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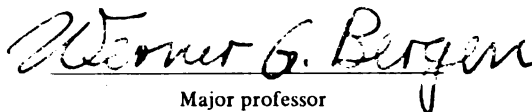
Inhibition of Fatty Acid Synthase  
by an Antisense Message in TAL Cells

presented by

Michelle Kay Mater

has been accepted towards fulfillment  
of the requirements for

Masters degree in Animal Science

  
Major professor

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INHIBITION OF FATTY ACID SYNTHASE BY AN ANTISENSE MESSAGE IN  
TA1 CELLS

By

Michelle Kay Mater

A THESIS

Submitted to  
Michigan State University  
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## ABSTRACT

### INHIBITION OF FATTY ACID SYNTHASE BY AN ANTISENSE MESSAGE IN TA1 CELLS

By

Michelle Kay Mater

Potential attenuation of lipogenesis by fatty acid synthase (FAS) antisense messenger RNA in adipocytes was examined. A portion of rat FAS message in the sense and antisense orientation was ligated into the selective expression vector, pcDNA3. These antisense and sense plasmids, as well as two controls (pcDNA3 and pcDNA3/CAT), were transfected into TA1 cells using lipofectin, and stable polyclonal and monoclonal cell lines were established. Fatty acid synthase activity was measured in each stable cell line to determine whether FAS activity had occurred. Results indicated that the antisense polyclonal cells had lower FAS activity ( $P < .05$ ) while the monoclonal antisense, sense and CAT cells all had lowered FAS activity ( $P < .05$ ). Site of integration of the sense, antisense and control plasmids into the TA1 genome was not determined. Integration of sense and control plasmids into genomic sites involved in lipogenesis may explain attenuation of FAS in monoclonal cell lines.

Dedication

*my inspiration,  
Cindy*

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## INTRODUCTION

The meat animal industry continues to strive toward producing a leaner product. Although nutrition, health and breeding programs have increased lean gains, fat gains have not decreased (Bergen and Merkel, 1991). Some attempts to decrease fat accumulation in swine have been successful, including feeding beta-agonists and injecting growth hormones (Mersmann, 1991). However, these methods are not yet approved for public use and involve exogenous agents.

A more specific approach in lowering fat deposition needs to be created to meet consumer demands for a leaner, low fat product. One method of specifically inhibiting fat synthesis is to block a key enzyme in the fatty acid synthesis pathway. Another technique to block a specific gene product involves the use of an antisense message (antisense mRNA). Antisense mRNA is a specific complementary message against its normal cellular mRNA (sense mRNA). Due to this complementarity, it is thought that the mRNAs hybridize and prevent translation of the protein. The hybrid is then degraded to nucleic acids leaving no unnatural by-products in the cell (Izant and Weintraub, 1985).

This thesis project develops a system to determine if antisense fatty acid synthase (FAS) mRNA can inhibit fat synthesis in the adipogenic cell line, TA1. Three specific



aims were undertaken: create an antisense plasmid, establish antisense stable cell lines and determine if the FAS enzyme was inhibited. To this end, a vector containing antisense FAS1 (a 3' portion of the rat FAS mRNA from Witowski et al, 1987) as well as control vectors were stably transfected into TA1 cells. Following transfection, monoclonal and polyclonal cell lines were selected with the antibiotic geneticin. inhibition of fat synthesis was measured by FAS enzyme assays in each of the cell lines. The system developed will enable further investigation of antisense mechanisms and viability of its uses to inhibit lipogenic enzymes at the cellular level.

## Antisense Literature Review

Understanding gene expression and its regulation is the goal of many scientists today. The cell has numerous mechanisms to regulate these processes, many of which are only starting to be comprehended. The fact that DNA is transcribed to RNA, which is translated to protein is easily accepted but when, where and how much transcription and translation occurs is a complex process. Antisense messages, whether RNA or DNA, are examples of how cells can control these processes.

Transcription of DNA occurs in the 5' to 3' direction. Polymerases read the 3' to 5' antisense strand of DNA and 5' to 3' sense RNA is synthesized. Ribosomes initiate translation by binding to an AUG site present in mRNA. This AUG site is generally near the 5' end of the sense RNA. It is understood that most cellular mRNA is sense mRNA.

The term "antisense" therefore refers to the directionality of a nucleic acid sequence. The sequence of the sense strand of DNA is the 5' to 3' strand and is usually the only sequence reported in the literature. It is also the sequence of the RNA transcribed from that DNA sequence, i.e. the sense RNA. The antisense strand of DNA is the template for sense RNA transcription. If RNA were synthesized from the sense strand, the RNA produced would then be antisense RNA. The two possible strands of RNA synthesized from a DNA template are exactly complementary to

each other. This complementarity between sense and antisense nucleic acids theoretically could be used to block sense RNA translation or DNA transcription, thereby controlling gene expression (Figure 1).

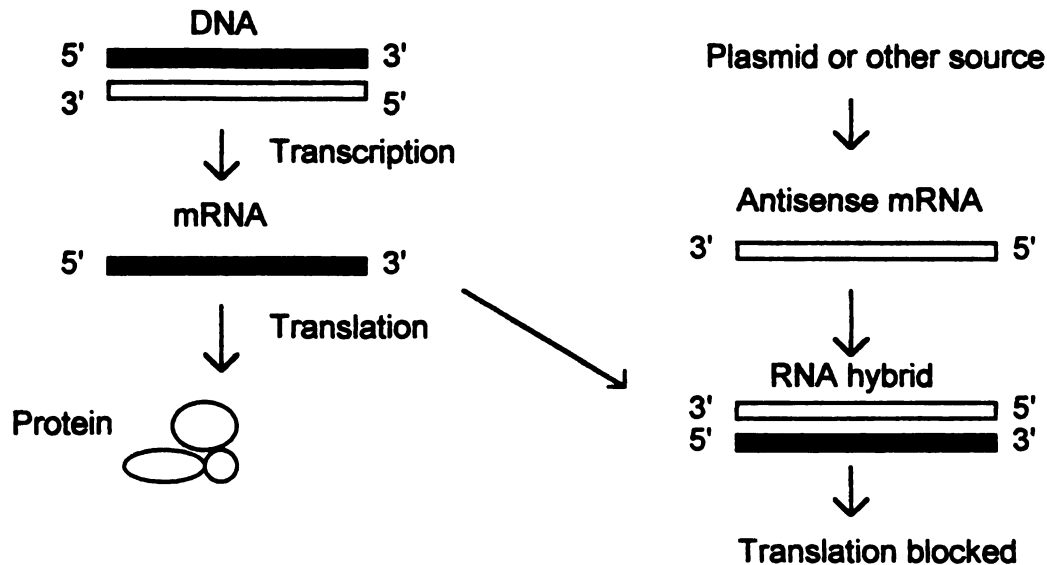


Figure 1. Antisense Action. Double stranded DNA contains both a sense and antisense strand but transcription results in only sense mRNA. The presence of antisense RNA could disrupt the translation process by preventing ribosomal binding, translocation of sense RNA into cytoplasm or inhibition splicing.

Antisense technology encompasses a wide scope of techniques. Prokaryotes and eukaryotes alike employ the use of antisense messages to block either transcription or translation by the hybridization of sense and antisense messages. Researchers have used antisense methods to create mutants and cure diseases like the flu and AIDS, as well as

develop new plants such as the Flavr Savr tomato and treat cancer patients.

### Mechanisms of Antisense Actions

In many systems, it is unknown how the antisense can inhibit a gene product. In theory, the action of antisense is based on the complementarity of sense and antisense nucleic acids. Eguchi et al (1991) defines antisense RNA as "an RNA that interferes with the activity of another RNA by binding to a complementary region of a target RNA and affecting its function". Antisense messages are specifically complementary to its sense target. This complementarity would allow the strands to hybridize in vivo. The hybrid would be unable to be transcribed (in rare cases of DNA:RNA hybridization) or be transported out of the nucleus and/or be translated into protein. This simple but elegant idea would not only be highly specific to one gene, but could also be transcriptionally regulated itself (Izant and Weintraub, 1985).

