# CONTROL OF RAT H-FABP EXPRESSION – REPORTER GENE STUDIES IN L6 MYOBLASTS

By

Hui (David) Qu
Bachelor in Medicine, The Fourth Military Medical University, 1995
Master in Surgery, The Fourth Military Medical University, 2000

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

In the
Department of Biological Sciences



SIMON FRASER UNIVERSITY

Spring 2006

# APPROVAL

**Name:** **Hui (David) Qu**

**Degree:** **Master of Science**

**Title of Thesis:** **Control of rat H-FABP expression – reporter gene studies in L6 myoblasts**

**Examining Committee:**

**Chair:** **Dr. Alton Harestad**
Professor / Department of Biological Sciences

---

**Dr. Norbert Haunerland**
Senior Supervisor
Professor / Department of Biological Sciences

---

**Dr. Lynne Quarmby**
Supervisor
Associate Professor / Department of Molecular Biology and Biochemistry

---

**Dr. Carl Lowenberger**
Supervisor
Assistant Professor / Department of Biological Sciences

---

**Dr. Julian Christians**
Public Examiner
Assistant Professor / Department of Biological Sciences

**Date Defended/Approved:** March 30, 2006

# ABSTRACT

Heart fatty acid binding protein (H-FABP) is a 14.5 kDa cytosolic protein which facilitates fatty acid solubility and transport. Its concentration in different muscles reflects their fatty acid oxidation capacity, and its expression is stimulated by increased fatty acid fluxes. In order to understand the mechanism of fatty acid-dependent gene expression of the rat H-FABP gene, an everted repeat was identified in its promoter as a potential fatty acid response element, based on its similarity to a previously identified element in an invertebrate FABP gene. Through gelshift analysis specific interaction between nuclear proteins and the everted repeat was demonstrated. Cultured myoblasts were transfected with a series of luciferase reporter genes constructs, and fatty acid induced changes in reporter gene expression were measured. However, since the changes were much smaller than observed with the invertebrate promoter, the reporter gene assay proved to be too insensitive, and unambiguous conclusions were not possible.

**Keywords:**

Fatty acid response element, rat heart fatty acid binding protein, gene regulation, reporter gene assay, molecular physiology

To my wife Fang and my parents.

# ACKNOWLEDGEMENTS

It is my great pleasure to acknowledge the help of many in making this thesis possible.

My greatest thanks go to my senior supervisor Dr. Norbert Haunerland for his continuous guidance and support for my study and research. His enlightening suggestions and help on the problems I encountered made my research life easier and meaningful. And his encouragement, understanding, and patience over the past four years led me out of many hard times.

I would like to thank my committee members, Dr. Lynne Quarmby and Dr. Carl Lowenberger for their consistent guidance and valuable advice. I would like to extend my appreciation to Dr. Julian Christians for serving as my examiner.

Special thanks to Dr. Jutta Rickers-Haunerland for her kindly help and family-like love, always. My thanks to Daniela Ginta for her grate help at the beginning of my research. Sincere thanks to my labmates, Jin Wu and Linda Cui for all the support, chat and laugh.

Last but not least, special thanks to my dear wife Fang for her continuous love and encouragement, and correcting my English; to my parents for their care and love through all these years.

# TABLE OF CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

# LIST OF ABBREVIATIONS

| | |
|---|---|
| BSA | Bovine serum albumin |
| BSA 20 | BSA solution; the number indicates the concentration (μM) |
| DMEM | Dulbecco's Modified Eagle medium |
| DR | Direct repeat |
| ER | Everted repeat |
| FA | Fatty acid |
| FA 180/20 | Fatty acid/BSA solution; the first number indicates the FA concentration (μM), the second number the BSA concentration (μM) |
| FABP | Fatty acid binding protein |
| FARE | Fatty acid response element |
| iLBPs | Intracellular lipid binding proteins |
| IR | Inverted repeat |
| PBS | Phosphate buffered saline (10 mM sodium phosphate pH 7.4, 0.9 % NaCl) |
| PPAR | Peroxisome proliferator activated receptor |
| PPRE | Peroxisome proliferator response element |

# CHAPTER 1: INTRODUCTION

## 1.1 Cellular fatty acid uptake and utilization

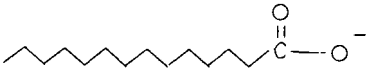
Lipids, such as neutral fats, phospholipids, and steroids, are grouped together based on the fact that they share one important trait: they have little or no affinity for water. The hydrophobic behavior of lipids comes from their molecular structures. Lipids contain fatty acids (FAs), organic acids composed of a hydrophilic carboxyl group followed by a long hydrocarbon chain of various lengths (Figure 1-1). Fatty acids, therefore, contain a hydrophilic head group and a hydrophobic tail, making them amphiphilic, i.e., both hydrophilic and lipophilic. Within lipids, the carboxy groups of fatty acids are connected, as an ester bond, to one or more of the alcohol groups of a glycerol molecule. The nonpolar hydrocarbon chains of fatty acids are the reason that lipids are hydrophobic.

Lipids play important roles in energy metabolisms and a variety of other processes. For example, lipids serve as membrane constituents, hormones, fat-soluble vitamins, thermal insulators, and biological regulators. Due to their hydrophobic properties, lipids can be stored easily as separate compartments (lipid droplets) within cells, and they contain far more energy than carbohydrates. On the other hand, the water insolubility makes their mobilization and transport more complex. Therefore carbohydrates, which are more quickly mobilized and metabolized, are generally used for immediate energy needs, such as for short bursts of muscle contraction. Lipids, however, are the preferred substrate for sustained muscle activities.

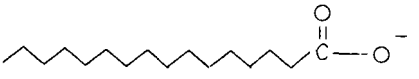
**Figure 1-1 Structures of fatty acids present in animals**

Fatty acids' name, chain length, and the number and positions of any double bonds are shown here along with their structures. For example, linoleic acid (18:2 $\Delta^{9,12}$) indicates it contains 18 carbons with 2 double bonds, which occur between 9 and 10, and 12 and 13.

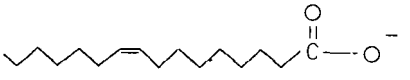
Myristic acid (14:0)



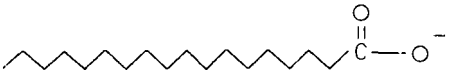
Palmitic acid (16:0)



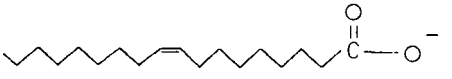
Palmitoleic acid (16:1 $\Delta^{9}$)



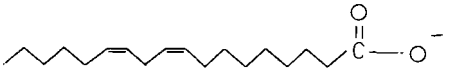
Stearic acid (18:0)



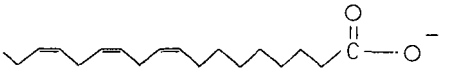
Oleic acid (18:1 $\Delta^{9}$)



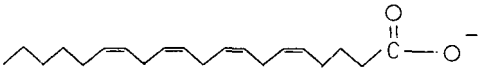
Linoleic acid (18:2 $\Delta^{9,12}$)



Linolenic acid (18:3 $\Delta^{9,12,15}$)



Arachidonic acid (20:4 $\Delta^{5,8,11,14}$)



In mammals, lipids account for between 5 % and 25 % of body weight. As much as 90 % of all lipids are present as triacylglycerols, i.e. esters of three long-chain fatty acids and one glycerol. The hydrolysis of triacylglycerol releases glycerol and free fatty acids, oxidation of which yields numerous ATP molecules. About 95 % of the energy derived from the oxidation of triacylglycerol is contained in the fatty acid molecules. Triacylglycerols are obtained from three primary sources: the diet; *de novo* biosynthesis, particularly in liver; and storage depots in adipocytes. Ingested lipids are transported from the intestine to the periphery in the form of lipoproteins (chylomicrons), which contain fatty acids and more complex lipid molecules combined with proteins. Liver-derived very low-density lipoproteins (VLDLs) are similar in structure and function, but their fatty acids are drawn from *de novo* synthesis in hepatocytes. Adipose tissue is the storage site for lipids; hormonally controlled lipases sequentially degrade triacylglycerol to glycerol and free fatty acids. Free fatty acids are commonly transported through the blood plasma in a complex with albumin, a 66 kDa, nonspecific lipid binding protein. All of these processes facilitate fatty acid delivery to cells and tissues most in need of sustained energy supply, particularly the heart.

To fuel their continuous contractions, heart muscles are able to oxidize a variety of substrates including FAs, glucose, pyruvate, lactate, ketone bodies, and several amino acids (Schaap et al., 1998). Under normal workload conditions, the energy requirement of the heart is primarily met by oxidation of long-chain fatty acids (LCFAs). Fatty acid oxidation contributes up to 70 % of the overall energy, while carbohydrates are the most important alternative fuels in healthy hearts (van der Vusse et al., 1992). Since cardiac

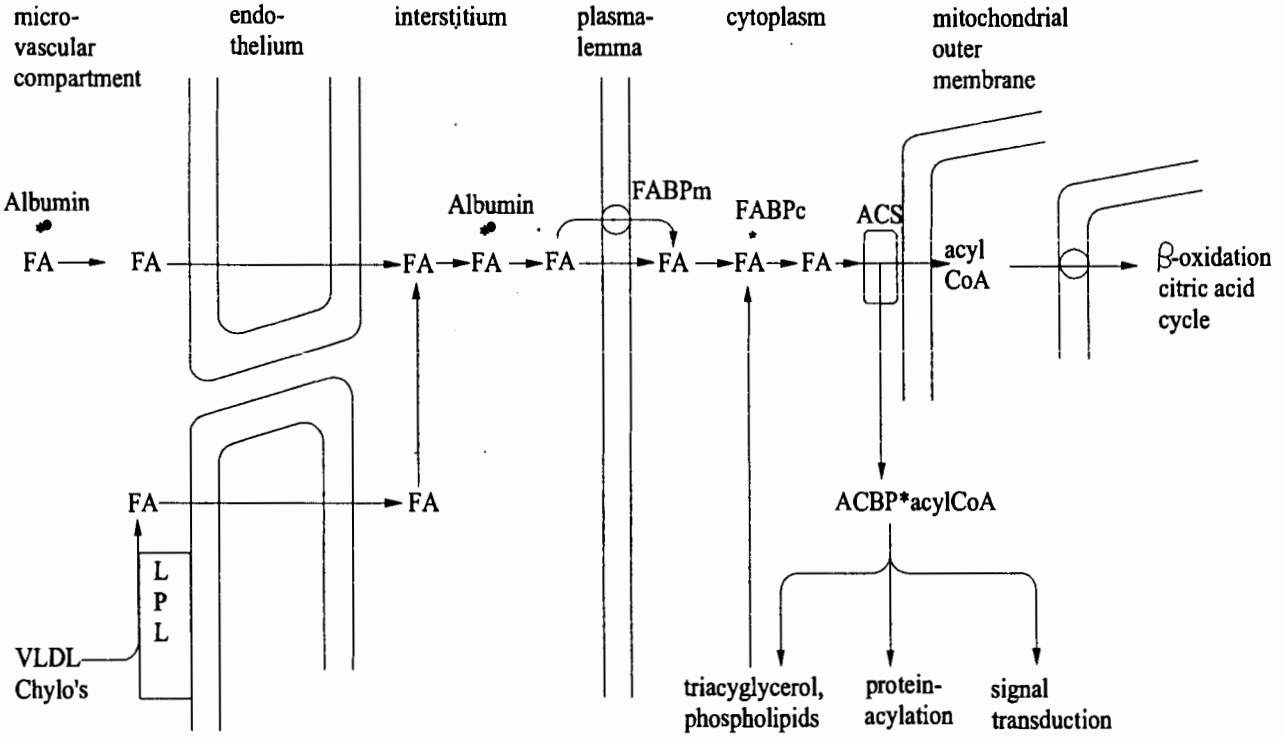
muscles are not capable of *de novo* synthesis of FA and possess limited amounts of FAs stored in the cytoplasmic compartment, they rely heavily on exogenous FAs.

These fatty acid are delivered to the heart either directly as free fatty acids, bound to serum albumin as described before, or in the form of triacylglycerols contained in the core of circulating chylomicrons and VLDLs. Prior to uptake by cardiomyocytes, fatty acids are liberated from these triacylglycerols by the catalytic process of lipoprotein lipase, an enzyme attached to specific sites at the luminal surface of the vascular endothelium. In both cases, the fatty acids are transferred through the endothelial cell and interstitial compartment. Subsequently, fatty acids have to penetrate the plasma membrane of the cardiomyocytes (sarcolemma) and cross the aqueous cytosol before they are converted into their CoA-esters at the outer mitochondrial membrane. In a carnitine-mediated process, the bulk of these fatty acid derivatives pass the inner mitochondrial membrane and are degraded via the β-oxidation pathway and citric acid cycle. The remaining part of the acyl moieties of fatty acyl-CoA are incorporated into the esterified lipid pool, mainly consisting of phospholipids and triacylglycerols (van der Vusse et al., 1992), as shown in Figure 1-2.

Due to the amphipathic nature of fatty acid molecules, both cellular membranes composed of a phospholipids bilayer, and the aqueous spaces such as the interstitial or the intracellular compartments represent constraints for FAs on their route from blood to the mitochondrial outer membrane. Circumstantial evidence shows that diffusion of FAs through the endothelial clefts is inconsequential in muscle tissue (Bassingthwaighte et al., 1989). The bulk of FAs most likely diffuses through the luminal membrane of the

**Figure 1-2 Schematic representation of long-chain fatty acid uptake and transport in organs**

FA--long-chain fatty acid; FABPc--cytoplasmic fatty acid-binding protein; FABPm--membrane-associnted fatty acid binding protein; ACS--acyl CoA synthetase; ACBP--acyl CoA-binding protein; VLDL--very low density lipoprotein; Chylo's--chylomicrons; LPL--lipoproteion lipase



endothelial cell, the endothelial cytoplasm, and subsequently through the abluminal membrane to be transmitted to albumin present in the interstitial compartment. Endothelial cytoplasmic fatty acid-binding protein, FABP, may play a role in the transport of FAs from the luminal to the abluminal endothelial membrane. Detailed information on the mechanisms underlying trans-endothelial transport of FAs in the heart and skeletal muscle is, however, lacking (van der Vusse et al., 1998).

Recent findings indicate that the sarcolemma of cardiac and skeletal muscle cells represents a major constraint for FAs bound to albumin in the interstitial compartment (Luiken et al., 2000). From the traditional view, fatty acids bind to protons of the cell membrane, and the uncharged FA molecules move across biological phospholipid bilayer membranes by passive diffusion driven by the concentration gradient (Zakim, 1996). Much of the recent evidence, however, suggests a protein-mediated transport mechanism. Several different membrane-associated proteins have been shown to bind fatty acids, and are believed to be involved in the overall transport of FAs from the interstitial compartment to the cytoplasm of the muscle cells. These membrane-associated fatty acid-binding proteins have been proven to bind fatty acids reversibly, thereby facilitating the fatty acids passage through the sarcolemma. At least three different proteins have been identified from various mammalian cells, including the plasma membrane fatty acid-binding protein (FABPpm, 40-43 kDa), fatty acid translocase (FAT/CD36, 88 kDa), and fatty acid transport protein (FATP, 63 kDa) (for a review, see van der Vusse et al., 2002). Although the relative importance of each transport mechanism is not known, it is generally believed that fatty acid uptake by the muscle involves both a passive diffusion and a protein-mediated transport mechanism.

In the cytoplasm, intracellular fatty acid-binding proteins (FABPs) appear to fulfil functions complementary to the plasma membrane associated binding proteins. These proteins may be indispensable for the bulk transport of FAs from the sarcolemma to the main intracellular site of conversion, the mitochondrial outer membrane, where fatty acyl CoA synthetase is located (Vork et al., 1997). In addition, intracellular FABPs may also act as the buffer for fatty acids, keeping the concentration of unbound fatty acids low in the cell. Large amounts of unbound free fatty acids may damage the membrane systems due to their detergent effects (Das et al., 1991), or through the enzymatic formation of lipid radicals (Yamomoto and Niki, 1990). Thus, it is widely accepted that FABPs are responsible for the intracellular transport of fatty acids in heart and skeletal muscle, for the purpose of ATP generation, notably through β-oxidation in the mitochondria.

## 1.2 Cytoplasmic fatty acid-binding proteins

### 1.2.1 Overview

More than 30 years ago, cytoplasmic fatty acid-binding proteins were first discovered in liver cells (Ockner et al., 1972). Since then, such FABPs have been found in several tissues of many different organisms including mammals, fish, birds, and insects; they are most prominent in tissues with an active fatty acid metabolism, such as heart, skeletal muscles and liver. All FABPs are members of a single multigene family of 14-16 kDa intracellular lipid binding proteins (iLBPs); members of this family include not only various FABPs, but binding prtoteins for other lipophilic ligands as well (Glatz and van der Vusse, 1996).

Initially, the individual members of this gene family were named after the tissue from which they were first isolated, or where they are found in greatest abundance (Table 1-1). It has become clear that most FABPs are not confined to a single tissue. For example, H-FABP (also referred to as cardiac FABP or muscle FABP) shows a wide distribution in heart, skeletal muscle, mammary, kidney, and lung, while liver contains both the liver and the epidermal FABP (L-FABP, E-FABP). This, however, does not necessarily mean that FAPBs are non-specifically expressed, as tissues always contain different cell types. Moreover, even some defined cells such as adipocytes express more than one FABP-type (Bernlohr, 1997).

FABPs contain an average of 135 amino acids, and the overall sequence identity between the different tissue-types ranges from 20 % to 70 %. Despite the considerable differences in their primary structure, the tertiary structure of all FABPs is highly conserved. The common structural feature is a 10-stranded β-barrel, made of two orthogonal antiparallel 5-stranded sheets that form the "clam"-shaped binding cavity, giving the protein a pocket into which a fatty acid is inserted (Banaszak et al., 1994). The opening of this clam, considered the portal domain, is framed on one side with the N-terminal helix-turn-helix domain, a further common structural motif of all iLBPs (Figure 1-3). In the binding pocket, the negatively charged carboxyl group of the fatty acid is generally buried inside the cavity for electrostatic interaction with one or two arginine residues. Hydrogen bonds with a tyrosine- or serine-OH and an ordered water molecule further stabilize the charge (Zanotti et al., 1992). In contrast, the hydrocarbon tail interacts with the side chains of hydrophobic amino acids facing the binding cavity. Fatty acid binding occurs always in a 1:1 stoichiometry, except in the case of liver FABP,

where it appears that two fatty acid molecules can be bound by one protein (Haunerland et al., 1984; Coe and Bernlohr, 1998).

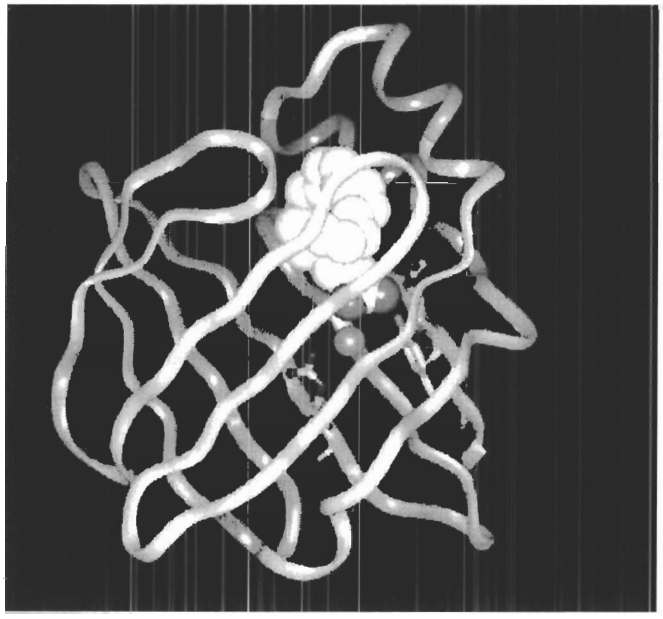
As mentioned before, the presence of cytoplasmic FABPs certainly increases the solubility of fatty acids and may thus protect cellular structures from damage by an excess of these amphipathic molecules. By the same token FABPs create a larger cytosolic fatty acid pool that enhances substrate availability to fatty acid metabolizing enzymes. FABPs enhance uptake of fatty acids into the cells by increasing their concentration gradient, which is approached through the minimization of unbound fatty acid in the cells (Weisiger, 1996). Consistent with these general roles in fatty acid transport and metabolism, the FABP content in most cells is generally proportional to the cells' rate of fatty acid metabolism. Furthermore, increased fatty acid exposure leads to a marked increase in FABP expression. It is also believed that FABPs are involved in fatty acid signaling and gene regulation, so as to affect cellular growth and differentiation (for a recent review, see Haunerland and Spener, 2004).

**Table 1-1 FABPs as members of the intracellular lipid binding protein family**

| iLBP-type | Alternatives names | Gene name | Expression | Ligands |
|---|---|---|---|---|
| *Subfamily I* | | | | |
| CRBP I, II, III and IV | | | Ubiquitous in mammalian cells | Retinol |
| CRABP I and II | | | Ubiquitous in mammalian cells | Retinoic acids |
| *Subfamily II* | | | | |
| L-FABP (liver) | | *Fabp1* | Liver, intestine, kidney, lung, pancreas | Long-chain fatty acids, acyl-CoAs, heme bile acids |
| I-BABP (intestinal) | ILBP, Gastrotropin | *Fabp6* | Ileum | Long-chain fatty acids |
| Lb-FABP (liver basic) | L-FABP | *Fabp10* | Fish and bird liver | Long-chain fatty acids |
| *Subfamily III* | | | | |
| I-FABP (intestinal) | | *Fabp2* | Intestine, liver | Long-chain fatty acids |
| *Subfamily IV* | | | | |
| H-FABP (heart) | M-FABP (muscle), MDGI | *Fabp3* | Heart, skeletal muscle, kidney, lung, mammary, placenta, testis, ovary, stomach | Long-chain fatty acids |
| A-FABP (adipocytes) | ALBP, aP2 | *Fabp4* | Adipose tissue | Long-chain fatty acids |
| E-FABP (epidermal) | KLBP, mal1 | *Fabp5* | Skin, adipose tissue, lung, brain, heart, skeletal muscle, testis, retina, kidney | Long-chain fatty acids |
| B-FABP (brain) | R-FABP | *Fabp7* | Brain, glia cell, retina | Long-chain fatty acids, docosahexacenoic acid |
| M-FABP (myelin) | mP2, Myelin P2 | *Fabp8* | Brain and Schwann cells | Long-chain fatty acids |
| T-FABP (testis) | | *Fabp9* | Testis | Long-chain fatty acids |

**Figure 1-3** **Structure of mammalian heart fatty acid binding protein**

**The common structural feature is a 10-stranded β-barrel, made of two orthogonal antiparallel 5-stranded sheets that form the "clam"-shaped binding cavity, giving the protein a pocket into which a fatty acid is inserted.**



### 1.2.2 H-FABP

H-FABP, which appears to be identical to mammary-derived growth inhibitor (MDGI) (Specht et al., 1996) and is also known as FABP3 or muscle FABP, consists of 132 amino acid residues and has a molecular mass of 14.5 kDa. It is primarily expressed in the mammalian heart but also found, with a lower content, in other tissues like skeletal muscle, intestine, lung and brain. However, this protein is considered to be the only FABP expressed in various muscle cells, in both vertebrate and invertebrate species. It contains a high-affinity fatty acid binding site and binds long-chain fatty acid with 1:1 ratio (Haunerland, 1994). The binding affinity and specificity appear to be related to fatty acid chain length and the degree of saturation. For the complex of human H-FABP with fatty acids, a binding constant (Kd) of 4 nM was reported for stearic acid (C18:0), 14 nM for palmitic acid (C16:0), 30-65 nM for linoleic acid (C18:2), and arachidonic acid (C20:4) (Richieri et al., 1996).

