
TADA2L	a component of chromatin-modifying complexes
TBE	tris-borate-EDTA
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylenediamine
Tet	tetracycline
Thr	Threonine
TK	thymidine kinase
Tm	melting temperature
TMB	3, 3, ‘5, 5’-tetramentylbenzidine
TNF	tumor necrosis factor
Tris	tris (hydroxymethyl)-amino-methane
tss	transcriptional start site
USF	upstream stimulating factor
UTR	untranslated region
UV	ultraviolet
V	voltage
WB	western blot
wt	Wild type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
X-phosphate	5-bromo-4-chloro-3-indolyl-phosphate (BCIP)
YFP	yellow fluorescence protein

Curriculum Vitae

DEMOGRAPHIC INFORMATION

Name:	Xuanming Shi
Gender:	Male
Nationality:	The People's Republic of China (P.R.China)
Birth:	Sep-17-1975, Anhui Province, P. R. China
Marital status:	Married, one son

EDUCATION INFORMATION

Ph.D. candidate (09/2005-Present)	Research Institute for the Biology of Farm Animals (FBN), Germany Major: Molecular biology
M.S (09/2002-07/2005)	Sichuan Agricultural University and Institute of Microbiology, Chinese Academy of Sciences, P.R.China Major: Biochemistry & molecular biology
B.S. (09/1995 -07/1999)	School of Life Science, Anhui University, P.R.China Major: Biochemistry

WORK EXPERIENCES

Technician 02/2002- 09/2002	Institute of Microbiology, Chinese Academy of Sciences. P.R.China
Assistant engineer 07/1999-08/2001	BBCA Biochemsitry Group Company. Anhui, P.R.China

Declaration on oath

I hereby declare that I myself prepared the work presented in this dissertation and that I did not use any other resources than indicated.

Xuanming Shi 2009