### Endogenous Antisense in Procaryotes

Natural occurrences of antisense messages have been detected in procaryotics and to a lesser extent in eucaryotics. After discussing examples of each of these,

the remainder of this section will review the variety of uses and methods of applying antisense technology.

A well studied example of a naturally occurring antisense mRNA used to inhibit a procaryotic gene product is in *E. coli*. ColE1, a common plasmid present in *E. coli*, has its number of copies per bacterium regulated to twenty to thirty by antisense RNA. To begin replication of ColE1, an RNA transcript is synthesized from a point 555 base pairs upstream of the origin and which has been designated RNA II. At the origin, RNase H cleaves this RNA II to give a 3'-OH end to be used as a primer for DNA replication. An antisense transcript, RNA I, is synthesized from the complementary strand of the DNA used to make RNA II. RNA I base pairs to the 5' end of RNA II, inactivating it. Since it is known that the primer can still be initiated and elongated at the 3' end, the antisense RNA I is thought to somehow prevent the RNase H cleavage and creation of the 3'-OH end. RNA I and RNA II have similar secondary structures predicting that they base pair in 3 loops, creating a conformational change at the cleavage site. This binding of RNA I to RNA II is sometimes referred to as a "kissing structure". RNA I therefore acts as a transcriptional regulator of ColE1 copy number (Lewin, 1990; Tomizawa 1981 and 1982).

Another example of a procaryotic antisense gene regulator is the micF gene (Figure 2). When osmolarity is increased in a bacterium's environment, the env2 gene is

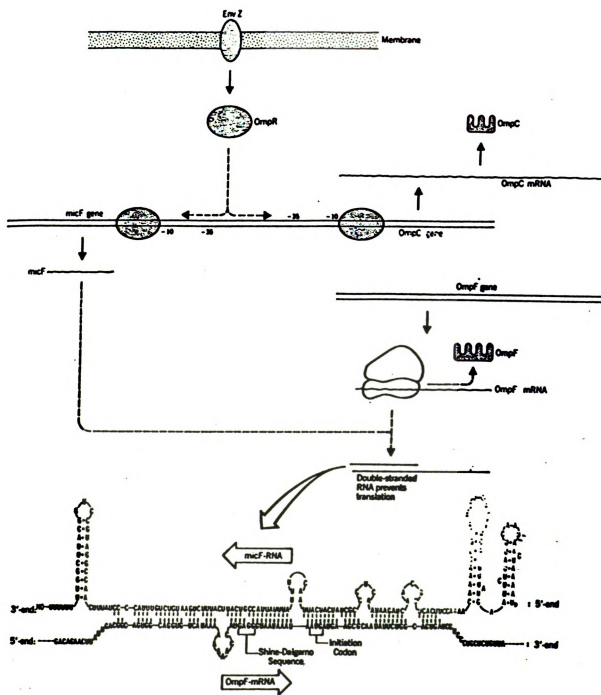


Figure 2. Action of micF RNA. Binding of micF RNA to OmpF inhibits the translation of OmpF protein. The lower portion of the diagram depicts the structure of the sense-antisense hybrid.

activated. This in turn activates *ompR* which increases levels of *on micF* and *ompC*. *OmpF*, a constitutive gene, is inhibited by *micF*. The *ompF* mRNA has a complementary region to the 174 base *micF* mRNA. This region in the *ompF* contains a ribosomal binding site. This suggests that the *micF* mRNA inhibits translation of *ompF*, or that the duplex formed is unstable and degraded by ribonucleases. *OmpF* and *ompC* proteins levels are known to be inversely related in the bacteria. This relationship of protein levels implies that regulation of one is tied to the other (Coleman, 1984; Mizuno, 1984).

In addition to the *micF* and *ColE1* antisense systems, other antisense examples exist in procaryotics. For instance, the protein transposase is regulated by an antisense mRNA in the transposon *Tn10* inhibiting translation of transposase and therefore inhibiting transposition (Simons, 1983; Eguchi et al, 1991). The *crp* gene in *E. coli* has a complementary region with an antisense transcript synthesized from the opposite strand. This antisense transcript inhibits transcription of *crp* by an action thought to be similar to rho-independent termination. Lambda and p22 phage as well as replication of *ColE2*, *IncF*, *IncI*, *R6K* and *pT181* plasmids are also thought to be regulated by antisense transcripts (Green, 1986; Eguchi et al, 1991).

Procaryotic organisms use antisense messages as transcriptional and translational regulators. These

examples have been used as models for using exogenous antisense in eucaryotic cells. Endogenous antisense messages in eucaryotics would be most desirable as references for using exogenous antisense in eucaryotics, but procaryotic antisense examples are both more numerous and better understood.

### Endogenous Antisense in Eucaryotes

The existence of eucaryotic antisense was predicted by Green et al (1986) based on the action of spliceosomes. Spliceosomes are comprised of snRNPs and protein. Splicing of mRNA requires the U1 RNA of U1 snRNP (small nuclear ribonucleoprotein) to bind to a complementary region at the intron-exon junction. If this complementary region is removed splicing can not occur (Green et al, 1986). Other occurrences of complementary genes appeared with the discovery of Alu sequences, studies of the c-myc gene, human e-globin and myosin heavy chain genes and sequencing of the erbA homologs.

An endogenous antisense messages to c-myc exists in eucaryotes. Early researchers had already inhibited the c-myc gene with exogenous antisense oligonucleotides before the endogenous message was detected. Cooney et al (1988) synthesized a twenty-seven base antisense oligodeoxyribonucleotide that was complementary to the -115 region of the c-myc gene. The oligonucleotide bound to the



DNA duplex to form a triple helix and repressed c-myc transcription *in vitro*. Postel et al (1991) continued this work by repeating the experiment *in vivo* with HeLa cells. Celano et al (1992) determined that an endogenous antisense transcript was produced when the human colon cancer cell (COLO 320) line was deprived of polyamines. The antisense transcript was shown to be highly homologous to the second exon of the c-myc gene and is thought to originate from the opposite strand in an intron of the c-myc gene. Interestingly, it was also shown that this antisense mRNA is homologous to regions in other genes such as N-myc, thymidine kinase and p53 suggesting that this antisense message may regulate other gene products as well (Celano et al, 1992).

McCarthy et al (1983) found that an endogenous antisense transcript existed in chick embryonic muscle. Translation of the chicken myosin heavy chain (MHC) mRNA is controlled by a 102 antisense nucleotide transcript. It binds specifically *in vitro* to MHC mRNA and blocks translation. McCarthy et al (1983) proposed that the area of binding of the two transcripts occurred in the poly(A) region although a sequence at the 5' end of the mRNA was also possible. The origin of the antisense transcript has not been identified.

Although a few other examples of natural antisense messages in eucaryotics exist, the mechanism of these endogenous antisenses are not yet known. One example is

Rev-ErbAa protein. Although this protein is a member of the thyroid/steroid hormone receptor superfamily, it does not form a dimer with retinoic acid or thyroid hormone. When the DNA sequence of Rev-ErbAa was characterized, it was found to contain a part of the C-erbAa gene. C-erbAa produces both r-erbAa-1 and r-erbAa-2 depending on how the mRNA is spliced. R-erbAa-2 has an antisense region complementary to Rev-ErbAa. Some tissues contain both the antisense and sense mRNAs suggesting that perhaps translation of one or both of the transcripts could be inhibited (Lazar et al, 1989). Miyajima et al (1989) determined that two erbA homologs ear-1 and ear-7 were synthesized from overlapping exons. Ear-7 was further processed to ear-71 and ear-72. Ear-71 is complementary to c-erbA (the T<sub>3</sub> receptor). Both the c-erbA and ear-71 mRNAs are made into similar functioning proteins. It is unknown if their expression is somehow regulated using this complementarity (Miyajima et al, 1989).