H-FABP is highly conserved, even between insects and mammals, and is found in all muscles that metabolize fatty acids. The overall identity of amino acid sequences is greater than 86 % among mammals, and decreases to 64-74 % between mammals and fish (Wu, 2001). The H-FABP primary structure of the locust, *Schistocerca gregaria*, is about 43 % identical to mammalian H-FABP, with a high percentage of conservative substitutions in the remaining residues (Haunerland et al., 1990). Heart FABP from vertebrates and invertebrates are much more similar than their paralogs in the same organism, likely because muscle structure and function are also very similar.

It has been well established that the H-FABP content of a particular muscle correlates with its fatty acid oxidation capacity in a linear fashion (Haunerland, 1994).

Smooth muscle that relies largely on carbohydrates possesses very low levels of this FABP, while various red muscles contain far larger amounts of H-FABP, roughly following the higher β-oxidation capacity. Cardiac tissue, which relies mostly on lipid for energy supply and encounters the highest β-oxidation rates of all mammalian muscles, also has the highest FABP content in mammals (up to 5 % of all cytosolic proteins). Muscles with even higher metabolic rates are found in birds and insects. A classical example is the flight muscle of the desert locust, which can sustain metabolic rates three times higher than mammalian heart. A beta-oxidation capacity of almost 1 μM of fatty acid per minute and gram of tissue has been reported; equally, the H-FABP (~ 18 % of all cytosolic proteins) is more than three times larger than in mammalian heart.

Given its evolutionary conservation, high expression rates, and essential role in fatty acid transport and metabolism, it was surprising to see no dramatic effects in H-FABP knock-out mice (Schaap et al., 1999). The absence of H-FABP did not result in phenotypic differences and muscle histological changes. However, fatty acid uptake was reduced remarkably in cardiac tissue and isolated cardiomyocytes, and those mice displayed an overall switch to glucose as an energy source, which can provide sufficient energy only in resting states. When exercised at higher metabolic rates, H-FABP null mice were unable to adapt this challenge. These results lend support to the essential role of H-FABP in metabolism.

In various muscles, elevated levels of H-FABP expression have also been observed as a consequence of endurance training or otherwise increased fatty acid utilization. For instance, locust flight muscle shows a marked increase in FABP expression, over and above its already extreme H-FABP content, after either sustained

flight training or increased fatty acid supply alone (Chen and Haunerland, 1994). Chronic electrical stimulation in rat soleus muscle also increases H-FABP expression (~ 30 %, Veerkamp and Moerkerk, 1993). Apparently, the cell senses increasing levels of fatty acids, and responds by increasing the expression of their binding protein. This up-regulation occurs at the level of gene transcription.

## 1.3 Regulation of FABP gene expression

### 1.3.1 FABP gene structure

Given the structural and functional similarities among all FABPs, it is not surprising that these proteins share an identical gene structure of four conserved exons and three introns of variable sizes (Bernlohr et al., 1997). The exon/intron boundaries are in identical positions in most FABPs, four exons coding for nucleotides from 1 to 76, 77 to 252, 253 to 354, and 355 to 407, respectively. There is only one exception that the second intron has been lost in several, but not all insect FABPs (Wu et al., 2001).

All FABP promoters contain a classical TATA box. Currently, the elements that control the tissue-specific expression of FABP are barely understood, but potential enhancer sequences have been characterized for several genes. These include two HNF1α regulatory elements in the L-FABP (Akiyama et al., 2000), a fat-specific enhancer required for A-FABP expression in adipocytes (Ross et al., 1990), and several binding sites for members of the POU transcription factor family that control B-FABP expression (Josephson et al., 1998). A concise promoter region that contained an atypical MEF2 binding site was shown to be responsible for the muscle-specific expression of H-FABP (Qian et al., 1999).

### 1.3.2 Regulation of FABP expression induced by fatty acids

The experimental results outlined above show an indirect correlation between higher H-FABP level and enhanced fatty acid uptake and metabolism (Veerkamp and Moerkerk, 1993; Glatz et al., 1994). It has been reported as well that the induction of H-FABP expression, in response to higher intracellular fatty acids under diabetes and fasting (Carey et al., 1994) or endurance training (Chen and Haunerland, 1994), results from increased gene transcription, as measured by run-on assays and RT-PCR (Zhang and Haunerland, 1998). The genes for various other FABP types are known to be transcriptionally regulated by fatty acids as well (Haunerland and Spener, 2004). Such an up-regulation requires that a nuclear receptor senses fatty acids, binds to a response element in the promoter regions, and enhances the transcription rates of the gene under its control. The best known of such transcription factors, peroxisome proliferator activated receptor (PPAR $\alpha$, $\beta/\delta$, or $\gamma$), is so called because of its activation by certain drugs (peroxisome proliferators) in rodents (Desvergne and Wahli, 1999). Polyunsaturated long-chain fatty acids and certain eicosanoids are considered as their natural ligands. PPARs bind as heterodimers with the subtypes $\alpha$, $\beta$, $\gamma$ of the retinoic acid receptor (RXR) to peroxisome proliferator response elements (PPREs) in the promoter region of the genes that they regulate. The PPRE consensus sequence (5'AGGTCA N AGGTCA 3') consists of a direct repeat of the nuclear receptor consensus half-site AGGTCA, separated by a single base; thus it is also called DR-1. In addition, similar elements are also the target for binding of other nuclear hormone receptor complexes, involving transcription factors such as HNF4, thyroid, or estrogen receptors (Jump and Clarke, 1999).

While circumstantial evidence suggests that PPARs are involved in the regulation of various FABP genes, proof has been provided only for A-FABP and L-FABP (Tontonoz et al., 1994; Issemann et al., 1992). In reporter-gene and transactivation assays Tontonoz et al. have shown that the mouse A-FABP gene is regulated by the binding of PPARγ2 and RXRα to a direct-repeat element 5.2 kb upstream of the FABP gene. The expression of the rodent L-FABP gene in liver is under the control of PPAR α bound to a PPRE around 110 bp upstream of the transcriptional start site; interestingly, its expression in intestinal cells is controlled by PPAR β, which binds to the same response element as PPAR α in liver (Poirier, 2001).

Although PPARs are involved in the regulation of A- and L-FABP genes, they may not play a role in the fatty acid mediated up-regulation of H-FABP gene expression. Of all mammalian H-FABP genes, only the rodent genes contain direct repeat sequences that have similarity to the consensus PPRE element (Zhang et al., 1999; Treuner et al., 1994). Moreover, this PPRE-like sequence found upstream of rodent genes appears to be non-functional. Recent data by Schachtrup et al (2004) and Kawabe (2005) demonstrated that PPARs binding with RXR appear not to be involved in the regulation of the mouse H-FABP gene. While some reports claim PPAR involvement based on the fact that H-FABP expression can be induced by PPAR agonists e.g. Wy 14,643 (van der Lee et al., 2000), it should be noted that this could be the consequence of increased fatty acid influx due to the induction of membrane transporters such as FAT/CD36, which are known to be under the control of PPAR α. Thus, the involvement of a different, yet unknown fatty acid response element (FARE) has been proposed (Wu and Haunerland, 2001).

Invertebrates do not express PPARs (Detera-Wadleigh and Fanning, 1994), but the ortholog of H-FABP can be induced by fatty acids (Chen and Haunerland, 1994). It is interesting to note that a different fatty acid response element (FARE) has been identified in the promoter of the H-FABP gene from locust muscle through gel-shift and reporter gene assays (Wu and Haunerland, 2001). Unlike PPRE, the locust FARE is an inverted repeat element (IR-3), a palindromic sequence containing two hexanuleotide half-sites (AGTGGT, ATGGGA), separated by three nucleotides reminiscent of a steroid hormone response element. Therefore, it is very likely that there is more than one way by which fatty acids can control gene expression.

Given the absence of a PPRE in at least some of the mammalian H-FABP promoters, and the presence of nuclear proteins that bind to the fatty acid response element characterized in the locust promoter, it may well be possible that the mammalian genes are regulated by similar elements. The conserved H-FABP genes may also include conserved fatty acid response elements in their promoters of all muscle FABP genes.

## 1.4 The research objectives of thesis project

As mentioned above, it has long been known that up-regulation of mammalian H-FABP expression is induced by fatty acid exposure. However, the mechanism by which this happens has not yet been elucidated. The aim of this thesis was to characterize a potential fatty acid response element in the rat heart-FABP gene.

To approach this objective, an initial analysis of all known mammalian H-FABP gene promoter regions is proposed to identify possible fatty acid response elements. The high degree of conservation found for the H-FABP genes may also include regulatory

elements in their promoters. Subsequently, the specific interaction between putative DNA elements and nuclear extract proteins needed to be analyzed by electrophoretic mobility shift assays (EMSA, also called by gel-shift). Functional analysis of these elements required the construction of reporter genes and the measurement of their expression in control and fatty acid exposed cultured L6 myoblasts.

# CHAPTER 2: ANALYSIS OF THE RAT H-FABP GENE AND EMSA OF THE POTENTIAL FARE

## 2.1 Analysis of the rat H-FABP gene

### 2.1.1 Overview of eukaryotic gene regulation

The fundamental doctrine of molecular biology is that DNA is transcribed into RNA which in turn is translated into proteins. Hence there are several levels of gene regulation available to eukaryotic cells: the level of inducible mRNA synthesis (transcription), the level of RNA stability, and the level of protein synthesis (translation), which is usually controlled through retention of mRNA in the nucleus, interference with ribosome binding and other mechanisms. The level of mRNA synthesis is regulated mostly by limiting the access of the gene promoter to RNA polymerase. Since DNA must first be transcribed into an RNA product before this product can be further processed and serve as template for translation, transcription initiation is often considered to be the most important regulatory point in gene expression.

The synthesis of RNA from DNA is mediated by the RNA polymerases, and the control over their activity forms the basis of the control of transcription rates. Eukaryotic cells contain three different RNA polymerases: RNA polymerase I and III are used for synthesis of ribosomal, transfer RNA and small nucleolar RNA; the vast majority of eukaryotic messenger RNA is synthesized by RNA polymerase II.

The upstream region of eukaryotic genes contains several important sequences for appropriate regulation. Sequence-specific DNA-binding proteins bind to these promoters

to regulate the transcription rate. Promoters are generally divided into three functional segments:

(i) The core promoter, which is located close to the site of initiation (generally within 50-100 bp), contains sequences necessary for transcription initiation. The core promoter elements bind to, and control assembly of, the preinitiation complex, which contains Pol II (the general transcription factor) and coactivators. In the meantime, they position the transcription start site, direct transcription, and respond to proximal or distal activators and repressors in a cell. In most cases, the core promoter elements do not work directly on regulating transcription. The core promoter alone is generally inactive *in vivo* as it needs to work in conjunction with a regulatory promoter, but *in vitro* it can bind to the general machinery and account for the "basal" levels of transcription. The core promoters are typically represented by a TATA box (consensus TATA(A/T)A(A/T)) and surrounding GC-rich sequences which are recognized by the TATA-binding protein subunit of TFIID (Burley and Roeder, 1996).

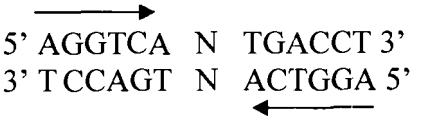
(ii) The regulatory promoter, which contains elements to form the targets to specific transcription factors in response to specific external stimuli, is usually upstream of the core promoter within several hundred nucleotides. Transcription regulation in response to extra-cellular signals can take different forms, but the endpoint is always the same. A previously inactive transcription factor is specifically activated by a signal molecule (ligand) and then binds to specific response elements located in the promoters of target genes, thereby activating their transcription. In the absence of the ligand, the receptor remains unable to bind to the elements, perhaps by direct inhibition of its DNA binding domain (Blackwood and Kadonaga, 1998).

(iii) The enhancer sequences near to further upstream, perhaps even more than 2 kb, bind a specific class of regulatory proteins to increase the basal level of transcription which is initiated through the core promoter elements. Their functions are independent of their orientation and, to some extent, independent of their distance from the genes they regulate as well. Elements such as E-boxes, bound by muscle-specific transcription factors, and HNF4, bound by liver-specific transcription factors, control specific and developmental stage regulation of gene expression.

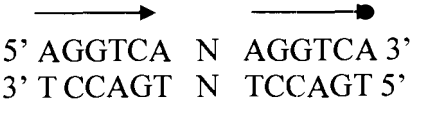
Transcriptional regulation is controlled by the binding of sequence-specific DNA-binding proteins to regulatory promoters and enhancers. Specific sequences located within the regulatory promoter are widely variable in sequence, but several general patterns can be outlined. They are usually small sequences containing short consensus motifs arranged in a specific combination of patterns. Each consensus sequence is referred to as a "half site" and the arrangement of the "half site" is often as important in establishing the element's function as the consensus sequence itself. Common binding sites are direct repeats (DR), inverted repeats (IR, also called palindromic sequences) and everted repeats (ER) as show in Figure 2-1. A hexamer (AGGTCA) is proposed as the consensus half repeat, but many elements identified do not match this sequence perfectly, and the spacing between the two repeats may also be variable for different nuclear receptors. Usually, the two half-sites bind two transcription factor molecules either as homo- or heterodimers.

**Figure 2-1 Different patterns arranged by the consensus half repeat**

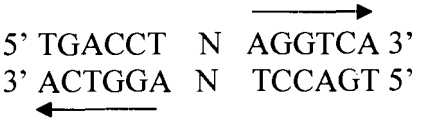
**A nuclear receptor binding site consensus hexamer 5'AGGTCA 3' is indicated as an inverted repeat (palindrome), a direct repeat, or an everted repeat.**




Inverted repeat
(Palindrome)




Direct repeat




Everted repeat

### 2.1.2 Potential FARE element

As discussed in the previous chapter, throughout the animal kingdom H-FABP expression is up-regulated by a rise in intracellular fatty acid levels. *In vitro* studies with cultured muscle cells or their precursors confirmed this. Given the widespread similarities in muscle function and energetics, as well as H-FABP structure and regulation, one can expect that the regulation of the H-FABP gene also follows similar mechanisms in insects and mammals.

If similar control mechanisms are at work, we reason that the suspected fatty acid response element (FARE) should be conserved as well, and it may be possible to identify candidate sequences of related control elements by comparing sequences of H-FABP promoters.

While the H-FABP genes from mammals and insects share the same overall gene organization, the introns vary widely in size, and the noncoding sequences show little similarity. However, a strikingly similar sequence was found within 1 kb of upstream sequence of all mammalian H-FABP genes investigated, but not found in other members of the iLBP family (Dawe, 2001; Table 2-1). This element contains two consensus hexanucleotide (AGAAGA) half sites in everted orientation, being separated by three nucleotides. Thus it is called everted repeat element (ER-3). In the upstream of rat H-FABP gene, there are two ER-3 elements, ER-3a locates between -528/-510 bp while ER-3b on -709/-691 bp.

Because this conserved sequence is found within the promoter region of all mammalian heart FABP genes and its similarity to previously characterized locust IR-3 elements in reverse orientation, it appears possible that it is a candidate for the fatty acid

response element that regulates mammalian H-FABP gene expression. However, previous work in our lab can not identify the potential rat heart-FABP ER-3 elements due to the large variation of results from the luciferase reporter gene assay. So the aim of this thesis was to optimize this luciferase reporter gene assay system, and then using it to prove this possible fatty acid response element.

More evidence was needed to support this hypothesis before carrying out a functional reporter gene assay. An electrophoretic mobility shift assay was therefore first performed to investigate the interaction between the everted repeat element and nuclear proteins.

**Table 2-1 Alignment of a potential regulatory element ER-3**

| Species | GenBank No. | Intron 1 | Intron 2 | Intron 3 | Putative FARE Sequences | Location | Type |
|---|---|---|---|---|---|---|---|
| Rat | AF144090 | 3.5 kb | 1.5 kb | 1.1 kb | 5′CC TCTTCT ACA AGAAGA▶GG 3′<br>3′GG◀AGAAGA TGT TCTTCT CC 5′ | -528/<br>-510 | ER-3 |
| | | | | | 5′CA TGTTCT CCC AGAGCA▶GG 3′<br>3′GT◀ACAAGA GGG TCTCGT CC 5′ | -709/<br>-691 | ER-3 |
| Mouse | U02884 | 3.4 kb | 1.5 kb | 1.1 kb | 5′CC TCTTCT ACA AGAAGA▶GG 3′<br>3′GG◀AGAAGA TGT TCTTCT CC 5′ | -538/<br>-520 | ER-3 |
| | | | | | 5′CA CGTTCT CCG CCAGCA▶GG 3′<br>3′GT◀GCAAGA GGC GGTCGT CC 5′ | -720/<br>-702 | ER-3 |
| Human | U17081 | 3.4 kb | 1.9 kb | 1.4 kb | 5′CC TCACTT GTA AAACGA▶GG 3′<br>3′GG◀AGTGAA CAT TTTGCT CC 5′ | -482/<br>-464 | ER-3 |
| Chicken | AY207009 | 0.6 kb | 2.2 kb | 0.1 kb | N/A | N/A | N/A |
| Fly | AAF54655 | 2.2 kb | -- | 0.1 kb | 5′GG AGTGGT▶GGC CCCCAT AG 3′<br>3′CC TCACCA CCG◀GGGGTA TG 5′ | -319/<br>-301 | IR-3 |
| Locust | AF244980 | 12.7 kb | -- | 2.9 kb | 5′GG AGTGGT▶AGT TCCCAT CC 3′<br>3′CC TCACCA TCA◀AGGGTA GG 5′ | -180/<br>-162 | IR-3 |
| | | | | | 5′GG AGTGGT▶GAT TTCCAT CC 3′<br>3′CC TCACCA CTA◀AAGGTA GG 5′ | -643/<br>-625 | IR-3 |
| | | | | | 5′GA GGTAGT▶ACT TCCCAT CC 3′<br>3′CT CCATCA TGA◀AGGGTA GG 5′ | -1102/<br>-1084 | IR-3 |

## 2.2 Electrophoretic mobility shift assay

The Electrophoretic Mobility Shift Assay (EMSA) has been used extensively for studying DNA-protein interactions. This assay is based on the fact that DNA-protein complexes migrate slower than unbound DNA in a native polyacrylamide or agarose gel, resulting in a "shift" in migration of the labeled DNA band. To verify that the everted repeat element, potential FARE, does bind to some nuclear transcription factors, EMSA was carried out with the ER-3 element and nuclear extracts from cultured myoblast cells.

### 2.2.1 Materials and methods

#### Cell culture

Rat skeletal muscle L6 myoblasts (American Type Culture Collection, Rockville, MD) were grown in Dulbecco's Modified Eagle medium (DMEM, Invitrogen, Burlington, ON) supplemented with 10 % fetal bovine serum (Invitrogen, Burlington, ON) , 150 U/ml penicillin, and 150 μg/ml streptomycin (Sigma, Oakville, ON).

#### Nuclear protein extraction

All steps were performed at 2-8 °C and buffers and equipment were procooled. L6 myoblasts were grown for 3 days to 80 % confluence in the 60×10 mm treated polystyrene dishes (Corning, Acton, MA). Monolayer cells were rinsed with phosphate buffered saline and scraped off the dishes. The pooled cells were centrifuged at 3000 rpm for 10 min, suspended in 3 volumes of homogenization buffer (10 mM Hepes-NaOH, pH 7.9, 0.35 M sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.15 mM spermine, 0.5 mM spermidine, 2 μg/ml leupeptin and 2 μg/ml aprotinin; all from Sigma, Oakville, ON), and processed further

according to the protocol of Nu-Clear Extraction Kit (Sigma, Oakville, ON). The nuclear protein concentration was determined by the method of Bradford with a kit from Bio-Rad (Richmond, CA, USA) using bovine serum albumin (Sigma, Oakville, ON) as a standard.

**Electrophoretic mobility shift assay**

The biotinylated, synthetic oligonucleotides (5' CGCCTCTTCT ACA AGAAGA GG 3') containing the palidromic everted repeat element (-529 — -510) was denatured at 100 $^{0}$C and slowly cooled down for a double-stranded probe. Non-radioactive gel shifts were carried out with the LightShift Chemiluminescent EMSA kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Labelled probe (40 fmoles) was incubated with L6 nuclear extracts for each reaction before electrophoresis. After electrophoresis, oligonucletides were blotted by capillary transfer onto nylon membranes, cross-linked, and visualized by chemiluminesence.

For the specific competition experiments, L6 nuclear extracts were pre-incubated with a 5, 10, 20 or 100-fold mole excess of unlabelled, double-stranded oligonucleotides of the putative element.

### 2.2.2 Results and discussion

When the probe containing the ER element was added to nuclear proteins extracted from L6 myoblasts, a distinct gel shift to lower mobility was observed (Figure 2-2). In addition, the unlabelled double-stranded DNA of the 19 bp element was able to compete with the labelled element, gradually eliminating this shift band by increasing amounts of the unlabelled double-stranded oligonucleotides (Figure 2-3). Therefore, it

clearly shows that the gel shift was a result of a specific interaction between the putative element, everted repeat element, and some nuclear proteins.

Further experiments in our lab concluded that antibodies to PPAR α did not induce a supershift with rat nuclear extracts in our gel shift assay, indicating that this transcription factor is not involved in the DNA-protein specific interaction (data not shown). Thus, this everted repeat element, specific binding with myoblast nuclear proteins, is proposed to be a possible novel fatty acid response element as it is different from any known nuclear receptor binding site. To examine the function of the everted repeat element in the native H-FABP gene promoter, the rat genomic sequence was cloned into a luciferase reporter plasmid for the fatty acid response.

**Figure 2-2 Gel shift assay of the everted repeat element binding to nuclear proteins**

**Nuclear proteins were extracted from L6 myoblasts and the rat probe was the double-stranded ER-3 element labelled with biotin. Gel shift assay was carried out with or without nuclear extract, as shown above the lanes.**

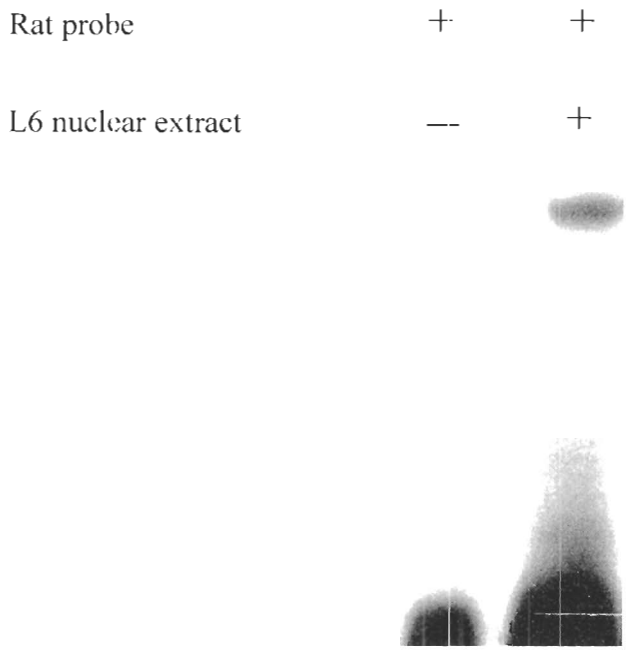
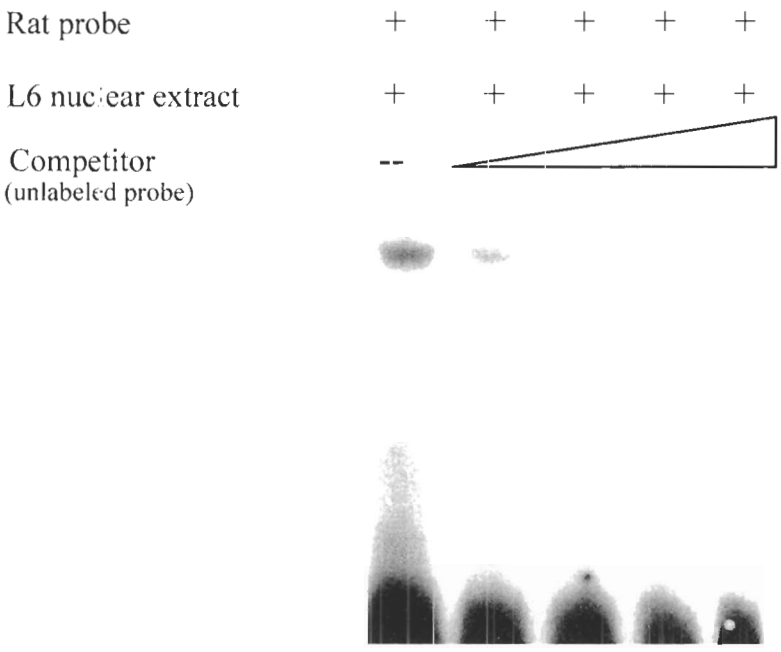


**Figure 2-3** **Specific binding between the everted repeat element and nuclear proteins**

**Nuclear proteins were extracted from L6 myoblasts and the rat probe was the double-stranded ER-3 element labelled with biotin. The unlabelled double-stranded ER-3 element was used to compete with the labelled element. Gel shift assay was carried out with gradually increasing amounts of the unlabelled double-stranded probe, as shown above the lanes.**



# CHAPTER 3: MODIFICATION OF THE DUAL LUCIFERASE REPORTER GENE ASSAY SYSTEM

## 3.1 Introduction

When initiating an analysis of a gene's transcriptional regulatory mechanism, a critical step is the development of an assay for measuring the activity of relevant control regions. With an appropriate functional assay, it is possible to assess a control region's ability to mimic the expression pattern of endogenous genes. In the absence of a functional assay, it is very difficult, if not impossible, to identify and assess the relevance of DNA sequence elements and proteins that may contribute to gene regulation.

Several types of functional assays have been used to study transcriptional regulation. The most common one is the transient transfection assay, in which plasmids containing the control region of interest are introduced by a transfection procedure into cells maintained in culture. Typically, the control region regulates transcription of a so-called "reporter gene", i.e., a known gene for which the levels of mRNA or protein can be easily measured. If the regulatory region of interest is recognized as a promoter, it is then placed immediately upstream of the reporter gene to drive reporter gene transcription. If the control region of interest is a regulatory sequence such as a hormone response element, that element is placed upstream of a universal promoter capable of base transcription rates. At a specific time point following transfection of cultured cells with the resulting plasmid, the activity of the control region is assessed by measuring mRNA or protein synthesis from the reporter gene. This assay is considered to be transient because the plasmids rarely integrate into the host genome. As such, mRNA or protein

production must be measured within a short time period, ranging from 1 to 3 days, even if the cells remain stable in culture; otherwise the plasmids will degrade or be diluted as the cells grow and divide. The transient assay is usually employed for an initial analysis of the *cis*-acting DNA sequences and *trans*-acting factors that regulate gene expression.

Luciferase is the reporter gene of choice in many laboratories. The luciferase assay is more sensitive than assays for chloramphenicol acetyl transferase (CAT) or other commonly used reporter genes. The high sensitivity of the luciferase assay allows analysis of weak promoter and use of smaller amounts of DNA and cells in transfection studies. The assay is linear over eight orders of magnitude (Promega, technical manual No.040, 2003). Therefore small amounts of transfected cell lysate can be assayed for enzyme activity and whole dish-to-dish comparisons made easier.

The dual luciferase reporter (DLR) assay system used in this thesis was purchased from Promega (Madison, WI). The term "dual reporter" refers to the simultaneous expression and measurement of two individual reporter enzymes within a single system. Typically, the "experimental" reporter is correlated with the effect of specific experimental conditions. In contrast, the activity of the co-transfected "internal control" reporter, pRL-TK, serves as the baseline response since it is under the control of the thymidine kinase promoter, which is a constitutively active promoter in all cells because this promoter controls common biochemical pathway. Normalizing the activity of the experimental reporter to the activity of the internal control minimizes experimental variability, as a result of differences in cell viability, transfection efficiency or other sources of variability. Thus, dual reporter assays often allow more reliable interpretation of the experimental data by reducing extraneous influences. The experimental reporters

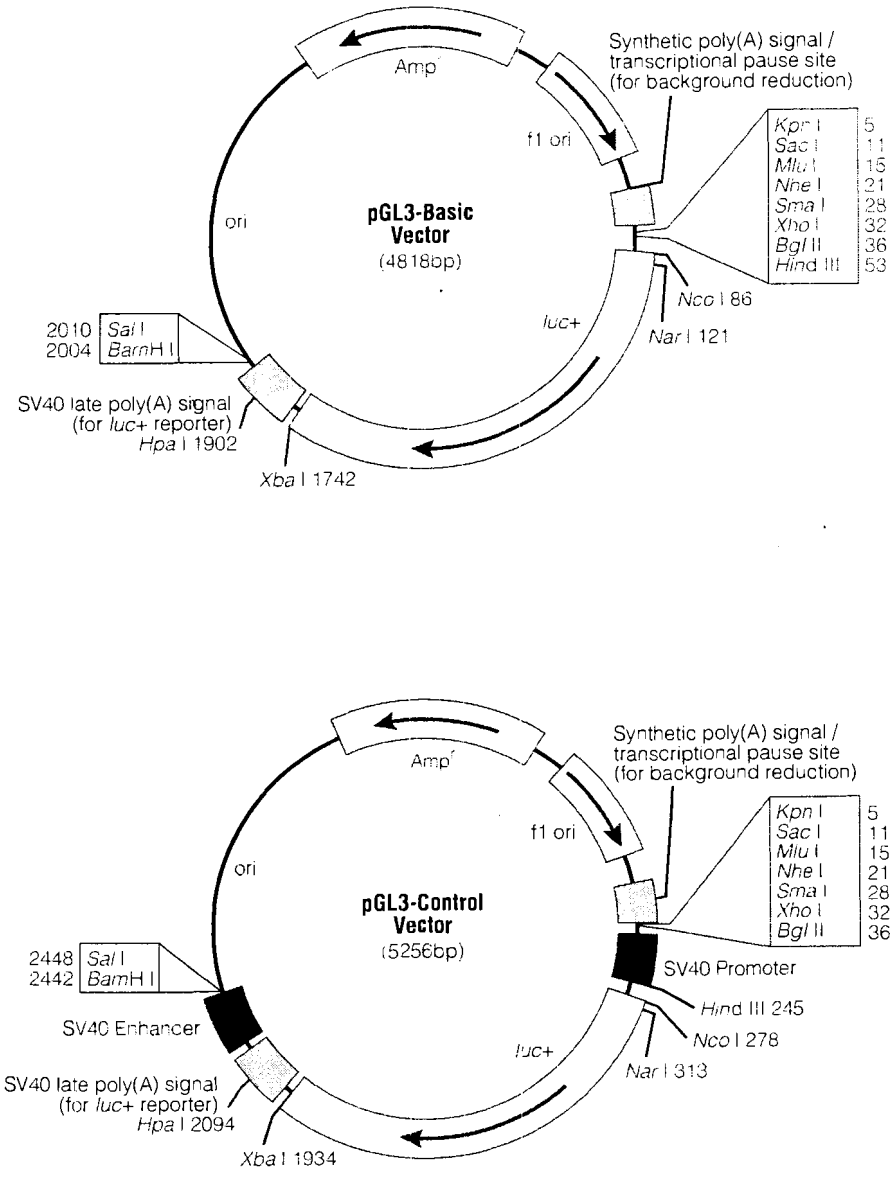
include pGL3-Basic supplied as a negative control as well as pGL3-Control as a positive control. The interest sequence of a putative promoter is inserted into the multiple cloning site of pGL3-Basic (Promega, technical manual No.040, 2003). Expression of luciferase activity in cells relies on insertion and proper orientation of a functional promoter upstream from the reporter gene (Figure 3-1).

In the DLR assay, the luciferase activities of both experimental and internal control reporters are measured sequentially from a single sample. This is made possible by the nature of the luciferase produced from each vector. The experimental reporter gene produces firefly luciferase that first was isolated from the firefly (*Photinus pyralis*), an enzyme which requires ATP and $O_2$ to produce photon emission. *Renilla* (*Renilla reniformis*, also known as sea pansy) luciferase encoded by the internal control vector does not need ATP, and emits a steady light signal followed by a long decay. This system allows the initial signal (the experimental reporter signal) to be measured and quenched by a second buffer, allowing sensitive detection of the internal control signal without necessitating a new assay. Results of luciferase activity are expressed as a ratio of both signals (Promega, technical manual No.040, 2003).

In this application, rat L6 myoblasts (ATCC, Manassaa, VA) were selected as the host for transfection. As not terminally differentiated muscle cell precursors, myoblasts are useful model system for the study of gene expression in muscle cells. Myoblasts expressed H-FABP as well, but in lower concentrations than differentiated myocytes. After a 10 h incubation with at least 60 μM fatty acid, a two-fold increase in FABP mRNA was observed in myoblasts. Cardiomyocytes responded to fatty acids treatment in

a similar way; hence, cultured L6 myoblasts are a suitable cell line for the study of H-FABP gene expression (Chang et al, 2001).

**Figure 3-1 The pGL3 vectors' circle maps**



## 3.2 Construction of luciferase reporter genes

The pGL-3Basic vector lacks a eukaryotic promoter and enhancer sequences, allowing maximum flexibility in cloning putative regulatory sequences. Expression of luciferase activity in cells transfected with these plasmids relies on insertion and proper orientation of a functional promoter upstream from *luc*+.

### 3.2.1 Materials and methods

#### 3.2.1.1 Reporter gene construction

A TA clone (Invitrogen, Carlsbad, CA) was constructed to contain the rat H-FABP promoter, from 900 bp upstream of the transcription start site to the ATG start codon and part of exon I (Wu et al., 2002). The sequence was confirmed by sequencing, and was used as template for further serial reporter gene construction.

To construct luciferase reporter genes, different regions of the promoter were generated by PCR reactions using different forward primers, each with an *Xho*I site, in combination with the same reverse primer containing a *Hin*dIII site located between transcriptional initiation site and the ATG start codon (Table 3-1). PCR products from these reactions and pGL3-Basic luciferase vector (Promega, Madison, WI) were double digested with *XhoI* and *Hin*dIII and resolved by agarose gel electrophoresis. Bands of the expected sizes were excised and purified using the Qiaquick gel extraction kit (Qiagen, Mississauga, ON). The purified DNA was inserted into pGL3-Basic luciferase vector in a ligation reaction. After an overnight ligation at 24 °C, the resulting DNA was transformed into One Shot Top10 chemically competent *E.coli* cells (Invitrogen, Carlsbad, CA) using a 42 °C heat-shock method. Then the transformed cells were grown overnight at 37 °C on LB-amp plates. The next day, the colonies were picked and grown

overnight at 37 °C in 5 ml LB-amp broth. Plasmids isolated by a plasmid mini-prep kit (Genelute, Sigma, St. Louis, MO) were first analyzed by restriction mapping, and then validated by sequencing.

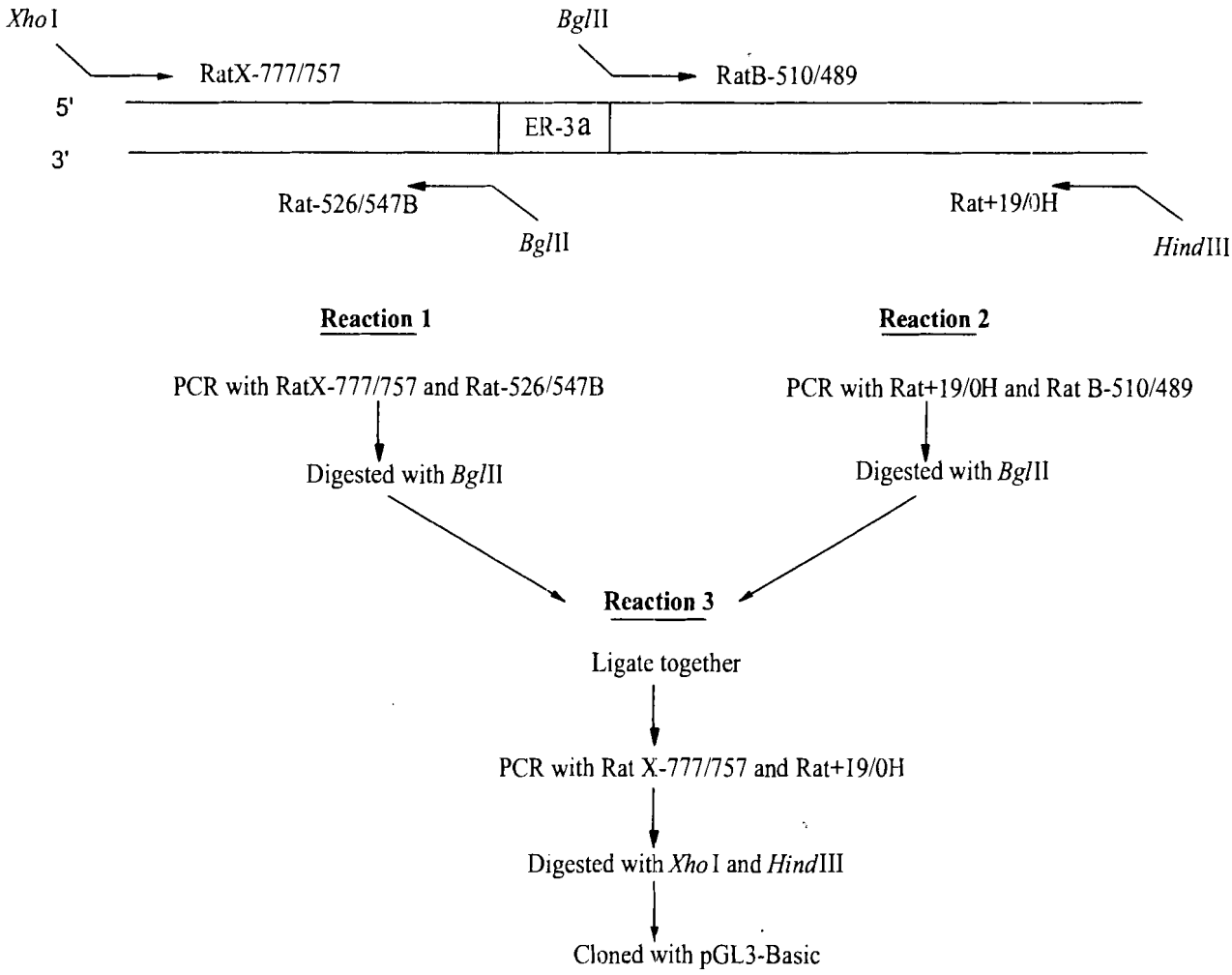
There were *NheI* sites on both -116/-111 bp of promoter upstream and the multiple cloning site in construct pGL3-777/+19. Therefore construct pGL3-116/+19 was produced simply through the following steps: pGL3-777/+19 digestion by *NheI*, right-size DNA band (~ 4,900 bp) selection from agarose gel electrophoresis, and purified DNA ligation reaction. The shortest construct was also confirmed by sequencing.

In addition, a special deletion mutant pGL3-777/+19dele was constructed in which nucleotides -528 to -510 were removed, as illustrated in Figure 3-2. Another deletion mutant pGL3-688/+19dele was developed through PCR with pGL3-777/+19dele as the template so that it contained less upstream sequence. Both mutants were verified by DNA sequencing.

**Table 3-1 Sequence and position of primers used to construct reporter genes**

| Primers | Sequence | Restriction site | Position | Construct |
|---|---|---|---|---|
| *Forward* | | | | |
| RatX-777/757 | ATCTCGAGAGGCGCAGGCAGGAGACATTC | *XhoI* | -777/-757 | pGL3-777/+19; pGL3-777/+19dele (deletion mutant) |
| RatX-688/671 | ATCTCGAGGCGCTGGGCAGCTCA | *XhoI* | -688/-671 | pGL3-688/+19; pGL3-688/+19dele |
| RatX-532/509 | ATCTCGAGCCGCGCCTCTTCTACAAGAAGAGG | *XhoI* | -532/-509 | pGL3-532/+19 |
| RatX-503/486 | ATCTCGAGGGGCCGTTGAAATGGGTC | *XhoI* | -503/-486 | pGL3-503/+19 |
| RatB-510/489 | ATAGATCTGGACATAGGGCCGTTGAAATGG | *BglII* | -510/-489 | pGL3-777/+19dele |
| *Reverse* | | | | |
| Rat+19/0H | ATAAGCTTGCGTGCAGGCAGTGAGAATC | *HindIII* | +19/0 | All reporter genes |
| Rat-526/547B | ATAGATCTGGCGCGGAGGCTCGAATGGGAA | *BglII* | -526/-547 | pGL3-777/+19dele |

**Figure 3-2 Schematic representation of the strategy to construct the deletion mutant pGL3-777/+19dele**



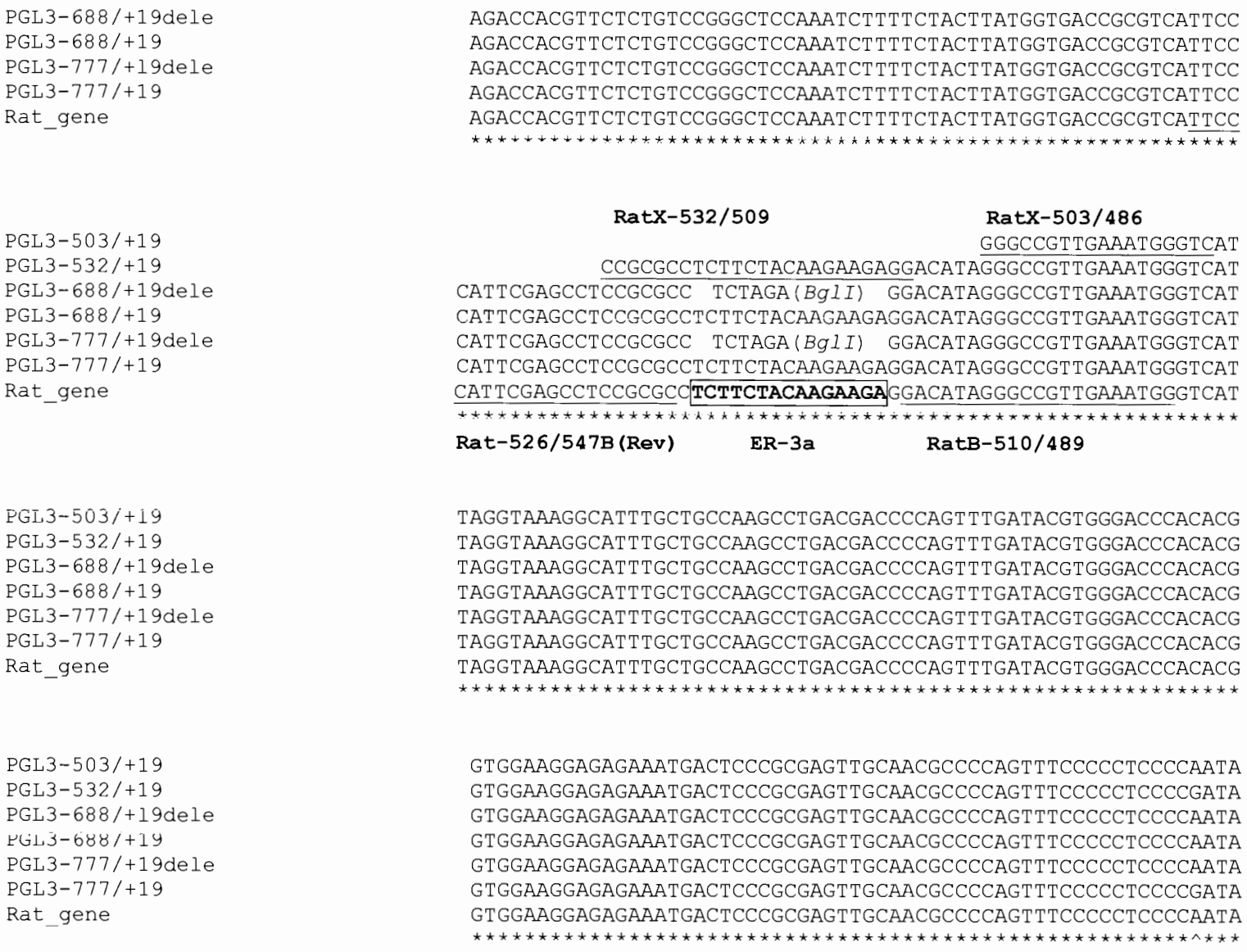
#### 3.2.1.2 Sequencing of reporter genes

All DNA sequencings were carried out using Applied Biosystems BigDye v3.1 Terminator Chemistry on the Applied Biosystems PRISM 377 sequencer at the Biotechnology Laboratory at the University of British Columbia. Each clone was sequenced in both directions using RV primer 3 (forward) and GL primer 2 (reverse, Promega) to confirm orientation and identity of the insert.