Wu et al (1990) found that human e-globin gene contained alternative transcripts originating from within an Alu repetitive sequence. These transcripts were transcribed in opposite directions, located only in the nuclei, not polyadenylated and found in mature K562 cells or embryonic red blood cells. Wu et al (1990) used a transient expression experiment to determine whether ε-globin could be down regulated using this antisense transcript and went on to suggest that the antisense transcript bound to the sense

mRNA as it was transcribing which somehow blocked the polymerase II from continuing its transcription. Other antisense transcriptions originating within Alu sequences were not unknown at the time. Adeniyi-Jones et al (1985) detected an antisense message to the alpha-fetoprotein, 210 nucleotides in length, perhaps being used to inhibit this protein either transcriptionally or translationally.

While all of these antisense transcripts have been detected, the mechanism of action of each has yet to be determined. Further antisense transcripts are likely to be detected as more sequencing is completed. Even as more antisense messages are found, the major focus of this type of research is using exogenous antisense rather than determining the purpose of the endogenous messages.

#### Early Use of Exogenous Antisense

Paterson et al (1977) first inhibited translation of rabbit  $\beta$ -globin by hybridization of antisense mRNA with its complementary cDNA. Though it was referred to as "hybrid arrested cell free translation", it is similar to the antisense method. Paterson et al (1977) used linearized adenovirus containing the rabbit  $\beta$ -globin gene in a cell free system with isolated rabbit globin mRNA. The nucleic acids were denatured, combined with formamide (to prevent DNA:DNA rehybridization) and incubated to allow for hybridization. Upon completion of the translation assay, it

was determined that  $\beta$ -globin mRNA was not translated in samples where hybridization could have occurred. It was also determined that heat denaturing the hybrid reinstated the ability to translate  $\beta$ -globin. Though Paterson et al (1977) used this method to locate the translatable sections of the viral genome, they went on to suggest that this work could be used to study organization and expression of RNA viruses.

Although Paterson et al (1977) may have initiated the use of exogenous antisense, the bridge between endogenous antisense to using exogenous antisense messages to manipulate gene function was built by Izant and Weintraub (1984). Their work resulted in several important discoveries about antisense specificity, target areas and methods of incorporation. These first experiments also fostered further research using antisense to attempt everything from inhibiting genes to treating cancer and AIDS.

The first experiments by Izant and Weintraub (1984 and 1985) determined the specificity of the antisense message. Using two plasmids (one containing an antisense fragment of the herpes simplex virus thymidine kinase gene (TK) and the other with the sense TK gene), they cotransfected the plasmids into LTK- cells (these cells produce no thymidine kinase). Not only did the antisense transcript block TK, it did so specifically. Further experimental results showed that antisense chicken TK inhibited only chicken TK and not

herpes TK. When the antisense fragment was put in front of the inducible promoter murine mammary tumor virus (MMTV) and the cells activated by dexamethasone, the antisense inhibited the TK but uninduced cells did not. Using a cell line that produced endogenous TK, the antisense plasmid lowered endogenous TK activity six-fold.

Izant and Weintraub (1985) also measured how much of the TK gene was needed to inhibit its target. Experiments with the TK gene showed that only a 52-base fragment from the untranslated 5' end of the gene was needed for the antisense transcript to inhibit TK. It did not contain the AUG start site. This work was repeated with antisense chloramphenicol acetyl transferase (CAT) expression vectors. CAT activity was lower in cells containing the antisense plasmids as compared to cells with other control plasmids.  $\beta$ -actin was also inhibited with an antisense transcript. Using pBR322 with antisense  $\beta$ -actin, transfected cells had reduced growth as a result of less actin production. Upon staining for actin filaments, only half of the antisense treated cells showed microfilaments. While an antisense fragment from the 3' or the 5' end of the CAT mRNA could block CAT, only the 5' antisense fragment from  $\beta$ -actin would inhibit it (Izant and Weintraub, 1985).

In conjunction with the above experiments, Izant and Weintraub (1985) used a variety of techniques to incorporate the antisense messages into the cells. They used microinjection, the DEAE transfection method for transient

expression and calcium phosphate transfection for selection of stable polyclonal cell lines. Each of these methods were successful. They determined that a ratio of 5:1 antisense to sense DNA was needed to block TK when transfection was used but 50:1 was needed when the antisense transcript was microinjected.

Izant and Weintraub (1985) summarized this early antisense work with what has become general guidelines for using antisense messages to inhibit a gene product. They observed that a 5' piece may be more efficient in inhibiting genes (which is supported by the finding that procaryotes use 5' antisense fragments). It was noted that the antisense fragment did not necessarily have to include the translation start site, AUG. Although Izant and Weintraub (1985) did not determine the stability of antisense transcripts *in vivo*, the intracellular site where the hybrids were located, or if different domains of RNA were more susceptible to antisense hybridization, they nevertheless proposed this is a very powerful technique for gene analysis.

#### Successful Use of Endogenous Antisense

Many genes have been successfully blocked with an antisense message. One common use is the creation of mutants in order to analyze cell function of a particular gene product. Florini and Ewton (1990) did this with

myogenin to determine if differentiation still occurred in the presence of IGF-1. Using an oligodeoxynucleotide against the first twenty-five nucleotides of the myogenin gene, Florini and Ewton (1990) blocked myogenin from being translated. Stimulation of antisense treated cells with IGF-1 resulted in no differentiation while mismatched oligomers (same bases as antisense oligomer but scrambled order) and control treated cells had normal differentiation. This effect was only seen when the antisense message was added at the same time as the IGF-1. Other cellular processes including proliferation were not changed in the treated cells. Using radioactive end-labelled oligomers, Florini and Ewton (1990) determined that incorporation of the antisense oligomers into the cells in culture continued slowly for seventy-two hours after addition. Thus, this antisense oligomer was highly specific and did not cause other cellular actions to be inhibited. Using antisense to create false "mutant" cells or animals has become an excellent way to study gene function.

Antisense can also be used as a therapeutic tool against many diseases. Simons et al (1992) used antisense oligomers against the c-myb gene to inhibit smooth muscle cell (SMC) growth. Proliferation of smooth muscle cells are implicated in the cause of atherogenesis, failure of bypass grafts and stenosis establishment after artery angioplasty. Using rats with injury to the carotid artery, Simons et al (1992) injected antisense c-myb oligomers into the artery

after a balloon angioplasty. The 1 mg/ml antisense solution (200  $\mu$ l total) gelled to the artery on contact. The antisense treatment resulted in reduced SMC growth as compared to sense, mismatched or gel treated. The reduction in growth occurred only in the area where the gel was placed; surrounding areas accumulated SMC. This technique may be a treatment for heart surgery patients in the future.

Antisense messages have also been used to treat cancer. Han et al (1991) used transgenic mice containing the antisense gene to the retroviral packaging sequence of the Moloney murine leukemia virus. Upon birth, mice were infected with the virus. All control mice got leukemia symptoms but antisense transgenic mice did not. While Han et al (1991) did inhibit the leukemia virus, no mechanism for inhibiting the virus was established. Virus could not be detected in supernatants of stable cell lines containing the antisense gene, implying the packaging of the virus must have been blocked as expected.