### 3.2.2 Results and discussion

When the sequences of reporter gene constructs were aligned with the published rat H-FABP sequence (Zhang et al., 1999), a few minor errors were observed in non-target regions and labelled in Figure 3-3. These minor errors were probably caused by single base pair substitution introduced through PCR cloning. Hence, it was considered that they did not affect the promoter function. The seven reporter gene constructs shown in Fig 3-4, as well as the control plasmids supplied by Promega (negative control pGL3-Basic, positive control pGL3-Control) were used in subsequent experiments. Construct pGL3-777/+19 was the longest structure including both ER-3 elements, the TATA box and transcriptional initiator, which functioned as the full length promoter. Construct pGL3-777/+19dele was very similar to pGL3-777/+19, but without the ER-3a element. Construct pGL3-688/+19 lacked ER-3b, but contained ER-3a. The pGL3-688/+19dele included neither ER-3 elements. Constructs pGL3-532/+19 and pGL3-503/+19 began immediately upstream and downstream of the ER-3a site, respectively. Construct pGL3-116/+19 contains TATA box and transcription initiator, but no other recognizable control elements; it is considered the minimal H-FABP promoter.

**Figure 3-3 Sequencing results of reporter gene constructs**


PGL3-688/+19dele
PGL3-688/+19
PGL3-777/+19dele
PGL3-777/+19
Rat_gene
RatX-532/509
RatX-503/486
PGL3-503/+19
PGL3-532/+19
PGL3-688/+19dele
PGL3-688/+19
PGL3-777/+19dele
PGL3-777/+19
Rat_gene
TCTAGA(BglI)
TCTTCTACAAGAAGA
Rat-526/547B(Rev)
ER-3a
RatB-510/489
PGL3-503/+19
PGL3-532/+19
PGL3-688/+19dele
PGL3-688/+19
PGL3-777/+19dele
PGL3-777/+19
Rat_gene
PGL3-503/+19
PGL3-532/+19
PGL3-688/+19dele
PGL3-688/+19
PGL3-777/+19dele
PGL3-777/+19
Rat_gene

```
PGL3-503/+19              AGCGCGCCTACACGTCCACGCACACTGTAATAAAACCACAGTGAAACAAGGCGCCAGAAGGATC
PGL3-532/+19              AGCGCGCCTACACGTCCACGCACACTGTAATAAAACCACAGTGAAACAAGGCGCCAGAAGGATC
PGL3-688/+19dele          AGCGCGCCTACACGTCCACGCACACTGTAATAAAACCACAGTGAAACAAGGCGCCAGAAGGATC
PGL3-688/+19              AGCGCGCCTACACGTCCACGCACACTGTAATAAAACCACAGTGAAACAAGGCGCCAGAAGGATC
PGL3-777/+19dele          AGCGCGCCTACACGTCCACGCACACTGTAATAAAACCACAGTGAAACAAGGCGCCAGAAGGATC
PGL3-777/+19              AGCGCGCCTACACGTCCACGCACACTGTAATAAAACCACAGTGAAACAAGGCGCCAGAAGGATC
Rat_gene                  AGCGCGCCTACACGTCCACGCACACTGTAATAAAACCACAGTGAAACAAGGCGCCAGAAGGATC
                          ************************************************************

PGL3-503/+19              AGGCGGCGTTTCCCCAGCGTGACGCCAGCTCAAGAGCGAGTTTCCTTTCAGTATGGCGGTGGGA
PGL3-532/+19              AGGCGGCGTTTCCCCAGCGTGACGCCAGCTCAAGAGCGAGTTTCCTTTCAGTATGGCGGTGGGA
PGL3-688/+19dele          AGGCGGCGTTTCCCCAGCGTGACGCCAGCTCAAGAGCGAGTTTCCTTTCAGTATGGCGGTGGGA
PGL3-688/+19              AGGCGGCGTTTCCCCAGCGTGACGCCAGCTCAAGAGCGAGTTTCCTTTCAGTATGGCGGTGGGA
PGL3-777/+19dele          AGGCGGCGTTTCCCCAGCGTGACGCCAGCTCAAGAGCGAGTTTCCTTTCAGTATGGCGGTGGGA
PGL3-777/+19              AGGCGGCGTTTCCCCAGCGTGACGCCAGCTCAAGAGCGAGTTTCCTTTCAGTATGGCGGTGGGA
Rat_gene                  AGGCGGCGTTTCCCCAGCGTGACGCCAGCTCAAGAGCGAGTTTCCTTTCAGTATGGCGGTGGGA
                          ************************************************************

PGL3-503/+19              TGGATGCTCTATTTGGGTTGCGGGGAGCGCCGGGCAATCGGGCAGGGATGGGTTAGAGGGCACC
PGL3-532/+19              TGGATGCTCTATTTGGGTTGCGGGGAGCGCCGGGCAATCGGGCAGGGATGGGTTAGAGGGCACA
PGL3-688/+19dele          TGGATGCTCTATTTGGGTTGCGGGGAGCGCCGGGCAATCGGGCAGGGATGGGTTAGAGGGCACC
PGL3-688/+19              TGGATGCTCTATTTGGGTTGCGGGGAGCGCCGGGCAATCGGGCAGGGATGGGTTAGAGGGCACC
PGL3-777/+19dele          TGGATGCTCTATTTGGGTTGCGGGGAGCGCCGGGCAATCGGGCAGGGATGGGTTAGAGGGCACC
PGL3-777/+19              TGGATGCTCTATTTGGGTTGCGGGGAGCGCCGGGCAATCGGGCAGGGATGGGTTAGAGGGCACA
Rat_gene                  TGGATGCTCTATTTGGGTTGCGGGGAGCGCCGGGCAATCGGGCAGGGATGGGTTAGAGGGCACC
                          ************************************************************^

                                                                              Nhe1
PGL3-116/+19                                                                  GCTAGCCCC
PGL3-503/+19              GGCGGGACCGCGGGCGCCGCTGACGTAGGCGCCCGGAGGGCTGTGGGGGATGGGCGCTAGCCCC
PGL3-532/+19              GGCGGGACCGCGGGCGCCGCTGACGTAGGCGCCCGGAGGGCTGTGGGGGATGGGCGCTAGCCCC
PGL3-688/+19dele          GGCGGGACCGCGGGCGCCGCTGACGTAGGCGCCCGGAGGGCTGTGGGGGATGGGCGCTAGCCCC
PGL3-688/+19              GGCGGGACCGCGGGCGCCGCTGACGTAGGCGCCCGGAGGGCTGTGGGGGATGGGCGCTAGCCCC
PGL3-777/+19dele          GGCGGGACCGCGGGCGCCGCTGACGTAGGCGCCCGGAGGGCTGTGGGGGATGGGCGCTAGCCCC
PGL3-777/+19              GGCGGGACCGCGGGCGCCGCTGACGTAGGCGCCCGGAGGGCTGTGGGGGATGGGCGCTAGCCCC
Rat_gene                  GGCGGGACCGCGGGCGCCGCTGACGTAGGCGCCCGGAGGGCTGTGGGGGATGGGCGCTAGCCCC
                          ************************************************************
```

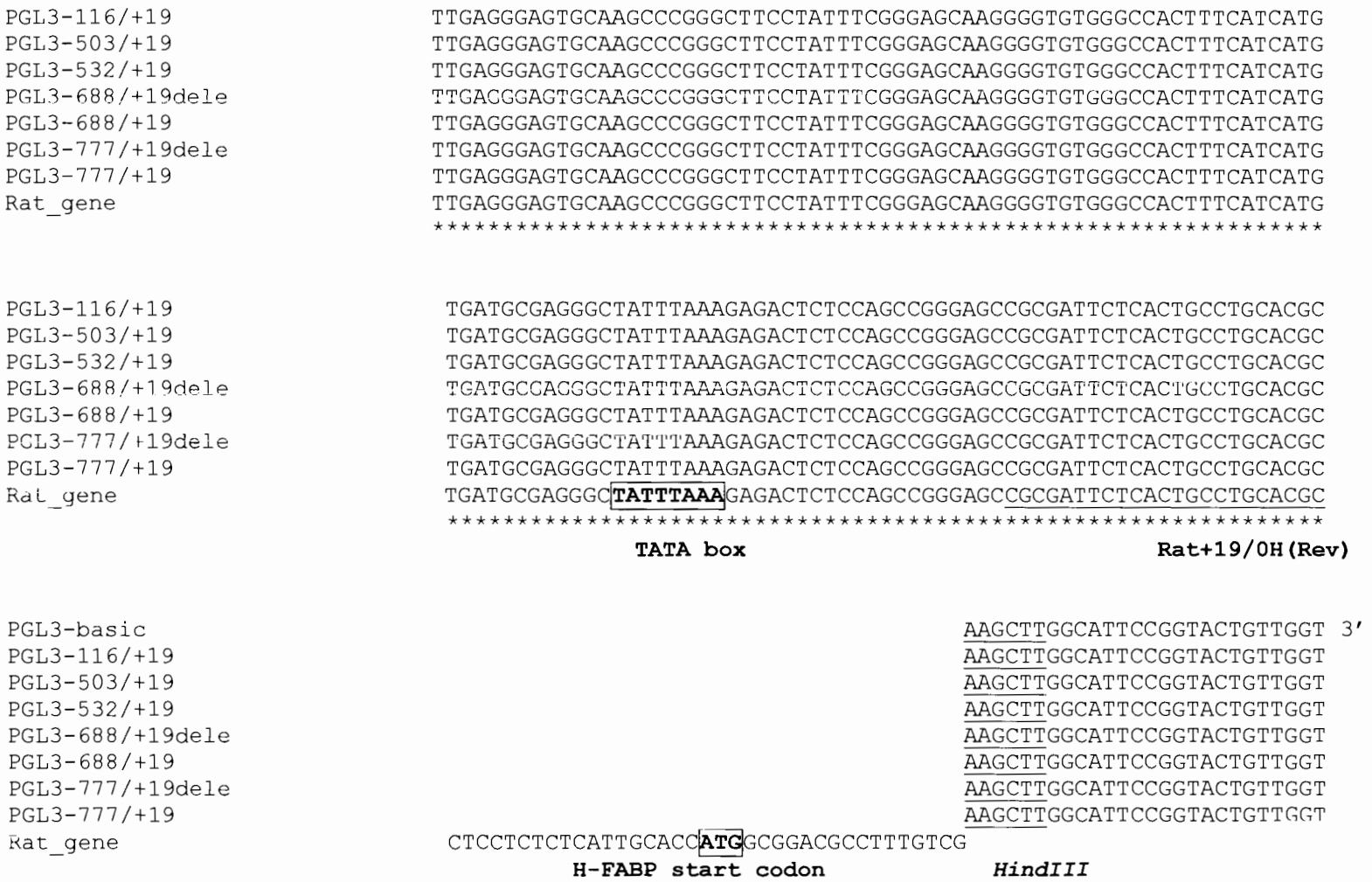
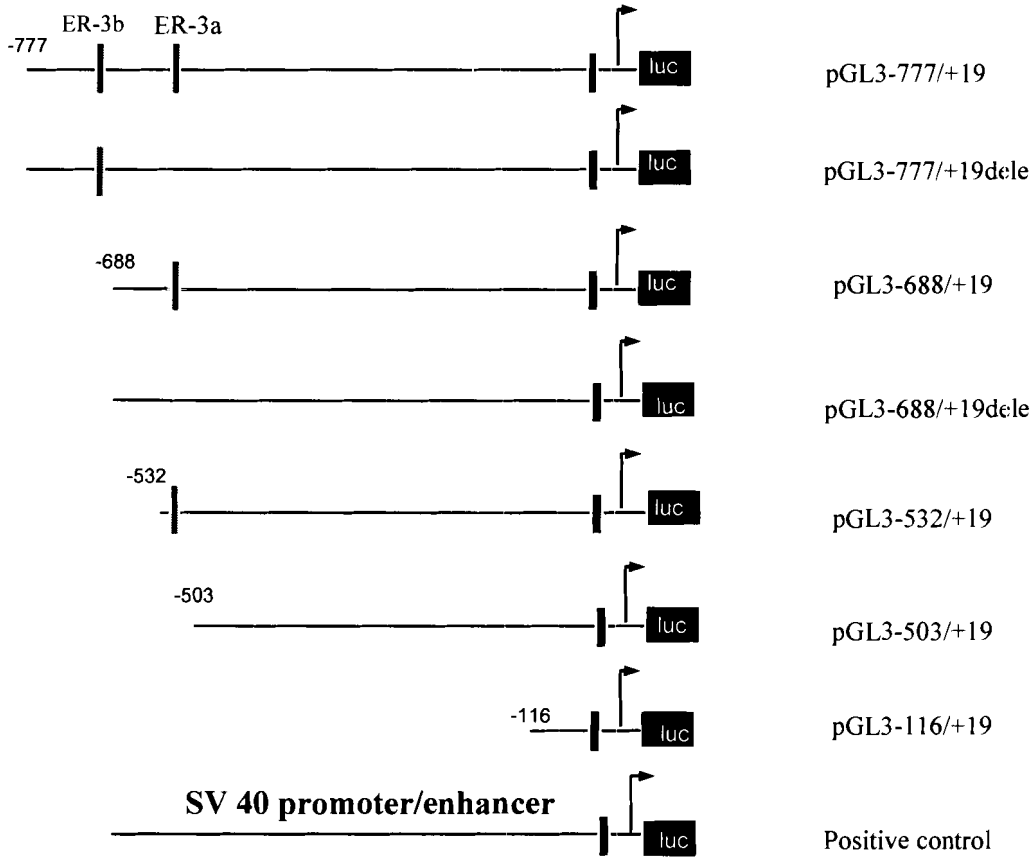

PGL3-116/+19 TTGAGGGAGTGCAAGCCCGGGCTTCCTATTTCGGGAGCAAGGGGTGTGGGCCACTTTCATCATG
PGL3-503/+19 TTGAGGGAGTGCAAGCCCGGGCTTCCTATTTCGGGAGCAAGGGGTGTGGGCCACTTTCATCATG
PGL3-532/+19 TTGAGGGAGTGCAAGCCCGGGCTTCCTATTTCGGGAGCAAGGGGTGTGGGCCACTTTCATCATG
PGL3-688/+19dele TTGACGGAGTGCAAGCCCGGGCTTCCTATTTCGGGAGCAAGGGGTGTGGGCCACTTTCATCATG
PGL3-688/+19 TTGAGGGAGTGCAAGCCCGGGCTTCCTATTTCGGGAGCAAGGGGTGTGGGCCACTTTCATCATG
PGL3-777/+19dele TTGAGGGAGTGCAAGCCCGGGCTTCCTATTTCGGGAGCAAGGGGTGTGGGCCACTTTCATCATG
PGL3-777/+19 TTGAGGGAGTGCAAGCCCGGGCTTCCTATTTCGGGAGCAAGGGGTGTGGGCCACTTTCATCATG
Rat_gene TTGAGGGAGTGCAAGCCCGGGCTTCCTATTTCGGGAGCAAGGGGTGTGGGCCACTTTCATCATG
PGL3-116/+19 TGATGCGAGGGCTATTTAAAGAGACTCTCCAGCCGGGAGCCGCGATTCTCACTGCCTGCACGC
PGL3-503/+19 TGATGCGAGGGCTATTTAAAGAGACTCTCCAGCCGGGAGCCGCGATTCTCACTGCCTGCACGC
PGL3-532/+19 TGATGCGAGGGCTATTTAAAGAGACTCTCCAGCCGGGAGCCGCGATTCTCACTGCCTGCACGC
PGL3-688/+19dele TGATGCGAGGGCTATTTAAAGAGACTCTCCAGCCGGGAGCCGCGATTCTCACTGCCTGCACGC
PGL3-688/+19 TGATGCGAGGGCTATTTAAAGAGACTCTCCAGCCGGGAGCCGCGATTCTCACTGCCTGCACGC
PGL3-777/+19dele TGATGCGAGGGCTATTTAAAGAGACTCTCCAGCCGGGAGCCGCGATTCTCACTGCCTGCACGC
PGL3-777/+19 TGATGCGAGGGCTATTTAAAGAGACTCTCCAGCCGGGAGCCGCGATTCTCACTGCCTGCACGC
Rat_gene TGATGCGAGGGCTATTTAAAGAGACTCTCCAGCCGGGAGCCGCGATTCTCACTGCCTGCACGC
TATA box
Rat+19/0H(Rev)
PGL3-basic AAGCTTGGCATTCCGGTACTGTTGGT 3′
PGL3-116/+19 AAGCTTGGCATTCCGGTACTGTTGGT
PGL3-503/+19 AAGCTTGGCATTCCGGTACTGTTGGT
PGL3-532/+19 AAGCTTGGCATTCCGGTACTGTTGGT
PGL3-688/+19dele AAGCTTGGCATTCCGGTACTGTTGGT
PGL3-688/+19 AAGCTTGGCATTCCGGTACTGTTGGT
PGL3-777/+19dele AAGCTTGGCATTCCGGTACTGTTGGT
PGL3-777/+19 AAGCTTGGCATTCCGGTACTGTTGGT
Rat_gene CTCCTCTCTCATTGCACCATGGCGGACGCCTTTGTCG
H-FABP start codon
HindIII

**Figure 3-4 Schematic representation of the reporter gene constructs**

**The grey boxes represent ER elements while the black boxes are the TATA box. The arrows show the transcriptional initiation site.**



## 3.3 Reporter gene transfection with Lipofectamine

A variety of strategies are available for the delivery of genes into eukaryotic cells. These techniques fall into three categories: transfection by physical methods, virus-mediated transduction, and transfection by biochemical methods. The choice of a particular transfection method is determined by the experimental goal (e.g., the type of assay to be used for screening, the ability of the cell line to survive the stress of transfection, and the efficiency requirement of the system).

There are three physical methods of transfection in common use: biolistic particle delivery, direct microinjection, and electroporation. The first two methods function through perforation of the cell membrane and subsequent delivery of the DNA into the cell. Electroporation uses brief electrical pulses to create transient pores in the plasmid membrane through which nucleic acids enter into cells. They perform well with some cell lines that remain impermeable to foreign DNA with other techniques. However, physical transfection methods require very special equipment and skilful operation to determine the optimum condition empirically in each laboratory and for each cell type.

Virus-mediated transduction is based on the use of viral vectors to transfer exogenous genes into target cells. These vectors have certain advantages, including high levels of transduction and efficient and stable integration of foreign DNA into the host genome. In addition to laboratory safety concerns, the usefulness of viral vectors is limited by a few factors such as host immune and inflammatory reactions, difficulty of large-scale production, size limit of the foreign DNA, random integration into the host genome, and the risks of inducing tumorigenic mutations and generating active viral particles through recombination (Clark and Hersh, 1999).

Biochemical transfection can be accomplished by various means. Early methods that are still used today include the combination of plasmid DNA with either DEAE-Dextran or calcium phosphate, and co-precipitation. By the 1980s, the method of mixing cationic lipids with plasmid DNA had been developed to efficiently transfer DNA and RNA into cells by a process called lipofection. The basic structure of cationic lipids consists of a positively charged head group and one or two hydrocarbon chains. The charged head group governs the interaction between the lipid and the phosphate backbone of the nucleic acid, and facilitates DNA condensation. Cationic lipids are usually formulated with a neutral co-lipid or helper lipid, followed by extrusion or microfluidization, which results in a unilamellar liposomal structure with a positive surface charge when formulated in water. The positive surface charge of the liposomes also mediates the interaction of the nucleic acid and the cell membrane, allowing for fusion of the liposome/nucleic acid (transfection complex) with the negatively charged cell membrane. The transfection complex is considered to enter the cell through endocytosis. Once entered into the cell, the complex needs to escape the endosomal pathway, diffuse through the cytoplasm, and enter the nucleus for gene expression. It is considered that cationic lipids facilitate transfection during the early steps of the process by mediating DNA condensation and DNA/cellular interaction.

Lipofectamine 2000 transfection reagent (Invitrogen, Burlington, Ontario) is a cationic lipid formulation that has been known to offer high transfection efficiencies and protein expression levels in a wide variety of adherent and suspension cell lines. It is a common transfection reagent for simple and effective nucleic acid delivery, and it had been used successfully for reporter gene transfection of myoblasts in previous research of

our laboratory (Wu and Haunerland, 2001). Therefore, Lipofectamine 2000 reagent was initially chosen to transfect the luciferase reporter gene into myoblasts.

It is worth mentioning here that pGL3-777/+19 was consistently used as a standard vector to verify the transfection efficiency under different conditions in the following sections of this chapter.

### 3.3.1 Materials and methods

Rat L6 myoblasts were cultured in Dulbecco's Modified Eagle medium (DMEM, Invitrogen, Burlington, ON) supplemented with 10 % (v/v) fetal bovine serum (Invitrogen, Burlington, ON), 150 U/ml penicillin and 150 μg/ml streptomycin (Sigma, Oakville, ON), as previously described in Chapter 2. Prior to transfection, cells were grown for 3 days to reach 90 % confluency in the 35×10 mm treated polystyrene dishes (Corning, Acton, MA). After removal of the medium, the cells were rinsed twice with phosphate buffered saline (10 mM phosphate, 0.9 % NaCl, pH7.4) and then 2 ml Opti-Mem I reduced serum medium (Invitrogen, Burlington, ON) was added to each dish. Cells were transfected transiently with pGL3-777/+19 and Lipofectamine 2000 reagent according to the manufacturer's instructions. For each dish, 5 μg luciferase reporter gene construct, 500 ng internal control vector pRL-TK and 12 μl Lipofectamine reagent were gently mixed in 400 μl Opti-Mem I medium and incubated at room temperature for 15 min. Subsequently, the transfection mixture was added into the cell dishes. After 6 h incubation at 37 °C, 5 % $CO_2$, the transfection medium was replaced by 1 % serum medium without antibiotics.