Plant and animal agriculture have benefitted from antisense as well. The Flavr Savr tomato just marketed contains an antisense gene to polygalacturonase. This gene is responsible for the softening of the tomato as it ripens. Smith et al (1988) originally reported the inhibition of softening by transforming tomatoes with a 730 bp fragment (containing the start site for translation). A plasmid containing an antisense ethylene gene was also transformed into tomatoes resulting in lower ethylene production



(Hamilton et al, 1990). Ethylene, thought to be involved in ripening, was reduced in the transgenic tomatoes. These tomatoes were less likely to become overripe or shrivel than nontransgenic tomatoes. Though an effect was seen, antisense RNA could not be detected. Hamilton et al (1990) explained this as a common occurrence in antisense systems and perhaps due to immediate degradation of the RNA hybrid or the antisense RNA inhibiting transcription. Hoffman (1993) reported on work done by Wong and Halawani using antisense prolactin. Prolactin is known to cause broodiness in turkeys which reduces further egg laying. After antisense prolactin was injected into pituitary cells from turkeys, prolactin levels are lowered. Further work is currently being conducted to create transgenic turkeys carrying this antisense gene. It is hoped that these turkeys will produce more eggs for the producer.

#### Using Exogenous Antisense Messages

Procaryotes and eucaryotes have provided a variety of examples of antisense messages regulating a gene product. Though the regulation of antisense is not well understood, many researchers have used these few examples to create exogenous antisense messages to interfere with a variety of genes. Exogenous antisense messages have become a powerful tool to create mutants and interfere with cellular processes

as well as potential therapeutic agents against a variety of diseases.

Three important points must be considered when using exogenous antisense messages to block a gene product. First, the target gene and its sequence for the antisense message must be known to create the antisense message. A variety of antisense messages can be used: DNA oligonucleotides, RNA oligonucleotides or plasmids containing a portion or the complete gene in the antisense orientation under the control of a promoter. This may depend on the cell, tissue or organism the antisense is used in. Finally, a method of incorporating the antisense message into the desired location must be ascertained.

The sense targets for hybridization include every part of a gene or mRNA. Antisense messages are targeted against the initiation site for translation, 5' end, 3' end, exons, introns, splice sites or the entire gene as well as combinations of the above. Moroni et al (1991) used plasmids containing either the 5' end, 3' end or the entire EGF receptor and found that each was effective at inhibiting translation of EGF receptor mRNA. Kim and Wold (1985) determined that both the 3' untranslated region as well as a 5' antisense fragment of thymidine kinase blocked TK production. DNA is also a target for antisense, creating a triple helix formation which prevents transcription (Cooney et al, 1988). Different areas in different genes are more susceptible to antisense messages but vary within each

system it is used in. Izant and Weintraub (1985) found that only 52 bases of the 5' end of thymidine kinase (TK) was needed to block the TK levels while a portion of the 5' or 3' end of the chloramphenicol acetyl transferase (CAT) gene inhibits this product. It is generally considered advantageous to use the 5' portion of the gene, one which contains the AUG translational start site, as the target for antisense (Izant and Weintraub, 1985; Kim and Wold, 1985). However, Denhardt (1992) suggests targeting several different areas of a gene and comparing the results of each.

The types of antisense created by researchers include a variety of structures and modifications of those structures. These include oligodeoxynucleotides, oligonucleotides and transfected plasmids or viruses containing antisense genes. Oligodeoxynucleotides are short strands of DNA while oligonucleotides are RNA strands (both are often called oligomers). Both of these short fragments have been synthesized with modified bases in attempts to increase stability and efficacy in the cell. For example, replacing an oxygen with a sulfur on the phosphate in the backbone of the nucleic acid (phosphorothioates) has been shown to increase the stability of the oligonucleotide in the cell (Akhtar et al, 1991). The length of the oligomer must be long enough to be specific while oligomers that are too lengthy are difficult to make and have reduced efficiency. An oligomer fifteen nucleotides long would occur naturally only once in a sequence 500 million base pairs in length

(Marcus-Sekura, 1988). Oligonucleotides have the advantage of being commercially available but the quantities needed for an experiment can be quite costly. Vectors containing an antisense gene are another type of antisense commonly used. Plasmids with an inserted gene or partial gene in the antisense orientation have been transfected transiently or stably into cells where they produce antisense mRNA. The length of the antisense transcript produced from the plasmid or virus ranges from as few as fifty bases to the entire length of a gene. Using a vector containing the antisense gene necessitates the use of a promoter. Strong promoters such as the cytomegalovirus (Han et al, 1991) or inducible promoters like the mouse mammary tumor virus (Izant and Weintraub, 1985) are some examples. Stable transfection of a plasmid with an antisense gene or gene fragment has an advantage over oligomers in vitro. Whether the vector uses an inducible or constitutive promoter, the antisense message is always present and more can be produced, unlike oligomers which have only a limited lifetime and must be continuously added to the system.

The delivery method of antisense transcripts depends on the target and the researchers as well as the type of antisense used. Oligonucleotides have been placed in media above cells to be incorporated naturally as well as microinjected into tumor cells (Moroni et al, 1992) or pumped subcutaneously into mice (Ratajczak et al 1992). Transgenic mice (Han et al, 1991; Pepin et al, 1992; Richard

et al, 1993) as well as transgenic plants like tomatoes (Smith et al 1988) and flowers (Moffat, 1991) have been created with the incorporation of antisense genes. Stable or transient cell lines are made by transfecting a plasmid containing the antisense gene. Though calcium phosphate precipitation and protoplast fusion are commonly used for transfection in animals and plants respectively, many different methods are used including lipofection (Yeoman et al, 1992), DEAE-dextran (Han et al, 1991) and electroporation (Kaiser et al, 1992). After transfection with a plasmid containing a resistance gene such as neomycin, selective medium is utilized to obtain the transgenic cells line(s). Lin and Lane (1992) used electroporation to stably transfect 3T3-L1 cells with an plasmid containing antisense CCAAT/enhancer-binding protein. This plasmid also had the neomycin resistance gene. After transfection monoclonal cell lines were selected with geneticin (cells transfected with plasmids containing the neomycin gene are resistant to the antibiotic geneticin). Although many researchers isolate monoclonal cell lines, Kim and Wold (1985), Kaiser et al (1992), Wu et al (1992) and Izant and Weintraub (1985) used polyclonal cell lines. Direct microinjection of *in vitro* transcribed RNA has also been used in oocytes (Rosenburg et al, 1985; Melton et al, 1985, Fire et al, 1991) and embryos (Bevilacqua et al, 1988).

## Problems with Exogenous Antisense Messages

Izant and Weintraub (1985) seemed to suggest that any gene product could be target for antisense messages and therefore be inhibited. Unfortunately, antisense inhibition is not as easy as Izant and Weintraub (1985) proposed it could be. Stability of the antisense transcripts, proper levels to use and detecting the messages have been causes of concern. While antisense works in some systems very well, in others it does not or requires high amounts of the antisense message.

One of the difficulties of using antisense messages is the stability of transcripts. Oligonucleotides modified with a sulfur (phosphorothioates) or methyl groups (methyl phosphonates) or alternating methyl/phosphodiester groups have all been determined to be more stable than unmodified oligonucleotides (Akhtar et al, 1991). Krieg et al (1993) added a cholesteryl moiety to the oligonucleotide and found that these associated to the lipid bilayer via low density lipoproteins and increased efficiency of the antisense message. The presence of serum also destabilizes oligonucleotides. Calf serum in particular was the most destabilizing serum type when compared to human serum, nuclear extract and cytoplasmic extract (Akhtar et al, 1991).

Antisense RNA, transcribed *in vitro* then injected, also has problems with stability. Bevilacqua et al (1988)

determined that when twenty picograms of antisense  $\beta$ -glucuronidase RNA (more than 20 picograms killed the embryos) was injected into embryos, only 20% of it remained after 36 hours. The sense-antisense hybrid was also made in vitro then injected into cells to measure the hybrid stability. It was determined that the hybrid was stable for only five hours. Even with such a short half life, they were able to block  $\beta$ -glucuronidase in the embryos.