Fatty acid treatment of transfected cells was carried out as described previously (Chang et al, 2001). Briefly, complexes of linoleic acid/BSA (1800 μM 18:2/200 μM BSA or 2400 μM 18:2/200 μM BSA) were prepared by dissolving fatty-acid-free BSA (Sigma, Oakville, ON) and the sodium salt of linoleic acid (18:2, Sigma, Oakville, ON) in 0.9 % NaCl. The fatty acid/BSA complex solution was then sterile filtered and added to cell culture media following the 6 h transfection period. For fatty acid treatment experiments, the linoleic acid/BSA complex solution was added to transfected cells at final concentrations of 180 μM 18:2 in 20 μM BSA, or 240 μM 18:2 in 20 μM BSA. As a control, the culture medium only with 20 μM BSA was used at the same time. The cells continued to incubate for another 12, 18 or 24 h. For each treatment, three dishes were employed and evaluated separately.

Cells were harvested and lysed with 200 μl passive lysis buffer (Promega, Madison, WI). The Dual Luciferase Reporter Assay System (Promega, Madison, WI) was used to evaluate the relative luciferase activity by a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Each sample was measured twice or three times and then an average of these readings was taken as the value of the sample.

### 3.3.2 Results and discussion

Transfection of rat myoblasts with the full-length rat H-FABP promoter construct resulted in strong expression of luciferase under this condition (Figure 3-5). The addition of BSA had no influence on the reporter gene expression, and the luminescence did not change greatly between 12 and 24 hours post-transfection. Therefore, it was concluded that the rat H-FABP promoter was functional in this expression system.

When transfected cells were treated with fatty acids, the inducible expression of luciferase activity was measured. Compared with the control conditions, treatment with 180 μM fatty acid made no measurable increase in gene expression. In contrast, the larger fatty acid concentration (240 μM) showed up to 60 % higher luciferase activity. It is obvious that the selected transfection protocol using Lipofectamine 2000 operated effectively for luciferase reporter genes with the rat H-FABP promoter. An 18 h incubation period after 6 h transfection (for a total treatment time of 24 h) with 240 mM linoleic acid appeared optimal.

However, the cells treated with 240 μM 18:2/20 μM BSA looked severely damaged under microscope (Figure 3-6), and the number of living cells was dramatically reduced. Most likely, the combination of transfection reagent and immediate fatty acid treatment changed cell membrane structure, worse than either of them alone, and thereby cells lost their normal morphology and functions.

**Figure 3-5 Luciferase assay of myoblasts transfected with Lipofectamine**

**L6 myoblasts were transfected with pGL3-777/+19 by Lipofectamine, and then L6 myoblasts were treated with different media for 12, 18, or 24 h. The results are the average of at least three independent determinations ± SD.**

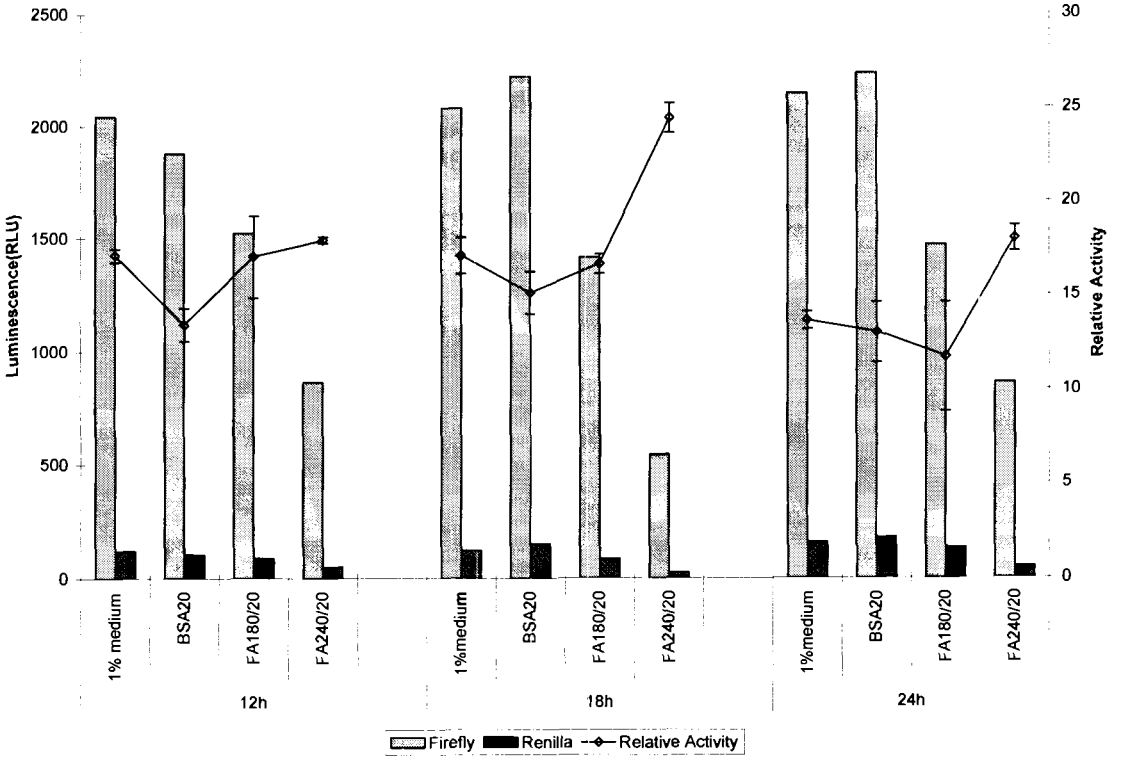
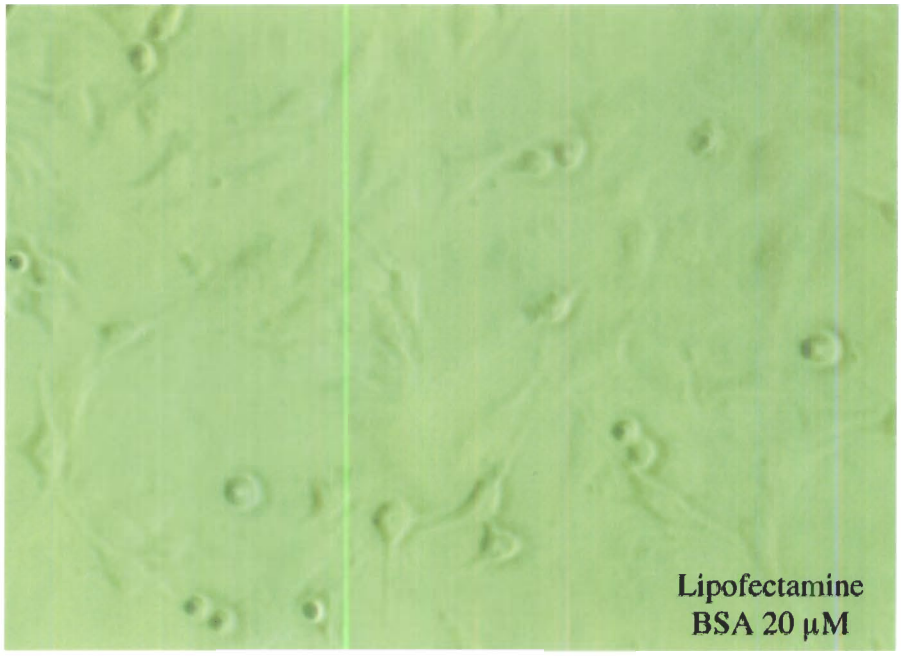
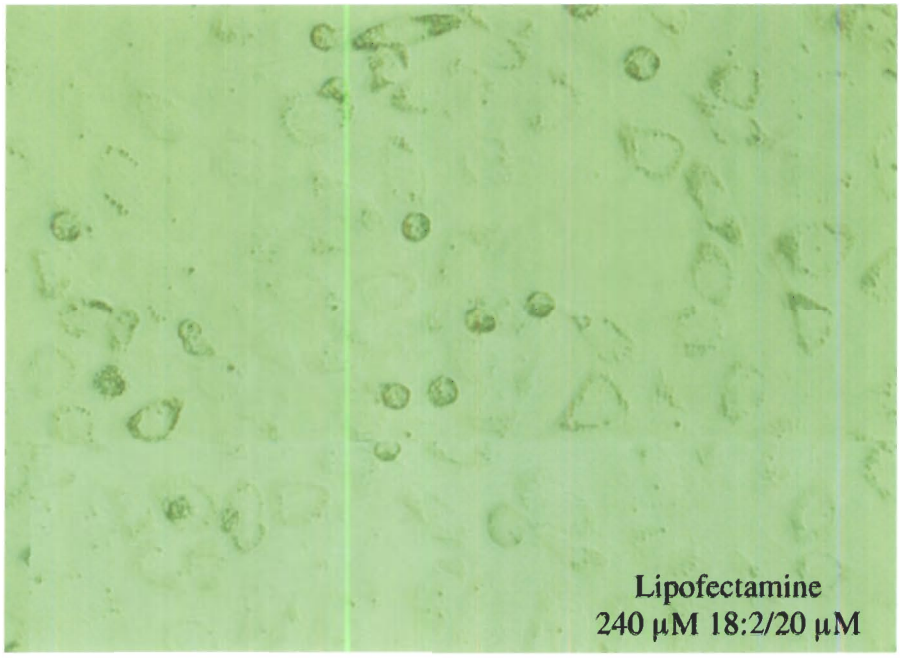


**Figure 3-6** **Morphological change of transfected myoblasts under FA treatment**

**Upper photo: L6 myoblasts were treated with 20 μM BSA in 1 % serum medium for 18 h after transfection with Lipofectamine. Bottom photo: L6 myoblasts were treated with 240 μM 18:2/20 μM BSA in 1 % serum medium for 18 h after transfection with Lipofectamine.**

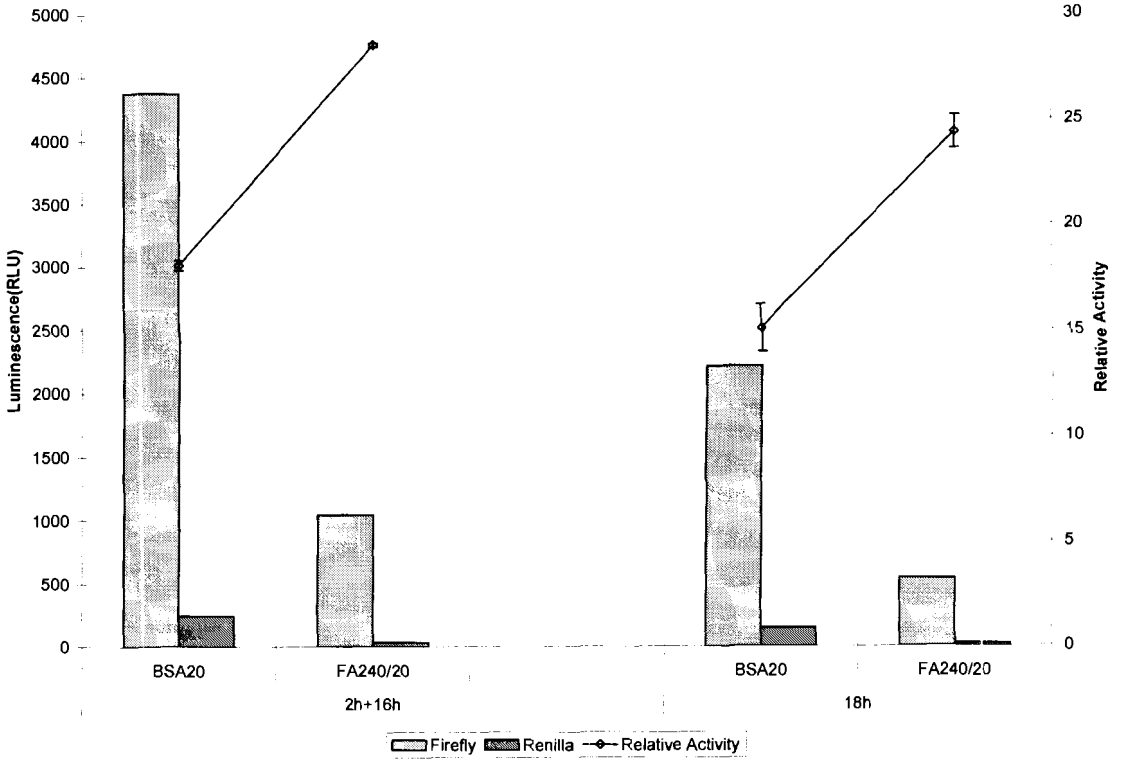


To improve the rate of cell survivals, various modifications of the current protocol were attempted. It was found that adding a 2 h recovery interval with fresh medium after the 6 h transfection considerably improved cell viability. In order to keep the overall treatment period as short as possible, the fatty acid incubation step was reduced to 16 h, so that the total treatment was kept at 24 h. These results are shown in Figure 3-7. While reporter gene expression was larger (up to 2-fold), myoblasts were still severely damaged. This raised the possibility that the observed higher relative luciferase activity is not due to specific induction of the reporter gene promoter, but a more pronounced decrease of the control *Renilla* luciferase. Given that such a scenario would make the interpretation of the data difficult, especially with respect to the identification of the fatty acid response element, it was decided to search for a gentler transfection protocol that would improve the rate of myocyte survivals.

**Figure 3-7** **Luciferase assay of myoblasts with 2h interval between transfection and FA treatment**

**Left group: as a 2 h recovery interval was added with fresh 1 % serum medium after the 6 h transfection, the fatty acid or BSA incubation step were reduced to 16 h. Right group: the 18 h incubation for FA or BSA treatment without 2 h interval. The results are the average of at least three independent determinations ± SD.**



## 3.4 Reporter gene transfection with Fugene 6

Fugene 6 is a multi-component lipid-based and non-liposomal transfection reagent. According to the manufactures (Roche Applied Science), it allows delivery of a broad range of nucleic acid, from plasmids (up to 10 kb) to oligonucleotides, into various cell types such as L6 myoblasts. Since Fugene 6 was introduced by Boehringer-Mannheim Biochemicals in 1997, its popularity has increased due to its ease of use, reduced cytotoxicity, and the high level of transfection in many different cell lines.

Fugene 6 has the advantage of transfecting equally well in the presence or absence of serum. Thus, any serum stimulation effects that might influence the interpretation of the effects of the transfected genes can be avoided. Fugene 6 was shown to be gentle on the cells including many primary cell types. Adherent cells can be trypsinized and transfected by the DNA-Fugene 6 complex prior to plating. Elimination of toxic side effects combined with high transfection efficiency led to accurate physiological results in more than 700 cell types (Refer to http://www.roche-diagnostics.com/products_services/fugene.html).

### 3.4.1 Materials and methods

Rat L6 myoblasts were cultured in the same way as previously described in section 3.3.1. Prior to transfection, cells were grown to reach 70 % confluency in the 35×10 mm dishes. After removal of the medium, the cells were rinsed twice with phosphate buffered saline (10 mM phosphate, 0.9 % NaCl, pH 7.4) and then 2 ml Opti-Mem I reduced serum medium (Invitrogen, Burlington, ON) was added into each dish. Cells were transfected with Fugene 6 according to the manufacturer's instruction. For

each dish, 1 μg pGL3-777/+19 reporter gene construct, 100 ng internal control vector pRL-TK and 3 μL Fugene 6 were gently mixed in 100 μl Opti-Mem I medium and incubated at the room temperature for 15 min. Subsequently, the transfection mixture was added into the cell dishes. After 6 h of incubation at 37 °C, 5 % $CO_2$, the transfection medium was replaced by 1 % serum medium without antibiotics and incubated for another 2 h. At the same time, other dishes of L6 cells were transfected with Lipofectamine 2000 as the control experiment to Fugene 6 following the previous method.

Fatty acid treatment of transfected cells was carried out as in the previous section. In brief, the linoleic acid/BSA complex solution was added to transfected cells at the final concentration of 180 μM 18:2/20 μM BSA or 240 μM 18:2/20 μM BSA. BSA groups were treated only with 20 μM BSA in the medium. All cells were incubated for 16h. For each treatment, three dishes were used in parallel and evaluated separately.

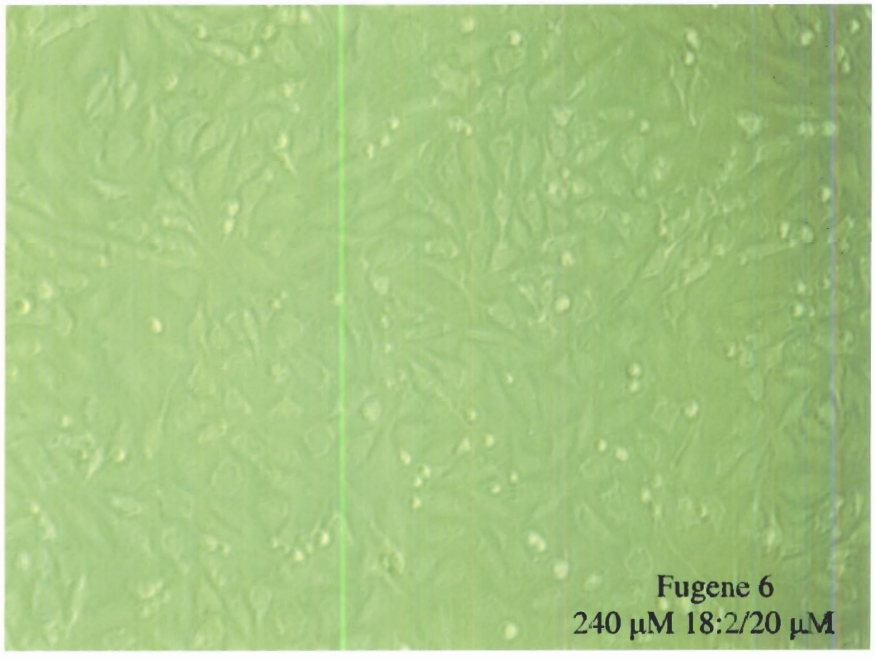
Cells were harvested to evaluate the relative luciferase activity using the same method. Each sample was measured twice and then an average of these readings was taken as the value of the sample.

### 3.4.2 Results and discussion

From Figure 3-8, it seems that Fugene 6 is much gentler than Lipofectamine 2000. Although cells in Fugene 6 groups appeared to be abnormal, their rate of survival had been improved considerably and morphological changes were less pronounced.

**Figure 3-8** **Improved morphological observation of myoblasts transfected with Fugene 6 compared with Lipofectamine**

**Upper photo: L6 myoblasts were transfected with Fugene 6 and then treated with 240 μM 18:2/20 μM BSA for 16 h. Bottom photo: L6 myoblasts were transfected with Lipofectamine and then treated with 240 μM 18:2/20 μM BSA for 16 h.**



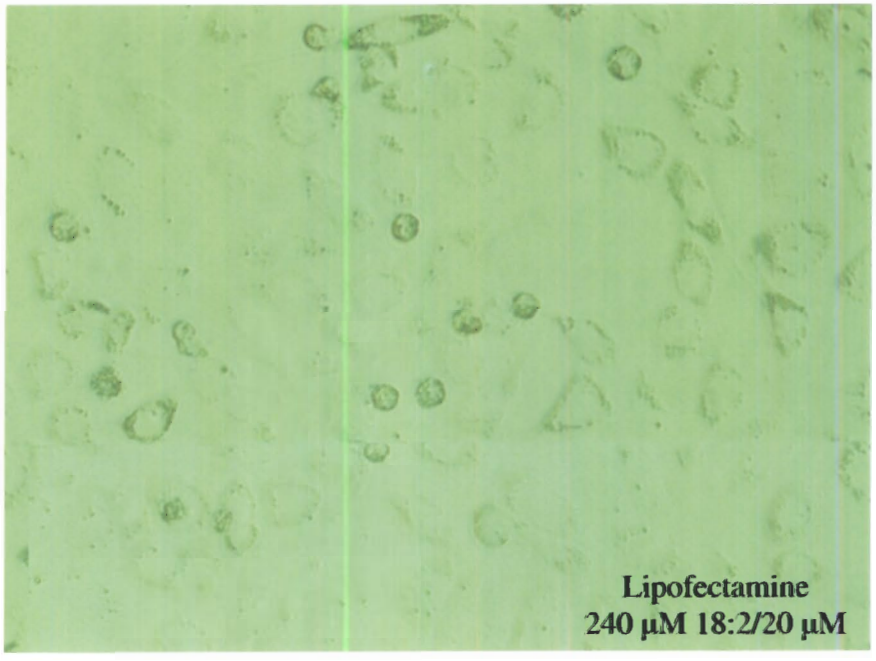
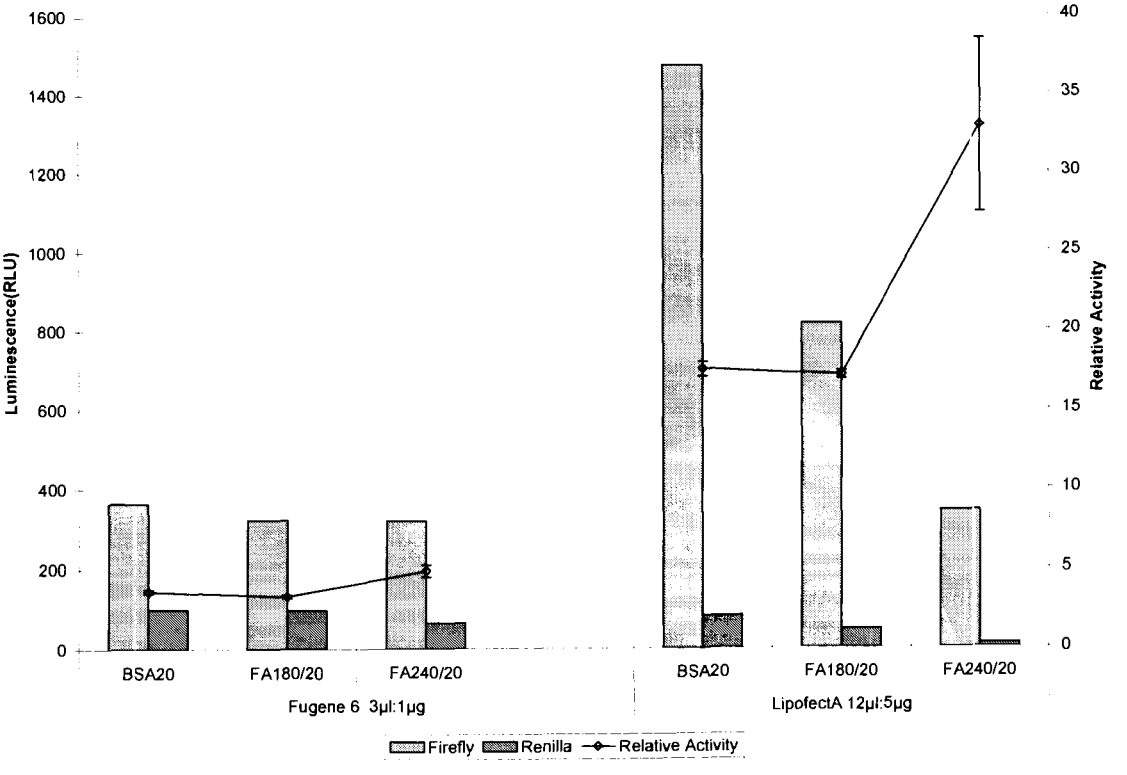



**Figure 3-9** **Luciferase assay of myoblasts transfected with Fugene 6 or Lipofectamine**

**Left group: L6 myoblasts were transfected with Fugene 6 and then treated with different media for 16 h. Right group: L6 myoblasts were transfected with Lipofectamine and then treated for 16 h. The results are the average of at least three independent determinations ± SD.**



As the Fugene transfection protocol uses much less reporter gene construct (20 % of the amount used for Liopfectamine), the raw numbers measured for firefly luciferase in the BSA controls were much lower (360 vs 1474, Figure 3-9). It is worth pointing out that *Renilla* luciferase readings in all Fugene 6 groups were notably higher than those in Lipofectamine groups. Moreover, the luciferase activities of Fugene 6 transfected cells remained high even after fatty acid exposure. This supports the conclusion from the morphological observation that rate of cell survival was greatly improved after fatty acid exposure in Fugene 6 transfected cells.