How much antisense message to use is different in every system. Izant and Weintraub (1985) reported that up to a fifty fold excess of antisense was required to yield a twenty fold inhibition of genes in *Xenopus* oocytes but only a one to one ratio was needed to block  $\beta$ -galactosidase transcription. The required amount also varied depending on the method of introduction. Only a five fold excess of antisense thymidine kinase was required to block the gene in transfected cells but a fifty fold was needed in microinjected cells (Izant and Weintraub, 1985). Huge amounts of antisense oligonucleotides are often needed to get the effect desired. For example, Lemaitre et al (1987) used 100 nM of antisense oligomers to vesicular stomatitis virus N-protein to inhibit the virus while Offensperger et al (1993) used 1.5  $\mu$ M antisense oligomers to duck hepatitis B virus to inhibit viral replication. Wang et al (1992) used 30  $\mu$ M antisense alpha subunit to a G-protein to reduce its expression.

Secondary structure of the antisense messages may play a role in their stability. The secondary structure of the RNA 1:RNA II hybrid has been determined in the ColEI system (Lewin, 1990). The binding structure is comprised of three stem loops and is thought to aid in the hybridization of the remainder of the molecules. Eguchi et al (1991) discusses the presence of a protein, Rom, that stabilizes the hybrid by lowering the dissociation rate by a factor of one hundred. It is thought that Rom recognizes the structure of the hybrid, not the sequences involved. Other proteins in the cellular environment may affect stability in other systems as well. The sequences within the RNA hybrids may also affect stability of each RNA as well as the hybrid. While computer programs can predict secondary structure and the energy of the molecule, it is difficult to use these methods on large antisense molecules. Though mutating one base pair in RNA II of ColEI results in the same predicted secondary structure as the nonmutant, the mutant has a dramatic loss in function and a different susceptibility to RNases. To complicate things even further, tertiary and other structures cannot be accounted for in computer programs (Eguchi et al, 1991).

Locating antisense transcripts in target cells has been a further challenge. Northern blots do not always reveal the antisense message which has resulted in the use of RNase protection assays. Celano et al (1992) used a protection assay to detect antisense c-myc synthesized in COLO 320



cells. Fernandez et al (1993) and Sklar et al (1991) also used nuclease protection assays to detect antisense messages. Fire et al (1991) used both a nuclease protection assay and PCR to amplify messages in order to detect antisense RNA. Pepin et al (1992) used PCR to amplify mRNA messages from transgenic mice as they were unable to find the antisense message in a Northern blot. Khokha et al (1989) were unable to detect any antisense mRNA in three out of four of their monoclonal cells lines by Northern blot analyses. Kim and Wold (1985) originally were unable to detect any appreciable level of antisense thymidine kinase message. They suggested that perhaps the antisense mRNA was being processed or transported incorrectly. After changing the promoter in their vector and the selection method, antisense RNA was detected with a protection assay. Although this implies antisense messages are hard to detect, Han et al (1991), Hamilton et al (1990) and Kaiser et al (1992) were able to detect antisense mRNA using either total RNA or mRNA in Northern blots with riboprobes.

Finding antisense oligonucleotides *in vivo* is rarely attempted since these molecules are so small. Grigoriev et al (1993) UV crosslinked oligodexonucleotides *in vitro* to the interleukin 2 receptor DNA to show a triple helix formed and prevented transcription. Holt et al (1988) end labelled oligonucleotides and using a S1 nuclease protection assay, showed that a hybridization did form between the antisense oligonucleotide and c-myc mRNA *in vivo*.

Once again, these differences in antisense systems have made it difficult to provide a single model for using antisense. Methods of detecting and measuring stability of antisense messages are sometimes not specific enough to determine how the antisense works. Even so, these problems have not halted the popularity of this relatively new technique to control gene expression.

#### Variations of results when using Antisense Messages

Antisense messages have not always worked as expected or not worked at all. The controls can also cause some inhibition as shown in some of the following examples.

Leiter et al (1990) is a good example of confusing antisense results. Leiter et al (1990) tried to inhibit the flu virus (strain A and C) with an antisense phosphorothioate oligomer. As controls, mismatched oligonucleotides were used as well as unmodified (phosphodiester) antisense and mismatched oligonucleotides. The phosphodiester oligomers did not inhibit either the C or A flu virus even at concentrations of 80  $\mu$ M. Both C and A viruses were inhibited with phosphorothioate oligomers but at 20  $\mu$ M and 1.25  $\mu$ M concentrations respectively. Virus A was even inhibited with the mismatched sequences. These results show that antisense is not the same in each case, even when similar genes are targeted.

Ratajczak et al (1992) used an antisense phosphorothioate oligomer to inhibit c-myc. C-myc proto-oncogene synthesizes proteins needed in leukemia cell growth. Inhibiting this gene would theoretically lower leukemia cell proliferation. Ratajczak placed tiny pumps into the paraspinal space of leukemic mice. Antisense oligomers were pumped into the mice at 1  $\mu$ l/hour at 4.2  $\mu$ g/ $\mu$ l (total dose 100  $\mu$ g/day) for 3 days. Although death was delayed, by day eleven all of the mice were dead. The experiment was repeated for seven days then again for fourteen days. In both cases, mice receiving antisense oligomers all lived longer than control, sense or mismatched oligomer treated mice. Eventually (by day 42) all mice had died. The authors suggested that antisense should perhaps be used in combination with other conventional treatments when treating cancer.

Antisense therapy has been suggested as a method to treat AIDS. Several researchers have tried to use antisense to inhibit the various viral genes involved [Matsukura et al (1987), Agrawal et al (1988), Gilboa et al (1994)]. Lisziewicz et al (1993) tried antisense against the tat, gag and rev genes in cell culture. Although the tat antisense did not inhibit the virus, both the gag and rev antisense inhibited virus replication for more than eighty days when cells were treated twice a week with the antisense. If the oligomers were removed, virus was detected after only four

passages. The antisense therefore inhibited replication of virus, but did not get rid of it.

Antisense oligonucleotides have been used in humans to treat cancer. Recently, an acute myeloblastic leukemia patient received antisense oligomers to p53, a tumor suppressor gene but involved in cancerous cell proliferation. This antisense message was successful in inhibiting p53 in cell culture even after treatment with the antisense was discontinued. The patient received 0.05 mg/kg/hour of phosphorothioate oligonucleotide for ten days (total dose 700 mg). The only side effect seen was higher levels of gamma glutamyl transpeptidase which returned to normal after treatment was complete. The patient did not respond to treatment though cells taken at day six and eleven had reduced growth *in vitro*. This is one of the few examples in which toxicity of the phosphorothioate modified antisense oligonucleotide was mentioned. Phosphorothioate modified nucleotides are released into the cell when the oligomer is broken down. Theoretically, these altered bases could be used in the synthesis of DNA. As there was no major toxicity detected in this case, this provides a definite advantage for using antisense oligonucleotides as a therapeutic (Bayever et al, 1992).

Another unexpected finding is the fact that sense messages, usually used as controls in antisense experiments, sometimes also inhibit the target gene (Jorgensen, 1990 and Nellen et al, 1993). Jorgensen (1990) was working on

genetically engineering flowers with different colors. After adding an extra copy of the gene of the anthocyanin (purple pigment), his flowers turned white. Thus an extra sense gene knocked out the endogenous gene. These new colored flowers may be making money for the plant industry, but it is adding to the confusion about antisense.

### Conclusion

Antisense RNA or DNA has been shown to affect many processes in gene expression including transcription, splicing, transport or translation. The function of endogenous antisense messages remains questionable in eucaryotes, but antisense undeniably has become very useful as an exogenous gene regulator. Further understanding of how these messages work may enable even more use of antisense as a treatment for diseases or as means to disrupt expression of other undesirable gene products. As of now, the action of antisense remains as Denhardt reports the "sometime inhibition of transcription, processing, transport or translation".