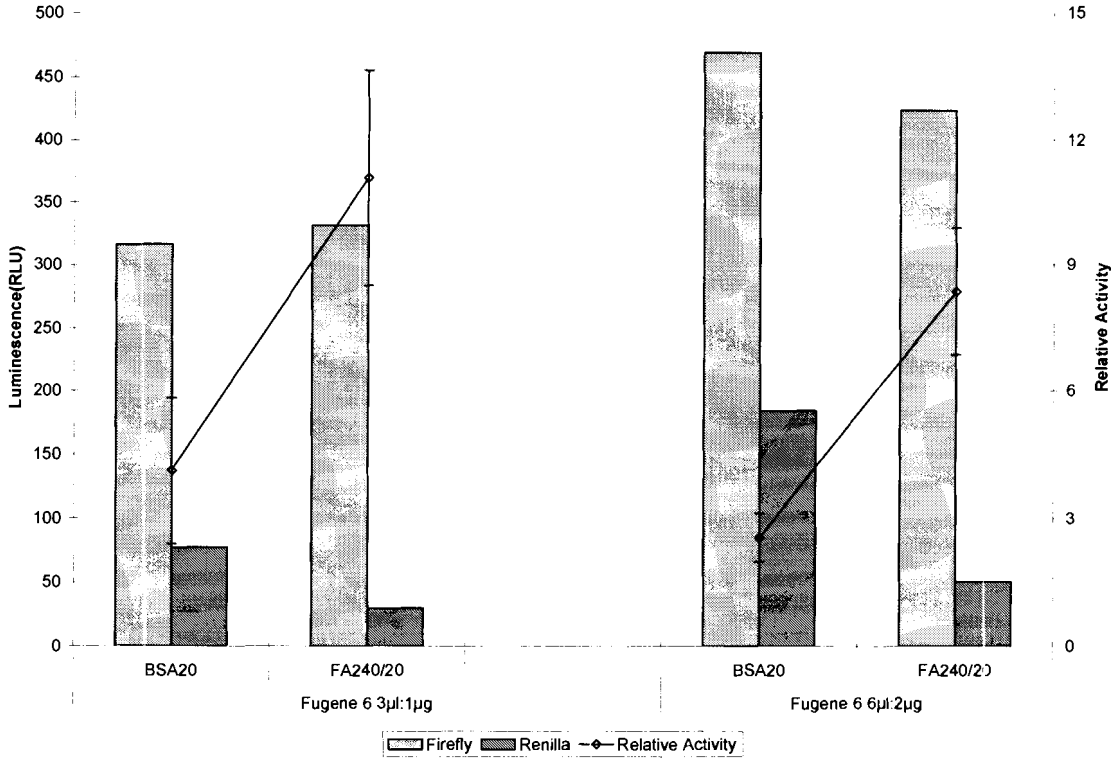
While the Fugene 6 transfection reagent is much more effective and mild than Lipofectamine 2000, the relatively low raw luciferase activity limits the reliability of the assay. To improve the enzyme activity, the amounts of reporter gene and Fugene 6 were doubled (2 μg/6 μl). However, the luciferase readings did not increase significantly as expected (Figure 3-10), indicating that the amount of plasmid used in the transfection is not the only reason of the lower luciferase expression observed in these cells.

It was noted that the myoblasts used in the Fugene experiments had been cultured for more passages than those in the earlier Lipofectamin experiments, raising the possibility that a change of myoblast properties contributed to the lower transfection efficiency. In fact, the distributor of the cell stock mentions on its website (http://www.atcc.org/common/catalog/numSearch/numResults.cfm?atccNum=CRL-1458 #22581) that some components of myoblasts may be depleted following several cell passages. This "culture drift" results from the phenotypic and genotypic changes that accumulate over time in most cell lines as they age and evolve in culture. It is advisable therefore to freeze a substock of myoblasts at low passage and periodically re-clone these

cells to reduce alterations in culture characteristics. Robinson et al. (1993) reported that L6 cells were carried as myoblasts for no more than 10 passages in growth medium, before they de-differentiated. Other work in our laboratory also indicated that the mRNA levels for H-FABP declined with increasing passage, while adipocyte FABP increased (Rickers-Haunerland, unpublished observation). The latter protein is normally not expressed in muscle cells, but characteristic for adipocytes. Therefore, we decided to prepare substocks of myoblasts and always use identical passages of cells in future experiments.

**Figure 3-10 Luciferase assay of myoblast transfected with more vector DNA**

**Left group: old protocol of Fugene 6 3 µl/vector 1 µg. Right group: new protocol used double amount of pGL3-777/+19 for transfection, Fugene 6 6 µl/vector 2 µg. The results are the average of at least three independent determinations ± SD.**



## 3.5 3rd passage myoblasts transfected with Fugene 6

The first passage of myoblast was defined as the original sample ordered from ATCC. It was plated and subcultured into 2nd passage during its exponential growth phase (log phase). The 2nd passage cultures were cultured until they reached the log phase, and then preserved in aliquots in liquid nitrogen. Transfection always started from the stored 2nd passage myoblast substock; for all subsequent experiments, the subcultured 3rd passage cells were used.

### 3.5.1 Materials and methods

Except for using 3rd passage myoblasts, all other details of experiment were the same as last section: 2 μg pGL3-777/+19 reporter gene construct, 200 ng internal control pRL-TK, and 6 μL Fugene 6 were added into each cell dish for 6 h transfection. The transfection medium was replaced by 1 % serum medium for a 2 h interval. Fatty acid treatment of transfected cells was carried out at the final concentration of 240 μM 18:2/20 μM BSA for another 16h.

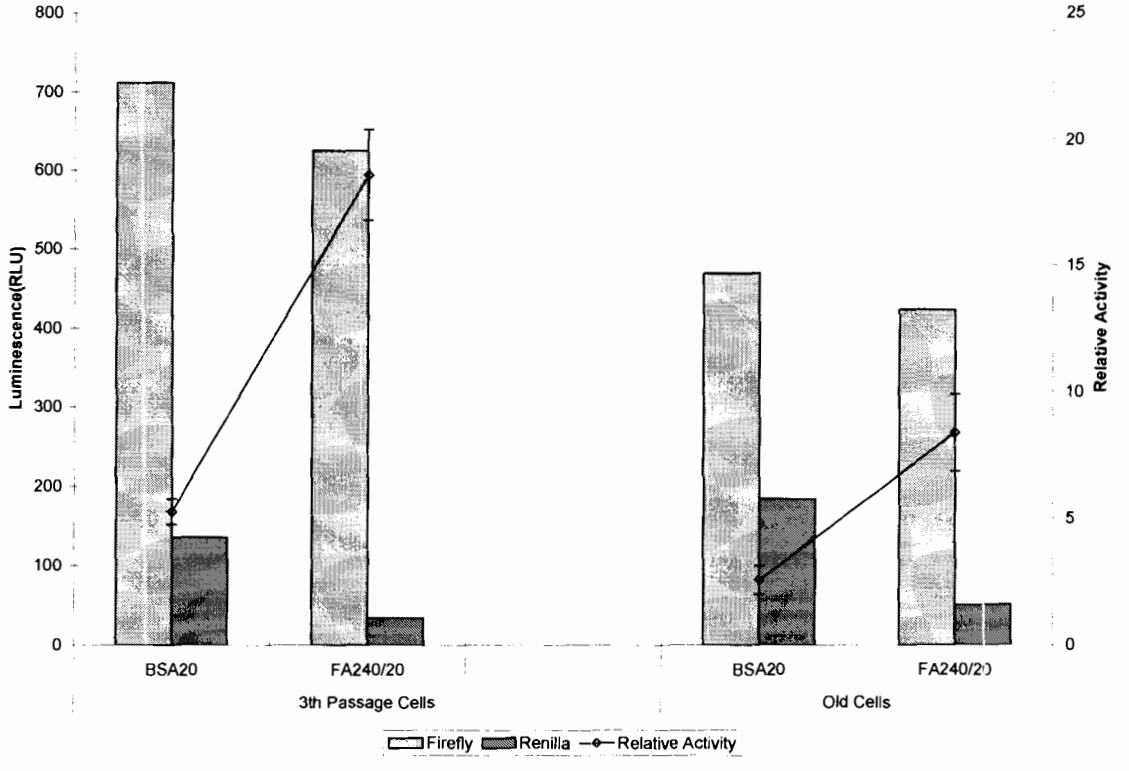
### 3.5.2 Results and discussion

Compared with older myoblasts, there was a limited improvement on luciferase activity in the 3rd passage cell group (Figure 3-11). On the other hand, cells did appear to be affected more by fatty acid treatment, as judged from visual inspection. In contrast, untreated cells observed healthy and grew rapidly. Since most factors in the reporter gene protocol had been modified, such as the transfection reagent, the amount of plasmids, treatment time point, and cell passage, the only other possible reason for the low cell viability appeared to be the fatty acid solution itself. Coincidently, it was also found from

the recorded images that cells were always able to keep the normal morphological appearance until fatty acid exposure, which generally resulted in obvious cell damage. Therefore, the certain modifications in the fatty acid solution were attempted, as described in the following section.

**Figure 3-11** $3^{rd}$ **passage myoblasts transfected with Fugene 6**

**Left group: $3^{rd}$ passage myoblasts were transfected with Fugene 6, and then treated with 240 μM 18:2/20 μM BSA for 16h. Right group: except for using old passage myoblasts, all other details of experiment were the same as left group. The results are the average of at least three independent determinations ± SD.**



## 3.6 Different molar ratios of fatty acid/BSA for treatment

Reviewing previous experiments from our laboratory, up to 240 μM linoleic acid had been used to treat L6 myoblasts to investigate the regulation of H-FABP mRNA expression (Chang et al, 2001); however, the ratio between linoleic acid and BSA may have varied. Usually, 60 μM 18:2/20 μM BSA (ratio 3:1) was used to treat transfected myoblasts in a subsequent study on locust muscle FABP (Wu and Haunerland, 2001). The low concentration was sufficient to induce reporter gene expression from the stronger locust promoter, but this solution cannot induce any change of luciferase activity for the rat promoter in the previous work (data not shown). In an unrelated study in isolated rat cardiac myocytes, Luiken et al (1997) concluded that both the fatty acid and the molar ratio between fatty acid and BSA are important for maintaining viable cells under physiologically relevant conditions. Apparently, the correct ratio of fatty acid to BSA is essential for myoblasts treatment. It is possible that at the high ratio of fatty acid to BSA, which is used in our studies (240 μM 18:2/20 μM BSA, 12:1), too many fatty acids are present in low affinity binding sites that can be rapidly released and cause cell damage. Hence, the effect of an increased BSA content (240 μM 18:2/80 μM BSA, 3:1) was investigated.

### 3.6.1 Materials and methods

The 3$^{rd}$ passage myoblasts were transfected for 6 h in the same protocol as described in last section. After 2 h rest, transfected cells were treated with 240 μM 18:2/80 μM BSA in 1 % serum medium for another 16 h. Cells were harvested and evaluated by the luciferase assay following the previous method.

### 3.6.2 Results and discussion

From myoblast morphology shown in Figure 3-12, it was very obviously that cells under the new treatment (240 μM 18:2/80 μM BSA) grew almost as healthily as the BSA controls, in a major improvement over the treatment with the lower BSA content. Indeed, the ratio between fatty acid and BSA appears to be the major reason for the cell damage at higher fatty acid concentration.

The higher the ratio of fatty acid/BSA, the more free fatty acids are in the medium. This may create a larger concentration gradient to drive more free fatty acids into myoblast cytoplasm at the beginning of the treatment. Free fatty acids may damage the membrane systems due to their detergent effects (Das et al., 1991) or through the enzymatic formation of lipid radicals (Yamomoto and Niki, 1990).

Under these conditions, luciferase levels were greatly increased (eg. from 700 to 5000). It has also been proved that the ratio of fatty acid/BSA was the key point for cell survival during the treatment. However, the two individual experiments under the same conditions still showed a significant variation in luciferase readings (Figure 3-13). While the reason for this variability was not clear, it was speculated that variation in the individual plasmid concentration used for transfection may be partially responsible. Detecting and quantitating small amounts of DNA is extremely important in a wide variety of biological applications. The most commonly used technique for measuring nucleic acid concentration is the determination of absorbance at 260 nm (A260). The major disadvantages of the absorbance method are the large relative contribution of nucleotides, single-stranded nucleic acids and proteins to the signal, the interference caused by contaminants commonly found in nucleic acid preparations, the inability to

distinguish between DNA and RNA, and the relative insensitivity of the assay (an A260 of 0.1 corresponds to a 5 μg/ml dsDNA solution). Because the plasmids were used at very limited amount for transfection, just 2 μg/dish, the small measurement bias of concentration could lead to big changes of DNA amount at the step of transfection. Therefore, we attempted to increase the sensitivity by using fluorometry instead of a UV spectrophotometer to measure the DNA concentration, and repeat the experiments with more accurately determined plasmids.

PicoGreen dsDNA Quantitation Reagent (Molecular Probes, Eugene, OR) is an ultrasensitive fluorescent nucleic acid stain for quantitating double-stranded DNA (dsDNA) in solution. The TD-700 Laboratory Fluorometer (Turner Designs, Sunnyvale, CA) in conjunction with Picogreen dsDNA quantitation kit was chosen to measure the concentration of plasmid DNA because it can quantify as little as 25 pg/ml of dsDNA (50 pg dsDNA in a 2-ml assay volume). Moreover, the linear detection range of the PicoGreen assay extends over more than four orders of magnitude in DNA concentration - from 25 pg/ml to 1000 ng/ml. This linearity is maintained in the presence of salts, urea, ethanol, chloroform, detergents, proteins and agarose compounds that commonly contaminate nucleic acid preparations. The assay protocol has been developed to minimize the fluorescence contribution of RNA and single-stranded DNA (ssDNA).

Since the ratio of Fugene 6 to plasmid DNA plays an important role in transfection, the exact ratios were determined fluorometrically. The whole protocol of transfection and treatment was carried out under the newest condition. The ratio of Fugene 6 to plasmid, as determined by the fluorometer of 6 μl/1μg, gave the best luciferase activity level and inducible change (Table 3-2). As the result, the TD-700

Laboratory Fluorometer combined with Picogreen dsDNA quantitation kit was chosen as the routine method of DNA concentration measurement, and 6 μl Fugene 6 per μg of plasmid was used in all subsequent experiments.

**Figure 3-12 Morphological observation of myoblasts treated by fatty acid/BSA (3:1)**

**Upper photo: L6 myoblasts were treated with 80 μM BSA in 1 % serum medium for 16 h after transfection with Fugene 6. Bottom photo: L6 myoblasts were treated with 240 μM 18:2/80 μM BSA (3:1) in 1 % serum medium for 16 h after transfection.**

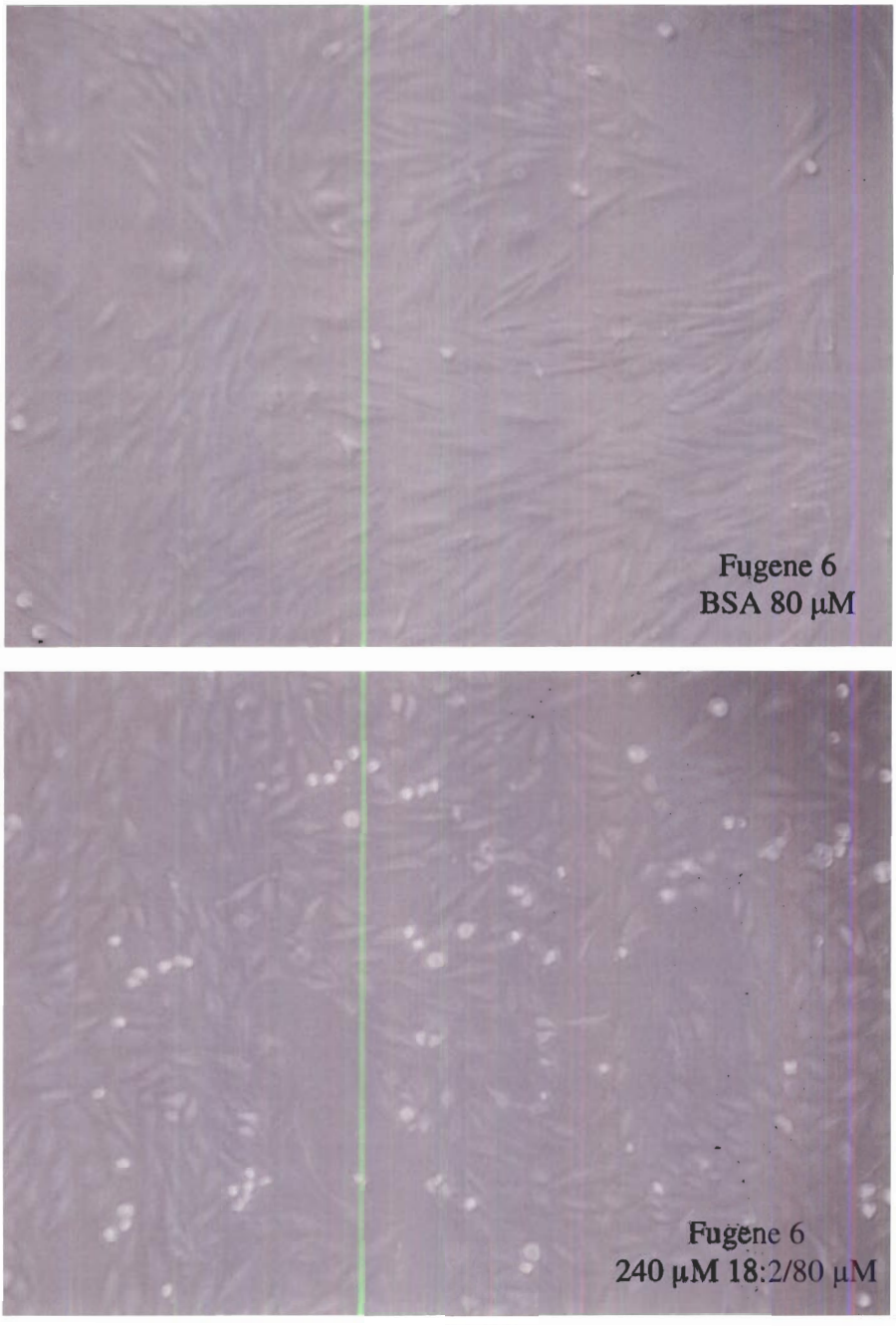


**Figure 3-13 Luciferase activity variation between two experiments with the same condition**

**$3^{rd}$ passage myoblasts were transfected for 6 h with Fugene 6. After 2 h interval, transfected cells were treated with 80 μM BSA or 240 μM 18:2/80 μM BSA in 1 % serum medium for another 16 h. The results are the average of at least three independent determinations ± SD.**

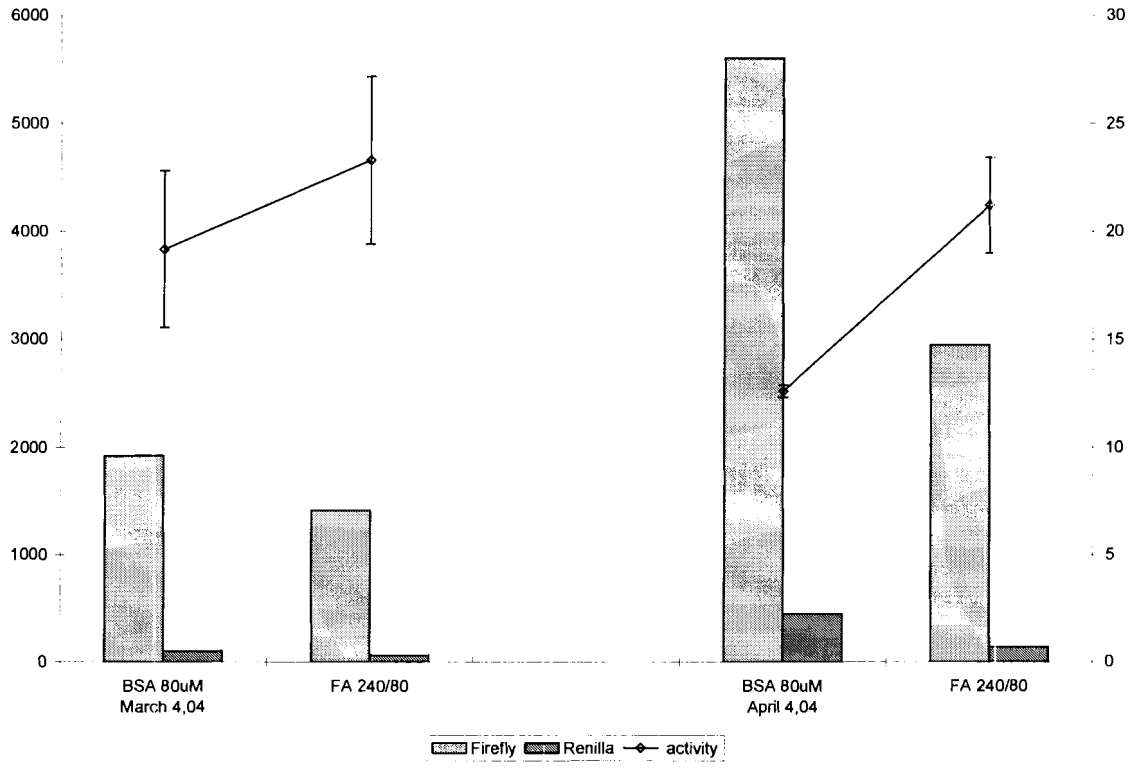


**Table 3-2 Luciferase assay of myoblasts transfected with different ratios of Fugene 6/vector DNA determined by fluorometer**

**The results are the average of at least three independent determinations ± SD.**

| Fugene6/DNA | Treatment | Firefly | *Renilla* | Relative activity | Change |
|---|---|---|---|---|---|
| 6µl:2µg | BSA80 | 52.49 | 93.07 | 0.563±0.063 | -- |
| | FA240/80 | 48.75 | 87.19 | 0.561±0.420 | -- |
| 6µl:1µg | BSA80 | 2081 | 412.2 | 5.055±0.016 | -- |
| | FA240/80 | 1696 | 246.1 | 6.929±0.018 | +37% |
| 6µl:0.5µg | BSA80 | 2116 | 310.3 | 6.905±0.981 | -- |
| | FA240/80 | 1429 | 165.3 | 8.654±0.496 | +25% |
| 3µl:1µg | BSA80 | 26.21 | 34.10 | 0.773±0.225 | -- |
| | FA240/80 | 13.93 | 30.24 | 0.547±0.465 | -- |
| 3µl:0.5µg | BSA80 | 140.9 | 81.13 | 1.767±0.346 | -- |
| | FA240/80 | 95.69 | 55.55 | 1.748±0.363 | -1% |
| 3µl:0.25µg | BSA80 | 514.7 | 144.5 | 3.550±0.403 | -- |
| | FA240/80 | 382.2 | 135.1 | 2.819±0.456 | -21% |

## 3.7 Optimization of transfection conditions

As a comparatively new transfection reagent, Fugene 6 had been on market for only 4 years. During the course of this thesis work, improved protocols were being developed with the aim of optimizing transfection efficiency (Jacobsen et al, 2004). In light of these developments, experiments with several additional modifications were conducted on the particular myoblast cell line to further increase the level of transfection efficiency.