## Fatty Acid Synthase Literature Review

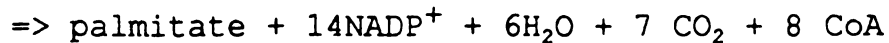
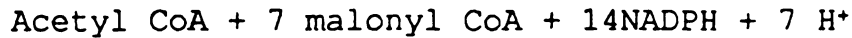
Fatty acid synthase (FAS) is a key enzyme in the synthesis of lipids in an organism. In this project, FAS was targeted in order to inhibit fattening in TAl cells in hopes that this method could be used to reduce fattening in a production animal. Due to the availability of the FAS1 fragment (Witowski et al, 1987) and the understanding of TAl cell culture (Dickerson et al, 1992), the enzyme activity was measured in order to determine if the method was successful. Outlined below is brief summary of this multidimensional enzyme.

FAS catalyzes the reactions needed to synthesize palmitate, the required fatty acid for further fatty acid biosynthesis. It is a very large protein, complex in its function, origin, sequence, gene structure and regulation. Each of these areas has been extensively studied.

### Overall Reaction

FAS synthesizes palmitate by the addition of two carbon units (from acetyl CoA) to malonyl CoA. As a new

fatty acid is elongated, carbon dioxide and water are released. FAS utilizes NADPH as a reductant throughout the reactions. The overall reaction is:



Acetyl CoA is converted to Malonyl CoA by acetyl CoA carboxylase and is the first committed step in *de novo* fatty acid synthesis. Malonyl CoA and acetyl CoA are then linked to the acyl carrier protein (ACP) subunit of FAS and fatty acid synthesis follows (Stryer, 1988).

Fatty acid synthesis occurs in the cytoplasm of eucaryotic cells. Each intermediate in the pathway is linked to ACP, a coenzyme, by a sulfur bond (Stryer, 1988). ACP has prosthetic group of phosphopantetheine which enables all the acyl groups of each intermediate to remain attached as the reaction progresses. The end product, palmitoyl CoA inhibits the enzyme while citrate, the source of acetyl CoA, activates it in the cytoplasm (Stryer, 1988).

#### Origin and Structure of FAS

One monomer of FAS is comprised of seven separate enzymes in three domains. The active protein functions as a homodimer in eucaryotes. The individual enzymes include  $\beta$ -

ketoacyl synthase (condensing enzyme), acetyl transacylase, malonyl transacylase,  $\beta$ -ketoacyl reductase, enoyl reductase, dehydrase, thioesterase and the acyl carrier protein (Stryer, 1988). In bacteria, the enzymes function as individual entities and as two separate units in fungi. It is thought that these individual enzymes became linked as animals evolved (Amy et al, 1992).

The monomer has a molecular weight of 260,000, with the three domains of each protein arranged in an antiparallel fashion. The first domain contains the  $\beta$ -ketoacyl synthase and both transacylases. The substrates enter in domain one and are elongated at the interface of domain I and domains II and III of the opposite monomer. Domain II contains the reductases, dehydrase and the ACP. The ACP prosthetic group is positioned into the center of the three domains. Domain III (the thioesterase) releases the palmitate. Overall Domain I from one monomer acts in conjunction with Domains II and III from the second monomer forming the homodimer (Wakil, 1989). In chicken FAS, domain I, II and III have a molecular weight of 127,000, 107,000 and 33,000 respectively (Mattick et al, 1983) and the rat domains are 125,000, 95,000 and 12,000 daltons respectively (Rangan et al, 1991).

The FAS amino acid sequence is also homologous among different species. Examination of homology between chicken, rat and yeast FAS was determined by Chang and Hammes (1989). Chicken and rat FAS amino acids were 67% similar. The highest degree of similarity was between active sites in



chicken and rat FAS. Yeast and chicken FAS was only 18.8% identical (Chang and Hammes 1989). Mildner and Clarke (1991) reported that rat and pig FAS was 68% homologous but pig and chicken had only 14% homologous amino acids. The thioesterase domains of FAS are similar in rats, mallards and rabbits. These similarities in the FAS protein also indicate a common point of origination (Witkowski et al, 1987).

#### Gene Structure of FAS

The rat FAS gene is a well studied example of eucaryotic FAS. This gene is a single gene over 18,000 base pairs in length and includes forty-two introns. Introns average 191 bases in FAS, unlike the 1127 base average in most vertebrate genes. Intron-exon connections match the established GT/AT standard. Two separate polyadenylation sites are present which give rise to two mRNAs of lengths 8.3 and 9.1 kilobases (Amy et al, 1992). The gene present only once in the eucaryotic genome. The rat FAS gene has been completely cloned including the transcriptional start site and regulatory region. Eighty-seven nucleotides present at the 5' end of the cloned gene are similar in lung, liver and mammary gland FAS mRNA. Sequence analysis revealed that the 5' end of the gene contains similar transcriptional regulatory sequences to the estrogen response element, glucocorticoid response element, thyroid

response element and the progesterone response element. Eight sequences of the cAMP transcriptional regulatory element are also present (Amy et al, 1990).

Joshi and Smith (1993) used a baculoviral vector to show that FAS could be synthesized, folded and dimerized in an insect host cell. The prosthetic group was also successfully attached to the ACP. The FAS cDNA was spliced together then cloned into the baculovirus. Forty-eight hours after transfection, FAS accounted for twenty percent of the cytoplasm. Further studies on FAS will benefit from this system (Joshi and Smith, 1993).

#### Regulation of FAS

FAS is an enzyme with an abundance of regulators. Dietary components and changes as well as hormones all affect the transcription of FAS.

Dietary changes can influence FAS transcription. Fasting an animal decreases transcription while feeding increases FAS levels. FAS mRNA levels were positively related to rate of FAS synthesis, i.e. transcriptional regulation is involved (Morris et al, 1984).

Fatty acids can inhibit lipogenic enzyme synthesis at the transcriptional level. Although polyunsaturated fatty acids in the diet can regulate transcription of FAS, monounsaturated or saturated fats seem to have no influence over FAS transcription (Armstrong et al, 1991).

Polyunsaturated fats have been shown to depress FAS mRNA levels in rat liver (Clarke et al, 1990a, Blake and Clarke, 1990). FAS mRNA levels also increased when the rats ate an elevated carbohydrate diet or glucose. High fat diets, however, dramatically lowered FAS mRNA levels. From these studies, it was estimated that FAS mRNA had a half life of eight hours (Clarke et al, 1990b).

Hormones can also modify FAS mRNA levels. FAS mRNA was decreased in both adipose and liver tissue in pigs injected daily with porcine somatotropin. Pigs fed higher levels of protein also had depressed FAS mRNA levels in the adipose but not in the liver (Mildner and Clarke, 1991).

In obese rats, FAS is overly abundant. Guichard et al (1992) determined that the rate of FAS transcription was much higher in obese rats as compared to lean rats. Amplification of the gene or mutations in the protein were ruled out. The difference in regulation of FAS between the lean and obese rats was not elucidated. The experiment did show that FAS was transcriptionally regulated in rat adipose tissue.

#### FAS in adipocyte cell culture systems

Several studies of FAS in cell culture have led to an increased understanding of FAS and its regulation. FAS mRNA levels dramatically increases in 3T3-L1 cells when they differentiate from preadipocytes to adipocytes. It has also

been shown that cAMP lowers and insulin raises FAS mRNA levels in these cells. Sequences present in the promoter region have been shown to be responsive to insulin (Moustaid et al, 1993; Paulauskis and Sul, 1988). Triiodothyronine has proven to increase transcription rates of FAS mRNA as well as increase its stability in 3T3-L1 as well (Moustaid and Sul, 1991). In TA1 cells, another adipogenic cell line, FAS activity increased upon differentiation with dexamethasone and indomethacin. The  $\beta$ -adrenergic agonists ractopamine and isoproterenol also affect FAS by depressing its activity in TA1 cells (Dickerson et al, 1992).