### 3.7.1 Materials and methods

The 3$^{rd}$ passage L6 myoblasts were cultured in DMEM supplemented with 10 % (v/v) fetal bovine serum, 150 U/ml penicillin, and 150 μg/ml streptomycin. Prior to transfection, cells were grown to reach 50 % confluency in 35×10mm dishes. Firstly, Fugene 6 was warmed up at room temperature for 10 min and vortexed for 3 seconds. Then 6 μl Fugene 6 was diluted into 94 μl Opti-Mem I reduced serum medium for each dish. The combination was vortexed afterwards and stood for 5 min at room temperature. Subsequently, 1 μg pGL3-777/+19 reporter gene construct and 100ng internal control vector pRL-TK were added into 100 μl diluted Fugene 6 solution. The mixture was vortexed again and it was then incubated at the room temperature for 20 or 40 min. During this period, the cells were rinsed twice with PBS (pH7.4). Then 2 ml DMEM, Opti-Mem I reduced serum medium, or 10 % serum medium were added into each dish. Subsequently, the incubated transfection mixture was added into the cell dishes. After 6 h incubation at 37 °C and 5 % $CO_2$, the transfection medium was replaced by 1 % serum medium without antibiotics and cells were incubated for another 2 h interval. As a

treatment without fatty acids in this optimization experiment, transfected cells were cultured with 1 % or 10 % serum medium for 16 h or 40 h before harvesting. For each group, three dishes were employed and evaluated separately.

After the incubation period, cells were rinsed twice with 2 ml PBS and lysed with 200 µl passive lysis buffer for 15 min at the room temperature. The cell lysates were collected into microcentrifuge tubes and then spun at 13200 rpm and 4 °C for 2 min. The supernatants were kept at -70 °C before luciferase measurement. The Dual Luciferase Reporter Assay System was used to evaluate the relative luciferase activity by TD-20/20 luminometer. Each sample was measured twice and then an average of these readings was taken as the value of the sample.

### 3.7.2 Results and discussion

The luciferase levels were greatly improved in all groups under the modified protocol (Table 3-3), and the cell morphology was normal. Firefly readings increased from 5000 to at least 10373, and *Renilla* luciferase from 450 to 2000 in most groups. These changes indicate a remarkably enhanced transfection with the reporter gene plasmids.

Compared with 70 % confluent cells, myoblasts at 50 % confluency can be maintained longer in an actively growing state, so that more efficient uptake of the complex of plasmid/Fugene 6 into myoblasts is possible. To reach this density more exactly and reproducibly, the cell density at 50 % confluency was measured and determined as $1.6 \times 10^5$ cells/ml (total volume per 35 mm dish was 2 ml).

Meanwhile, modifications in the Fugene 6 warm-up procedure and its incubation with plasmids greatly enhanced the transfection efficiency. These modifications ensured that Fugene 6 performed in the optimal condition and the transfection complex was formed at the best level prior to transfection. The optimal incubation time for complex formation was determined to be 20 min; longer incubation time at room temperature tended to cause gradual degradation and loss of activity of the transfection complex.

It was always necessary to enhance the transfection efficiency by vortexing Fugene 6 before use, the mixture of Fugene 6-Opti-Mem I medium, and the mixture of diluted Fugene 6–DNA. After a long period of storage, Fugene 6 may be slightly turbid due to the precipitation of some components and vortexing can help dissolve precipitation problem. On the other hand, insufficient mixing resulted in a layer of ethanol on top of the medium (Fugene 6 is supplied in 80% ethanol). Therefore DNA sometimes precipitated when it hit the ethanol layer. As a result, the optimal way was to perform a vortex of Fugene 6 with medium and vector DNA.

Finally, the procedure of cell lysis was also improved. The 15 min incubation with lysis buffer assured complete release of the luciferase enzyme from cells. Together with the subsequent centrifugation and freezing steps, it considerably increased the luciferase readings.

The most important factor for luciferase expression was the culture medium. The 10 % serum medium performed better than 1 % serum medium. This formula was most similar to the normal growth medium for myoblasts, and had all necessary nutrients for cell metabolism. Unlike other transfection agents, Fugene 6 had the ability to work in the

presence of serum. Hence, all the subsequent transfection and treatment steps were carried out with 10 % serum medium.

Although luciferase activities of 40 h treatment after transfection were somewhat higher than 16 h treatment in one group, other groups did not show remarkable differences between these two time points. Since cells would probably tolerate fatty acid less well with longer incubation time, and no consistent, obvious advantage of the extended incubation was visible, it was decided to continue using 16 h incubation in future experiments, as it was generally done in the earlier experiments.

By now, the optimal conditions are summarized in the following detailed protocol: 3$^{rd}$ passage L6 myoblasts are cultured in DMEM supplemented with 10 % (v/v) fetal bovine serum, 150 U/ml penicillin, and 150 µg/ml streptomycin. Prior to transfection, cells are grown to reach 50 % confluency ($1.6x10^5$/ml) in 35×10mm dishes. Firstly, Fugene 6 is warmed up to room temperature for 10 min and vortexed for 3 seconds. Then 6 µl Fugene 6 is diluted into 94 µl Opti-Mem I reduced serum medium for each dish. The combination is vortexed afterwards and stands for 5 min at room temperature. Subsequently, 1 µg reporter gene construct and 100 ng internal control vector pRL-TK are added into 100 µl diluted Fugene 6 solution, vortexed again and then incubated at the room temperature for 20 min. During this period, the cells are rinsed twice with PBS (pH 7.4) and then 2 ml 10 % serum medium without antibiotics is added into each dish. Then the incubated transfection mixture is added into the cell dishes for transfection. After 6 h incubation at 37 °C and 5 % $CO_2$, the transfection medium is replaced by 10 % serum medium without antibiotics and cells are incubated for another 2 h interval. Fatty acid treatment is carried out subsequently for 16 h. After the treatment period, cells are rinsed

twice with 2 ml PBS and lysed with 200 μl passive lysis buffer for 15 min at the room temperature. The cell lysates are collected into microcentrifuge tubes and then spun at 13200 rpm and 4 °C for 2 min. The supernatants are kept at -70 °C before luciferase measurement. The Dual Luciferase Reporter Assay System is used to evaluate the relative luciferase activity by TD-20/20 luminometer. Each sample is measured twice and then an average of these readings is taken as the value of the sample. For each group, three dishes are employed and evaluated separately.

**Table 3-3 Luciferase assay of myoblasts with different media for transfection and treatment**

**The results are the average of at least three independent determinations ± SD.**

| Transfection | Treatment | Firefly | *Renilla* | Relative activity |
|---|---|---|---|---|
| 20 m+ 10 % Medium | 1 % Medium +16 h | 23110 | 2741 | 8.434±0.016 |
| | 10 % Medium+16 h | 32269 | 5471 | 5.893±0.158 |
| | 10 % Medium+40 h | 50249 | 8129 | 6.201±0.345 |
| 20 m+ DMEM | 1 % Medium +16 h | 19010 | 1865 | 10.19±0.071 |
| | 10 % Medium+16 h | 25628 | 4115 | 6.236±0.220 |
| | 10 % Medium+40 h | 22249 | 4994 | 4.457±0.089 |
| 20 m+ Opti-Mem | 1 % Medium +16 h | 10373 | 972.0 | 10.67±0.028 |
| | 10 % Medium+16 h | 13733 | 2236 | 6.142±0.071 |
| | 10 % Medium+40 h | 13736 | 2438 | 5.635±0.158 |
| 40 m+ 10 % Medium | 1 % Medium +16 h | 17012 | 1759 | 9.672±0.308 |
| | 10 % Medium+16 h | 26348 | 3885 | 6.785±0.070 |
| | 10 % Medium+40 h | 29309 | 4348 | 6.740±0.016 |
| 40 m+ DMEM | 1 % Medium +16 h | 14684 | 1409 | 10.45±0.219 |
| | 10 % Medium+16 h | 19399 | 2898 | 6.688±0.387 |
| | 10 % Medium+40 h | 14609 | 3119 | 4.676±0.134 |
| 40 m+ Opti-Mem | 1 % Medium +16 h | 10794 | 874.0 | 12.36±0.431 |
| | 10 % Medium+16 h | 13139 | 2141 | 6.139±0.228 |
| | 10 % Medium+40 h | 10675 | 2085 | 5.116±0.135 |

## 3.8 Optimization of treatment conditions

Based on the optimal transfection protocol, further parameters of fatty acid exposure needed to be studied to optimize the treatment condition. The complex of 240 μM linoleic acid and 80 μM BSA (3:1) in 1 % serum that was used in the past still showed some cytotoxic effects. Furthermore, because it was prepared from stock-solutions, which has been stored for several weeks at -20 °C, the concentrations of either fatty acid or BSA may have been altered due to precipitation or interactions with the wall of the tube. Therefore, other fatty acid concentrations and preparations were used to determine if improvements over the previous conditions could be achieved when using the optimized transfection protocol.

### 3.8.1 Materials and methods

The transfection procedure was exactly the same as the optimized one in the last section. After 2 h rest period, fatty acid exposure for transfected myoblasts was carried out at the final concentration of 120 μM 18:2/40 μM BSA (3:1) in 1 % or 10 % serum medium for 16 h. Then cells were harvested and luciferase activities were evaluated following the optimized method.

Since the readings of luciferase activities were higher than 10000 under newly optimized transfection condition, it was considered necessary to adjust the luminometer sensitivity from 53.1 % down to 35.2 % from this time on; past luciferase readings should be reduced by a factor of 11.61 for comparison with readings at the lower sensitivity.

### 3.8.2 Results and discussion

Transfected myoblasts were tested with 120 μM 18:2/40 μM BSA in 10 % or 1 % medium for 16 hours and the corresponding result is as shown in Figure 3-14. In the 10 % medium firefly luciferase increased 40 %, while *Renilla* luciferase remained the same. It appeared that under the optimal conditions, the lower fatty acid concentration of 120 μM satisfactorily induced the up-regulation of reporter gene expression. A small amount of fatty acid may also be present in the 10 % fetal bovine serum, but it is unlikely to have a major effect on the experimental results. Repetitions of these experiments, however, showed considerable variations, likely due to the longer storage of the home-made stock solution (Table 3-4). Because it is not possible to accurately weight the small amounts of fatty acids needed for single treatments, and to prepare the complex with BSA reproducibly for each experiment, commercially prepared standard fatty acid solutions from Sigma were tested. Stabilized stock solutions were obtained as the complex of linoleic acid with BSA (molar ratio 2:1), the complex of a mix of linoleic acid and oleic acid with BSA (molar ratio 4:1), and the complex of linoleic acid, oleic acid, and arachidonic acid with BSA (molar ratio 6:1). After a serial dilution in DMEM, these fatty acids were used to treat transfected myoblasts in 10 % serum medium at different concentrations and molar ratios (Table 3-5). Among these experiments, the 2:1 complex of linoleic acid and BSA (120 μM 18:2/60 μM BSA) gave the clearest induction of gene expression, essentially confirming that 120 μM linoleic acid is the optimal compromise between gene induction and low cytotoxicity.

It was concluded here that the Sigma preparation of 120 μM linoleic acid with 60 μM BSA (2:1) in 10 % serum was the optimum condition of fatty acid treatment. After

the optimization of all factors of transfection and treatment conditions, the series of reporter gene constructs of the rat H-FABP promoter fragments was ready to be introduced into cultured L6 myoblasts to test the potential element function under fatty acid exposure.

**Figure 3-14 Luciferase assay of transfected myoblast with different treatment media**

**Transfected myoblasts were tested with 40 μM BSA or 120 μM 18:2/40 μM BSA in 10 % serum medium (left group), or in 1 % serum medium (right group) for 16 h. The results are the average of at least three independent determinations ± SD.**

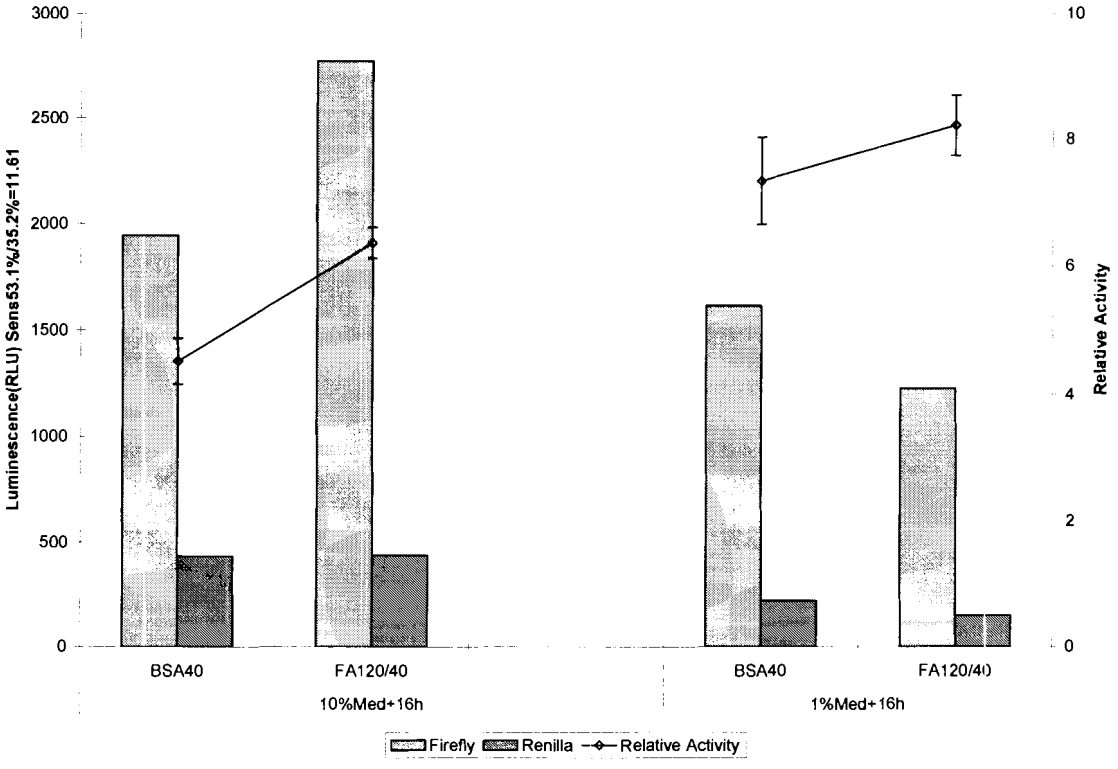


**Table 3-4 Luciferase activity variation under the optimized conditions**

**The results are the average of at least three independent determinations ± SD.**

| Date | FA/BSA(μM) | Firefly | *Renilla* | Relative activity | Change |
|---|---|---|---|---|---|
| Nov10,04 | BSA 40 | 1943 | 430.6 | 4.518±0.359 | -- |
| | FA 120/40 | 2773 | 435.5 | 6.362±0.243 | +41% |
| Dec13,04 | BSA 40 | 1323 | 262.8 | 5.032±0.368 | -- |
| | FA 120/40 | 1156 | 176.6 | 6.544±0.137 | +30% |
| Dec24,04 | BSA 40 | 1182 | 296.2 | 3.994±0.213 | -- |
| | FA 120/40 | 1222 | 279.0 | 4.389±0.172 | +10% |

**Table 3-5 Luciferase assay of myoblasts treated with different kinds of fatty acid solutions The results are the average of at least three independent determinations ± SD.**

| Fatty Acid | [Conc] (μM) | Firefly | *Renilla* | Relative activity | Change |
|---|---|---|---|---|---|
| | 10 % Medium | 1876 | 418.8 | 4.477±0.089 | |
| | BSA 40 | 1993 | 412.9 | 4.871±0.489 | |
| Home 3:1 | FA 120/40 | 1799 | 307.8 | 5.842±0.413 | +20% |
| Sigma2:1 | FA 80/40 | 2176 | 314.7 | 6.914±0.006 | +42% |
| Sigma4:1 | FA 160/40 | 2066 | 383.7 | 5.387±0.058 | +11% |
| Sigma6:1 | FA 240/40 | 1280 | 147.7 | 8.678±0.284 | +78% |
| Sigma2:1 | FA 120/60 | 2141 | 273.4 | 7.845±0.283 | +61% |
| Sigma4:1 | FA 240/60 | 2165 | 373.4 | 5.805±0.239 | +19% |
| Sigma6:1 | FA 360/60 | 909.9 | 101.3 | 8.978±0.669 | +84% |

# CHAPTER 4: LUCIFERASE REPORTER GENE ASSAY

As the optimal condition of transfection and treatment has been determined in the cultured L6 myoblasts, the serial reporter gene constructs with various fragments of the region upstream from the rat H-FABP gene were introduced to this dual luciferase reporter gene assay system to test the function of the potential fatty acid response element, ER-3, following fatty acid exposure.

## 4.1 Material and methods

The 3$^{rd}$ passage L6 myoblasts were cultured in DMEM supplemented with 10 % (v/v) fetal bovine serum, 150 U/ml penicillin and 150 μg/ml streptomycin. Prior to transfection, cells were grown to reach 50 % confluency ($1.6x10^5$/mL) in the 35×10mm dishes. Fugene 6 was firstly warmed up at room temperature for 10 min and vortexed for 3 seconds. Then 6 μl Fugene 6 were diluted into 94μl Opti-Mem I reduced serum medium for each dish. The combination was vortexed afterwards and allowed to stand for 5 min at room temperature. Subsequently, 1 μg of each reporter gene construct and 100 ng internal control vector pRL-TK were added into 100 μl diluted Fugene 6 solution. The mixture was vortexed and incubated for 20 min at room temperature. During this period, the cells were rinsed twice with PBS (pH 7.4) and then 2 ml 10 % serum medium without antibiotics were added into each dish. Finally, the transfection mixture was added into the cell dishes. After 6 h incubation at 37 °C and 5 % $CO_2$, the transfection medium was replaced by 10 % serum medium without antibiotics and incubated for an additional 2 h interval.

Fatty acid treatment was performed with the Sigma linoleic acid/albumin standard solution (2:1) at the final concentration of 120 μM 18:2/60 μM BSA in 10 % serum medium without antibiotics. As controls, the same culture medium with 60 μM BSA was used at the same time point. Then the transfected cells were treated for 16 h.

After the incubation period, cells were rinsed twice by 2 ml PBS and lysed with 200 μl passive lysis buffer for 15 min at room temperature. The cell lysates were collected into microcentrifuge tubes and then spun for 2 min at 13200 rpm and 4 °C. The supernatants were kept at -70 °C before luciferase measurement. The Dual Luciferase Reporter Assay System was used to evaluate the relative luciferase activity by TD-20/20 luminometer. In this step, each sample was measured twice and then an average of these readings was taken as the value of the sample. For each group, three dishes were employed and evaluated separately.

## 4.2 Results and discussion

The Sigma linoleic acid/albumin complex (120 μM 18:2) was used to treat all reporter genes under optimal conditions. All cells observed were very healthy and luciferase activities were at a very high level as well (Table 4-1). All experimental reporter constructs were examined but there were no remarkable induced changes in firefly readings between fatty acid groups and BSA groups. However, the values for the *Renilla* luciferase dropped dramatically after fatty acid exposure, an unexpected result as the internal control vector should not be influenced by fatty acids. However, because of the unchanged firefly enzyme and reduced *Renilla* readings, the ratio of firefly to *Renilla* increased upon fatty acid treatment, for all constructs. At this point, no meaningful

conclusion can be drawn from those results. Again, it appears that the fatty acid treatment interfered with normal physiological functions of the cell.

To prevent such interference by fatty acid, all reporter constructs were transfected again and then treated with the same fatty acid solution at the half concentration, 60 μM 18:2/30 μM BSA (Table 4-2), but similar results were obtained. The changes didn't make a big difference.

Since the *Renilla* luciferase expression from the internal control pRL-TK plasmid was more easily influenced during the treatment, more pRL-TK was used to co-transfect into cells in an attempt to minimize the changes in its expression (Table 4-3). The DNA ratio between experimental reporter gene constructs and pRL-TK was increased from 1:0.1 to 1:0.5 μg, while the ratio of Fugene 6 / DNA stayed at 6:1 (9 μl/1.5 μg). Then fatty acid treatment was performed at two concentrations: 60 μM 18:2/30 μM BSA and 120 μM 18:2/60 μM BSA. In pGL3-777/+19, which contained the full length promoter construct of H-FABP, firefly readings increased a little while *Renilla* luciferase dropped much more following the rising of fatty acid concentration. It suggested that these fatty acid solutions could be too strong for pRL-TK to express *Renilla* luciferase, but not enough for experimental vector pGL3-777/+19 to induce the up-regulation of firefly luciferase expression. Alternatively, it is conceivable that all expression would be reduced upon fatty acid treatment, but the upregulation of the H-FABP promoter compensates for the reduction otherwise expected for the full-length reporter construct. However, even the minimal promoter in reporter gene pGL3-116/+19 promoter performed similarly. The firefly luciferase almost remained at the same level, while *Renilla* luciferase dropped steeply after fatty acid exposure. This construct does not

contain known regulatory elements, and should hence not be inducible by fatty acids, making the alternative explanation mentioned above unlikely. On the other hand, in the third tested construct, pGL3-control, which contains the strong universal promoter SV40 promoter/enhancer, both firefly and *Renilla* luciferase were reduced in a similar way upon fatty acid exposure. Consequently, the relative luciferase activities hardly changed.

**Table 4-1 Luciferase assay of all reporter genes under Sigma 120 μM 18:2/60 μM BSA treatment The results are the average of at least three independent determinations ± SD.**

| Construct | Treatment | Firefly | *Renilla* | Relative activity | Change |
|---|---|---|---|---|---|
| -777/+19 | BSA 60 | 3857 | 907.8 | 4.250±0.189 | -- |
| | FA 120/60 | 3658 | 620.0 | 5.901±0.114 | +33% |
| -777/+19dele | BSA 60 | 3896 | 809.8 | 4.810±0.117 | -- |
| | FA 120/60 | 3976 | 524.6 | 7.580±0.006 | +58% |
| -688/+19 | BSA 60 | 5259 | 797.7 | 6.600±0.347 | -- |
| | FA 120/60 | 4750 | 529.1 | 8.974±0.092 | +36% |
| -688/+19dele | BSA 60 | 4032 | 706.0 | 5.710±0.111 | -- |
| | FA 120/60 | 3864 | 451.7 | 8.555±0.046 | +50% |
| -532/+19 | BSA 60 | 4229 | 668.0 | 6.333±0.157 | -- |
| | FA 120/60 | 3784 | 380.5 | 9.955±0.237 | +57% |
| -503/+19 | BSA 60 | 1471 | 301.4 | 4.881±0.095 | -- |
| | FA 120/60 | 1381 | 203.8 | 6.791±0.528 | +39% |
| -116/+19 | BSA 60 | 1451 | 650.1 | 2.232±0.075 | -- |
| | FA 120/60 | 1334 | 414.0 | 3.220±0.221 | +44% |
| Control | BSA 60 | 3872 | 246.2 | 15.71±0.444 | -- |
| | FA 120/60 | 3463 | 184.2 | 18.82±0.512 | +20% |

**Table 4-2 Luciferase assay of all reporter genes under Sigma 60 μM 18:2/30 μM BSA treatment The results are the average of at least three independent determinations ± SD.**

| Construct | Treatment | Firefly | *Renilla* | Relative activity | Change |
|---|---|---|---|---|---|
| -777/+19 | BSA 30 | 2723 | 814.1 | 3.343±0.033 | -- |
| | FA 60/30 | 2802 | 559.3 | 5.006±0.221 | +50% |
| -777/+19dele | BSA 30 | 3097 | 758.2 | 4.082±0.084 | -- |
| | FA 60/30 | 3101 | 490.0 | 6.329±0.130 | +55% |
| -688/+19 | BSA 30 | 4363 | 985.7 | 4.426±0.012 | -- |
| | FA 60/30 | 4539 | 647.3 | 7.006±0.235 | +58% |
| -688/+19dele | BSA 30 | 4134 | 938.5 | 4.407±0.305 | -- |
| | FA 60/30 | 3686 | 565.3 | 6.533±0.388 | +48% |
| -532/+19 | BSA 30 | 4593 | 874.9 | 5.250±0.053 | -- |
| | FA 60/30 | 4373 | 567.5 | 7.706±0.056 | +47% |
| -503/+19 | BSA 30 | 1488 | 336.0 | 4.428±0.132 | -- |
| | FA 60/30 | 1501 | 251.4 | 5.970±0.051 | +35% |
| -116/+19 | BSA 30 | 1346 | 624.7 | 2.155±0.027 | -- |
| | FA 60/30 | 1349 | 374.5 | 3.608±0.165 | +67% |
| Control | BSA 30 | 3325 | 266.8 | 12.47±0.249 | -- |
| | FA 60/30 | 3416 | 229.1 | 14.91±0.005 | +20% |

**Table 4-3 Luciferase assay of myoblasts transfected with more pRL-TK**

**Under optimal conditions, L6 myoblasts were transfected with 5 fold internal control vector DNA, 0.5 μg pRL-TK. The results are the average of at least three independent determinations ± SD.**

| Construct | Reagent | Vector | FA/BSA | Firefly | *Renilla* | Relative activity | Change |
|---|---|---|---|---|---|---|---|
| -777/+19 | 9μl:1.5μg | 1μg:0.5μg | BSA 30 | 1690 | 730.2 | 2.314±0.035 | -- |
| | | | FA 60/30 | 1650 | 443.9 | 3.715±0.110 | +60% |
| | | | FA 120/60 | 1752 | 384.1 | 4.562±0.024 | +97% |
| -116/+19 | 9μl:1.5μg | 1μg:0.5μg | BSA 30 | 649.1 | 514.8 | 1.258±0.058 | -- |
| | | | FA 60/30 | 663.3 | 330.8 | 2.005±0.039 | +59% |
| | | | FA 120/60 | 652.2 | 260.7 | 2.519±0.303 | +100% |
| Control | 9μl:1.5μg | 1μg:0.5μg | BSA 30 | 2210 | 324.5 | 6.800±0.489 | -- |
| | | | FA 60/30 | 2065 | 265.2 | 7.785±0.043 | +14% |
| | | | FA 120/60 | 1716 | 209.4 | 8.206±0.275 | +21% |

At present, it is impossible to decide why the reporter gene experiments did not give unambiguouos results. Based on the great improvement of the dual luciferase reporter assay system developed in this thesis, it can be concluded that reporter genes containing the rat H-FABP promoter or fragments thereof indeed can direct the expression of luciferase reporters. However, no conclusion can be reached about fatty acid induction of the constructs, and of the role potential ER-3 element may play in this process. Possible reasons reducing this system efficiency is discussed below.

The L6 myoblast cell line has been used widely as a muscle model system, as it exhibits many of the properties of skeletal muscle and is easily maintained in the laboratory. In several studies, it has been demonstrated that myoblasts express H-FABP, but at much lower level than differentiated muscle cells (Rump et al., 1996, Chang and Haunerland, 2001). The latter authors demonstrated by quantitative PCR techniques that H-FABP mRNA in L6 myoblasts is clearly up-regulated by long chain fatty acids (2-fold); an earlier study by Prinsen and Veerkamp (1998), however, could not detect increases of FABP protein levels after fatty acid treatment. Hence, it may be possible that substantially larger levels of gene induction at the mRNA level are needed before changes at the protein level can be detected.

Indeed, the reporter gene studies with the locust FABP promoter may have been successful for this reason. In mature locust flight muscle, FABP mRNA increases 8-10 fold 10 h after increased lipid delivery, a fact that suggests that this gene has a very strong promoter. While it is not known whether the invertebrate promoter, and the fatty acid response element in particular, work equally well in the mammalian cell system, it should be noted that the luciferase levels found in un-stimulated myoblasts the earlier

studies (Wu and Haunerland, 2002) were comparable to those found here. Fatty acid treatment with as little as 60 μM linoleic acid, however, resulted in an up to two-fold increase of the reporter gene activity.

Many factors contribute to the ultimate levels of protein that is found after a gene is up-regulated. These include not only the frequency of transcription initiation (which is mediated by response elements), but also processing, mRNA stability, and translation and stability of the final gene product. Since FABP, as a highly expressed protein, is assumed to be a very stable protein, with a stable mRNA as well, a two-fold increase in mRNA after fatty acid exposure may well lead to increased H-FABP levels in vivo. Reporter genes, on the other hand, are designed to be less stable, so that gene expression changes can be detected quickly. Thus, the rat H-FABP promoter constructs tested in this thesis could indeed contain the functional fatty acid response element responsible for the gene induction in vivo. However, the reporter gene methodology employed here, in spite of the extensive optimization, appears to be too insensitive to identify such an element in the H-FABP promoter.

# CHAPTER 5: CONCLUSION AND FUTURE WORK

In this study, gel shift succeeded to demonstrate the specific interaction between the everted repeat element and nuclear proteins. These results suggested that ER-3 was the potential element to control rat H-FABP gene regulation. Then, the series of reporter gene construction were carefully developed and gene reporter assay system was greatly optimized in detailed steps. Unambiguous conclusions have not been drawn from the current results, however. Unlike in the earlier study with the locust FBAP promoter, where 60 μM linoleic acid were sufficient to induce a two-fold increase in luciferase activity, the larger concentrations necessary for the rat H-FABP promoter appear to be cytotoxic and negatively affect the expression of the *Renilla* control enzyme. Thus, the reporter gene assay developed here may not be useful for wild-type myoblasts. However, the reporter gene constructs and the assay system should work if higher levels of gene induction can be achieved.

Towards this goal, various studies in our laboratory seek to identify possible candidates for the transcription factors that were shown to bind to the putative response element. Once such factors have been identified, they can be introduced into the myoblast model system together with the reporter gene constructs. Their involvement in fatty acid mediated gene regulation, and the elements to which they bind, then can be studied with the reporter gene approach optimized in this thesis.

Ultimately, other suitable cell lines or primary cardiomyocytes may be used as hosts for transfection in order to avoid the disadvantage of undifferentiated myoblasts.

While these studies will require lengthy optimization as well, they can take advantage of the pilot studies carried out in this thesis, and thus avoid pitfalls and misguided approaches. Thus, we expect that this thesis will lay the groundwork for the ultimate identification and characterization of fatty acid activated receptors and their recognition sequences in promoters of genes involved in fatty acid transport and metabolism.

# REFERENCE LIST


Akiyama TE, Ward JM, and Gonzalez FJ: Regulation of the liver fatty acid-binding protein gene by hepatocyte nuclear factor 1 alpha (HNF1 alpha) – alterations in fatty acid homeostasis in HNF1 alpha-deficient mice. J. Biol. Chem. 275: 27117-27122, 2000

Banaszak L, Winter N, Xu A, Bernlohr DA, Cowan S, and Jones TA: Lipid-binding proteins: a family of fatty acid and retinoid transport proteins. Adv. Protein. Chem. 45: 89-151, 1994

Bassingthwaighte JB, Noodleman L, van der Vusse GJ, and Glatz JFC: Modeling of palmitate transport in the heart. Mol. Cell. Biochem. 88: 51-58, 1989

Bernlohr DA, Coe NR, Simpson MA, and Hertzel AV: Regulation of gene expression in adipose cells by polyunsaturated fatty acids. Adv. Exp. Med. Biol. 422: 145-156, 1997

Blackwood EM and Kadonaga JT: Going the distance: A current view of enhancer action. Science 281: 61-63, 1998

Burley SK and Roeder RG: Biochemistry and structural biology of transcription factor IID (TFIID). Annu. Rev. Biochem. 65: 769-799, 1996

Carey JO, Neufer PD, Farrar RP, Veerkamp JH, and Dohm GL: Transcriptional regulation of muscle fatty acid binding protein. Biochem. J. 298: 613-617, 1994

Chang W, Rickers-Haunerland J, and Haunerland NH: Induction of cardiac FABP gene expression by long chain fatty acids in cultured rat muscle cells. Mol. Cell. Biochem. 221: 127-132, 2001

Chen XM and Haunerland NH: Fatty acid binding protein expression in locust flight-muscle - induction by flight, adipokinetic hormone, and low-density lipophorin. Insect Biochem. Mol. Biol. 24: 573-579, 1994

Clark PR and Hersh EM: Cationic lipid-mediated gene transfer: current concepts. Curr. Opin. Mol. Ther. 1:158-176, 1999

Coe NR and Bernlohr DA: Physiological properties and functions of intracellular fatty acid-binding proteins. Biochim. Biophys. Acta 1391: 287-306, 1998

Das DK, Barua PK, and Jones RM: Release of fatty acid-binding protein from ischemic-reperfused rat heart and its prevention by mepacrine. Biochim. Biophys. Acta 1168: 307-314, 1991

Dawe I: Studies of the upstream control region of the gene encoding rat heart fatty acid binding protein. Master thesis, Simon Fraser University, Canada, 2001, pp 40-42

Desvergne B and Wahli W: Peroxisome proliferator-activated receptors: nuclear control of metabolism. Endocr. Rev. 20: 649-688, 1999

Detera-Wadleigh SD and Fanning TG: Phylogeny of the steroid receptor superfamily. Mol. Phylog. Evol. 3:192-205, 1994

Glatz JFC and van der Vusse GJ: Cellular fatty acid-binding proteins: their function and physical significance. Prog. Lipid Res. 35: 243-282, 1996

Glatz JFC, van Breda E, Keizer HA, Dejong YF, Lakey JRT, Rajotte RV, Thompson A, van der Vusse GJ, and Lopaschuk GD: Rat heart fatty acid binding protein content is increased in experimental diabetes. Biochem. Biophys. Res. Commun. 199: 639-646, 1994

Haunerland NH, Andolfatto P, Chisholm JM, Wang ZX, and Chen XM: Fatty acid binding protein in locust flight muscle – Developmental changes of expression, concentration and intracellular distribution. Eur. J. Biochem. 210: 1045-1051, 1992

Haunerland NH and Chisholm JM: Fatty acid binding protein in flight muscle of the locust *Schistocerca gregaria*. Biochim. Biophys. Acta 1047: 233-238, 1990

Haunerland NH, Jagschies G, Schulenberg H, and Spener F: Occurrence of two fatty acid-binding proteins in bovine liver cytosol and their binding of fatty acids, cholesterol, and other lipophilic ligands. Hoppe-Seyler's Z. Physiol. Chem. 365: 365-376, 1984

Haunerland NH and Spener F: Properties and physiological significance of fatty acid binding proteins. In: G.J. van der Vusse (ed.). Lipobiology. Elsevier, 2004, pp. 99-123.

Haunerland NH: Fatty acid binding protein in locust and mammalian muscle. Comparison of structure, function and regulation. Comp. Biochem. Physiol. B. 109: 199-208, 1994

Haunerland NH: Transport and utilization in insect flight muscles. Comp. Biochem. Physiol. B. 117: 475-482, 1997

Issemann I, Prince R, Tugwood J, and Green S: A role for fatty acids and liver fatty acid-binding protein in peroxisome proliferation. Biochem. Soc. T. 20: 824-827, 1992

Jacobsen LB, Calvin SA, Colvin KE, and Wright M: Fugene 6 transfection reagent: the gentle power. Methods 33: 104-112, 2004

Josephson R, Muller T, Pickel J, Okabe S, Reynolds K, Turner PA, Zimmer A, and McKay RD: POU transcription factors control expression of CNS stem cell-specific genes. Development 125: 3087-3100, 1998

Jump DB and Clarke SD: Regulation of gene expression by dietary fat. Annu. Rev. Nutr. 19: 63-90, 1999

Kawabe K, Saegusa H, Seto K, Urabe H, and Motojima K: Peroxisome proliferator-activated receptor alpha and its response element are required but not sufficient for transcriptional activation of the mouse heart-type fatty acid binding protein gene. Int. J. Biochem. Cell Biol. 37:1534-1546, 2005

Luiken JJ, Glatz JFC, and Bonen A: Fatty acid transport proteins facilitate fatty acid uptake in skeletal muscle. Can. J. Appl. Physiol. 25: 333-352, 2000

Ockner RK, Manning JA, Poppenhausen RB, and HO WK: A binding protein for fatty acids in cytosol of intestinal mucosa, liver, myocardium, and other tissues. Science 177: 56-58, 1972

Poirier H, Niot I, Monnot MC, Braissant O, Meunier-Durmort C, Costet P, Pineau T, Wahli W, Willson TM, and Besnard P: Differential involvement of peroxisome-proliferator-activated receptors alpha and delta in fibrate and fatty-acid-mediated inductions of the gene encoding liver fatty-acid-binding protein in the liver and the small intestine. Biochem. J. 355: 481-488, 2001

Prinsen CF and Veerkamp JH: Transfection of L6 myoblasts with adipocyte fatty acid-binding protein cDNA does not affect fatty acid uptake but disturbs lipid metabolism and fusion. Biochem. J. 329: 265-273, 1998

Promega: Dual-luciferase reporter assay system. Technical manual No. 040, 2003

Qian QH, Kuo L, Yu YT, and Rottman JN: A concise promoter region of the heart fatty acid-binding protein gene dictates tissue-appropriate expression. Circ. Res. 84: 276-289, 1999

Richieri GV, Ogata RT, and Kleinfeld AM: Equilibrium constants for the binding of fatty acids with fatty acid-binding proteins from adipocytes, intestine, heart, and liver measured with the fluorescent probe ADIFAB. J. Biol. Chem. 269: 23918-23930, 1996

Robinson R, Robinson LJ, James DE, and Lawrence JC: Glucose transport in L6 myoblasts overexpressing GLUT1 and GLUT4. J. Biol. Chem. 268: 22119-22126, 1993

Rump R, Buhlmann C, Börchers T, and Spener F: Differentiation-dependent expression of heart type fatty acid-binding protein in C2C12 muscle cells. Eur. J. Cell. Biol. 69: 135-142, 1996

Schaap FG, Binas B, Danneberg H, van der Vusse GJ, and Glatz JFC: Impaired long-chain fatty acid utilization by cardiac myocytes isolated from mice lacking the heart-type fatty acid binding protein gene. Circ. Res. 85: 327-337, 1999

Schaap FG, van der Vusse GJ, and Glatz JFC: Fatty acid-binding proteins in the heart. Mol. Cell. Biochem. 180: 43-51, 1998

Schachtrup C, Emmler T, Bleck B, Sandqvist A, and Spener F: Functional analysis of peroxisome-proliferator-responsive element motifs in genes of fatty acid-binding proteins. Biochem. J. 382: 239-45, 2004

Specht B, Bartetzko N, Hohoff C, Kuhl H, Franke R, Börchers T, and Spener F: Mammary derived growth inhibitor is not a distinct protein but a mix of heart-type and adipocyte-type fatty acid-binding protein. J. Biol. Chem. 271: 19943-19949, 1996

Tontonoz P, Hu E, Graves RA, Budavari AI, and Spiegelman BM: mPPAR-gamma-2 – tissue-specific regulator of an adipocyte enhancer. Genes Dev. 8: 1224-1234, 1994

Treuner M, Kozak CA, Gallahan D, Grosse R, and Müller T: Cloning and characterization of the mouse gene encoding mammary-derived growth inhibitor / heart-fatty acid binding protein. Gene. 147: 237-242, 1994

van der Lee KAJM, Vork MM, De Vries JE, Willemsen PHM, Glatz JFC, Reneman RS, van der Vusse GJ, and van Bilsen M: Long-chain fatty acid-induced changes in gene expression in neonatal cardiac myocytes. J. Lipid Res. 41: 41-47, 2000

van der Vusse GJ, Glatz JFC, Stam HCC, and Reneman RS: Fatty acid homeostasis in the normoxic and ischemic heart. Physiol. Rev. 72: 881-940, 1992

van der Vusse GJ, Glatz JFC, van Nieuwenhoven FA, Reneman RS, and Bassingthwaighte JB: Transport of long-chain fatty acids across the muscular endothelium. Adv. Exp. Med. Biol. 441: 181-191, 1998

van der Vusse GJ, van Bilsen M, Glatz JFC, Hasselbaink DM, and Luiken JJ: Critical steps in cellular fatty acid uptake and utilization. Mol. Cell. Biochem. 239: 9-15, 2002

Veerkamp JH and van Moerkerk HTB: Fatty acid binding protein and its relation to fatty acid oxidation. Mol. Cell. Biochem. 123: 101-106, 1993

Vork MM, Glatz JFC, and van der Vusse GJ: Modeling intracellular fatty acid transport: possible mechanistic role of cytoplasmic fatty acid-binding protein. Prostagl. Leukotr. Essent. Fatty Acids. 57: 11-16, 1997

Weisiger RA: Cytoplasmic transport of lipids: role of binding proteins. Comp. Biochem. Phys. B. 115: 319-331, 1996

Wu QW and Haunerland NH: A novel fatty acid response element controls the expression of the flight muscle FABP gene of the desert locust, *Schistocerca gregaria*. Eur. J. Biochem. 268: 5894-5900, 2001

Wu QW, Chang WH, Rickers-Haunerland J, Higo T, and Haunerland NH: Characterization of a new fatty acid response element that controls the expression of the locust muscle FABP gene. Mol. Cell. Biochem. 239: 173-180, 2002

Wu QW: Cloning and transcriptional regulation of the muscle fatty acid-binding protein gene from the desert locust, *Schistocerca gregaria*. Ph.D. thesis, Simon Fraser University, Canada, 2001, pp 12-13

Yamomoto Y and Niki E: Role of antioxidants in lipid peroxidation. In: VigoPelfrey C (ed.), Membrane lipid oxidation. Vol.1. CRC Press, Boca Raton, 1990, pp. 286-298

Zakim D: Fatty acids enter cells by simple diffusion. Proc. Soc. Exp. Biol. Med. 212: 5-14, 1996

Zanotti G, Scapin G, Spadon P, Veerkamp JH, and Sacchettini JC: Three-dimensional structure of recombinant human muscle fatty acid-binding protein. J. Biol. Chem. 267: 18541-18550, 1992

Zhang J and Haunerland NH: Transcriptional regulation of FABP expression in flight muscle of the desert locust, *Schistocerca gregaria*. Insect Biochem. Mol. Biol. 28: 683-691, 1998

Zhang J, Rickers-Haunerland J, Dawe I, and Haunerland NH: Structure and chromosomal location of the rat gene encoding the heart fatty acid-binding protein. Eur. J. Biochem. 266: 347-351, 1999