## Conclusions

These different examples demonstrate that FAS regulation remains as complex as the protein itself. Antisense may be an alternative method to study the regulation and function of FAS.

## **Objective**

The meat animal industry continues to strive toward developing methods to lower fat accumulation in food animals that can be regulated, produce no by-products and could be implemented in a production animal in the future. This experiment was designed to research a very specific method of inhibiting a lipogenic enzyme. The objective was to determine if a plasmid containing a portion of the FAS gene in antisense orientation can inhibit fatty acid synthase activity in stably transfected TA1 cells. Three separate aims were required to accomplish this goal: create an antisense plasmid, transfect the plasmids into TA1 cells and create stable cells and subsequently determine if FAS activity was inhibited.

## **Hypothesis**

Stably transfecting TA1 cells with a plasmid containing antisense fatty acid synthase can lower fatty acid synthase (FAS) activity.

### *Aim #1*

Synthesize a plasmid containing the antisense and sense piece of FAS.

### *Aim #2*

Establish stable TA1 cell lines with the antisense, sense and control plasmids.

### *Aim #3*

Determine if inhibition of endogenous FAS occurred in the stable antisense TA1 cell lines.

## Aim #1: Plasmids

### Methods for Plasmid Synthesis

The host vector required several characteristics: a strong constitutive promoter, a polyadenylation site, resistance genes for selection in bacteria as well in eucaryotes and the ability to integrate into a eucaryotic genome. For these reasons, pcDNA3 was purchased from Invitrogen, San Diego, California (Figure 3). This vector uses the cytomegalovirus as a strong constitutive promoter. It contains a polyadenylation sequence from the bovine growth hormone which immediately follows the multiple cloning site. Both the ampicillin and neomycin resistance genes are present for procaryotic and eucaryotic selection, respectively. The SV40 early promoter allows for integration into the host genome. Both T7 and SP6 promoters enable the plasmid to be used for *in vitro* transcription or for sequencing. The pcDNA3 plasmid, with the chloramphenicol acetyl transferase (CAT) gene ligated into the HindIII site, was also purchased from Invitrogen (Figure 4). Both of these plasmids were used as controls throughout the experiments.

FAS1 (Witowski et al, 1987), was ligated into the vector to create the antisense and sense plasmids (Figures 5 and 6). This fragment is 1200 base pairs in length and codes for the acyl carrier protein domain and parts of the

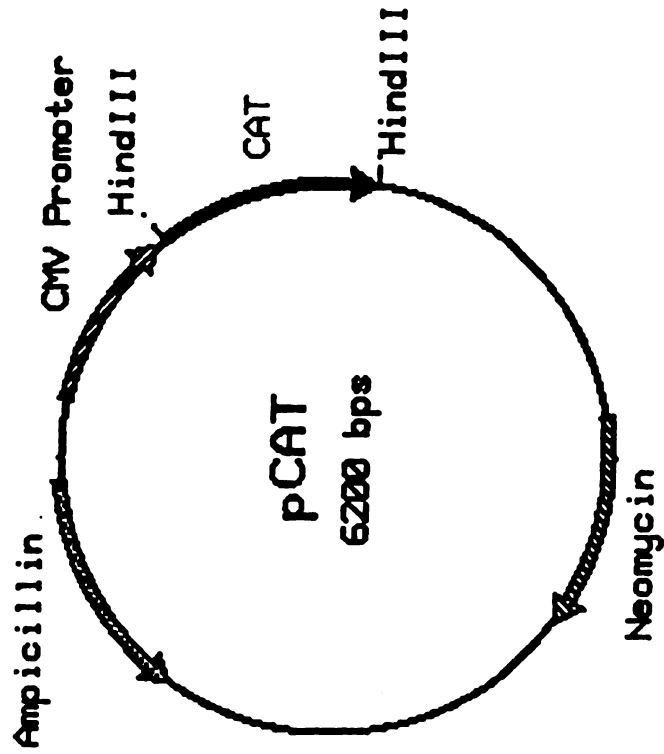


Figure 4. pCAT. CAT is ligated into the HindIII site of pCDNA3.

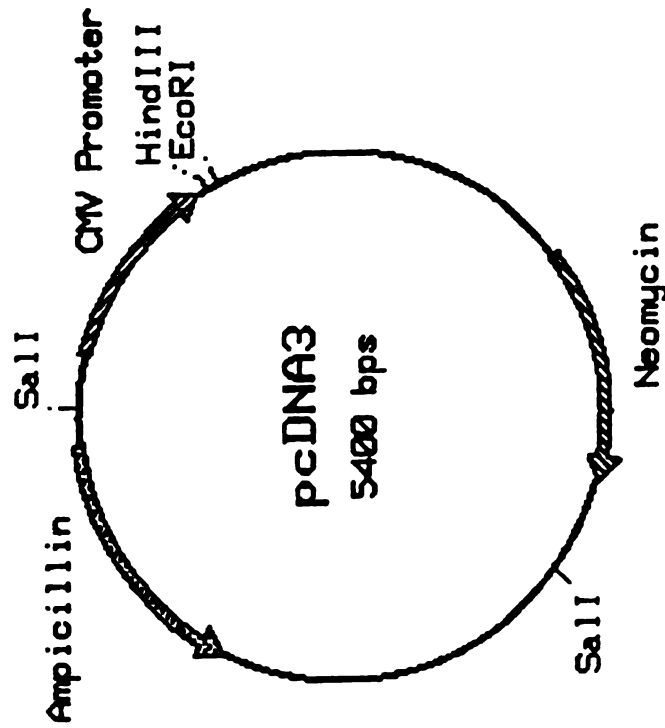


Figure 3. pCDNA3. The multiple cloning site is indicated by the EcoRI and HindIII sites.

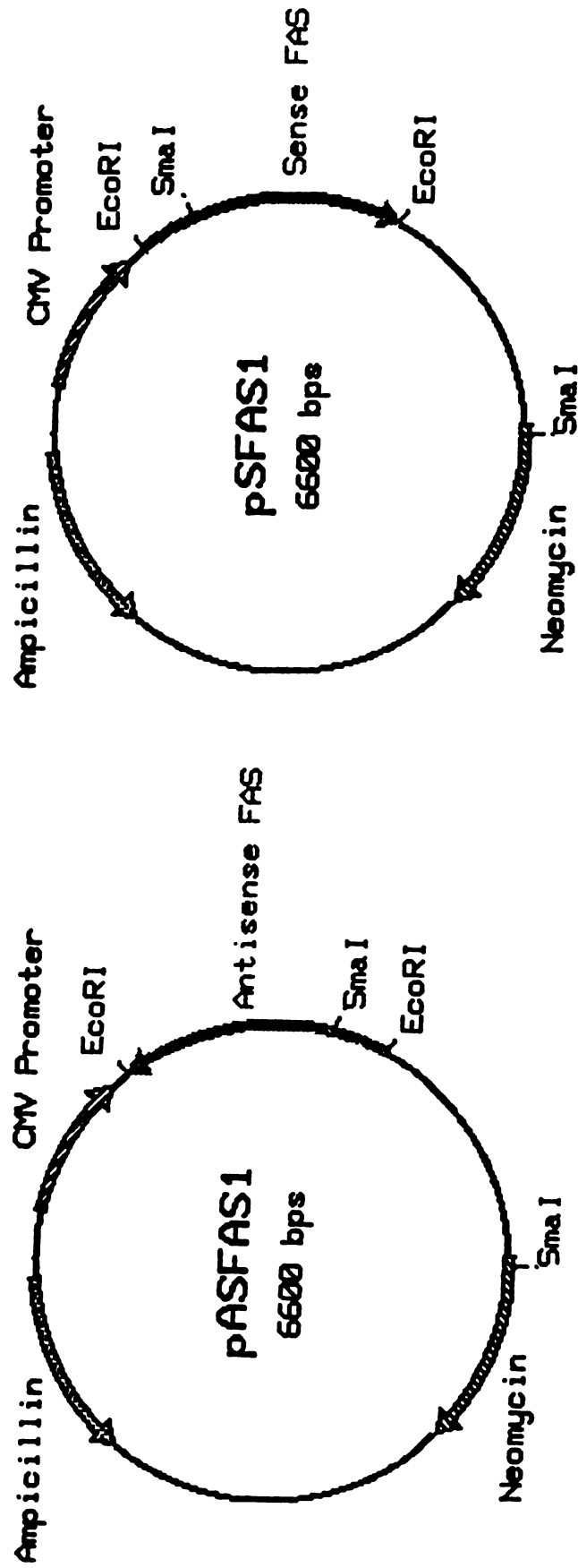


Figure 5. pASFAS1. FAS1 is ligated into the EcoRI site of pCDNA3 in antisense orientation.

Figure 6. pSFAS1. FAS1 is ligated into the EcoRI site of pCDNA3 in sense orientation.



thioesterase and ketoreductase subunits of FAS. This fragment was inserted into the EcoR1 site of pcDNA3, creating pASFAS1 and pSFAS1 for the antisense and sense plasmids respectively.

Immediately following ligation, the plasmids were all transformed into competent DH5 $\alpha$  bacteria. Upon positive colony formation, mini-preps on 12 colonies were performed using Promega's Magic Miniprep System. Antisense and sense clones were selected by restriction enzyme digests. Once both antisense and sense clones were detected and verified, large scale plasmid preparations provided large quantities of pASFAS1, pSFAS1, pcDNA3 and pcDNA/CAT for transfection (Sambrook et al, 1987). These methods are outlined in detail in Appendix A.

The plasmids pSFAS1, pASFAS1 and pcDNA3 were sequenced at the Plant Research Laboratory by Susan Lootens. The DyeDeoxy method was used using the T7 dyeprimer or SP6 dyeprimer, a system designed by Applied Biosystems, Inc. This method is similar to the Sanger dideoxy method, a "controlled interruption of enzymatic replication", (Stryer, 1988).

## Results and Discussion of Plasmid Creation

The antisense and sense FAS plasmids were created and designated pASFAS1 and pSFAS1 respectively. Restriction

digests of all four plasmids were done to verify each plasmid (Figure 7).

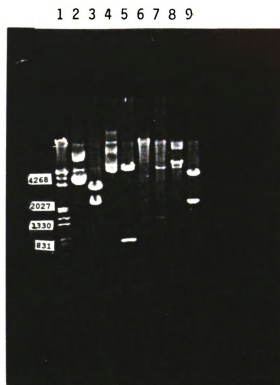


Figure 7. Orientation of plasmids. The gel pictured above contains each plasmid uncut and also cut by enzymes that verify the orientation of an insert if present. The uncut lanes may have more than one banding corresponding to the supercoiled, partially coiled and linear plasmids, the usual mixture in a plasmid preparation. Supercoiled plasmids travel farther in a gel than linear which travel farther than circular plasmids. Lane 1:Marker 3 with selected sizes noted, Lane 2:uncut pcDNA3, Lane 3:SalI digest of pcDNA3, Lane 4:pCAT uncut, Lane 5:pCAT cut with HindIII, Lane 6:pASFAS1 uncut, Lane 7:pASFAS1 cut with SmaI, Lane 8:pSFAS1 uncut, Lane 9:pSFAS1 cut with SmaI.

As shown, the correctly sized fragments were obtained in each lane (Table 1).

**Table 1.** Predicted Lengths of Restriction Fragments of each Plasmid.

Plasmid	Enzyme	Predicted Fragments
pcDNA3	SalI	2200 bp, 3200 bp
pCAT	HindIII	5400 bp, 800 bp
pASFAS1	EcoRI	5400 bp, 1200 bp
	SmaI	5200 bp, 1400 bp
pSFAS1	EcoRI	5400 bp, 1200 bp
	SmaI	4500 bp, 2100 bp

The sequence of pcDNA3 and pSFAS1 were successfully done with the DyeDeoxy method. The sequence chromatogram (Figure 8) of pSFAS1 nearly match the sequence of FAS1 (Figure 9). The sequence of pSFAS1 is identical to that of FAS1 starting at base number 59 in the chromatogram. The pASFAS1 plasmid was only partially sequenced, with short fragments matching the expected sequence.

The synthesis of the vectors was successful. All four plasmids gave the predicted digested fragments. The sequencing of pcDNA3 and pSFAS1 further verified the orientation of FAS1 in pSFAS1. Only one base within the first 100 bases varied between the FAS1 sequence reported by Witowski et al (1987) and the pSFAS1 sequence determined by Dyedeoxy sequencing in the pSFAS1 plasmid.

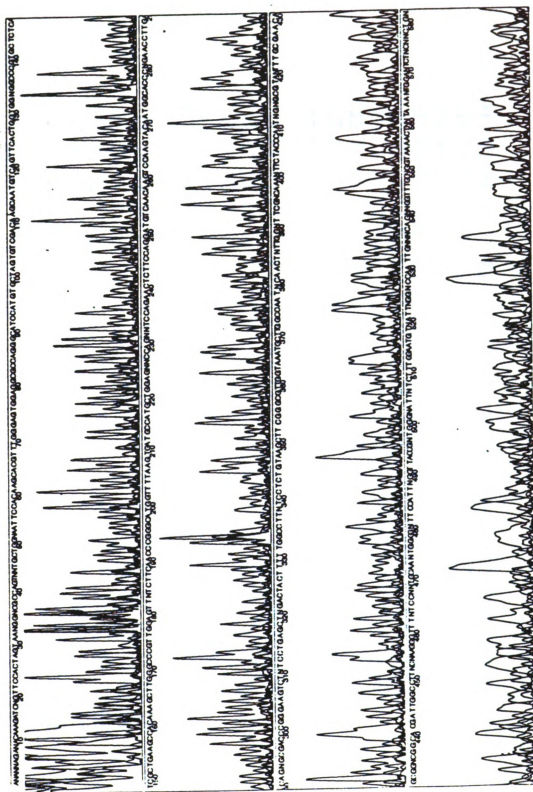


Figure 8. Sequence Chromatogram of pSFAS1.

```

3   AAG CAC GTT CCG CAG TCG AGG CCG CAG GCG ATC CAT GTG CTA GTG TCG ACA AOC AAT GTC AC
1   Iys His Val Arg Glu Trp Arg Arg Glu Gly Ile His Val Leu Val Ser Thr Ser Ser Val

63  AGT TCA CTG CAG GCG GCG GGT GCT CTC ATC GCT GAA GCG ACA AAG CTT GCG CCG GTT GGA
21  Ser Ser Leu Glu Gly Ala Arg Ala Leu Ile Ala Glu Ala Thr Lys Leu Gly Pro Val Gly

123 GGT GTC TTC AAC CTG GCG ATC CTT TTA AAG GAT GCG ATG CTG CAG AAC CAG ACT CCA GAA
41  Gly Val Phe Asn Leu Ala Ile Leu Leu Arg Asp Ala Met Leu Glu Asn Glu Thr Pro Glu

183 CTC TTC CAG GAT GTC AAC AAG CCG AAG TAC AAT GCG ACC CTG AAC CTT GAC AAG GCG ACC
61  Leu Phe Glu Asp Val Asn Lys Pro Lys Tyr Asn Gly Thr Leu Asn Leu Asp Arg Ala Thr

243 CCG GAA GCG TGT CCT GAG CTG GAC TAC TTT GTG GCG TTC TCG TCT GTA AGC TCG GCG GGT
81  Arg Glu Ala Gys Pro Glu Leu Asp Tyr Phe Val Ala Phe Ser Ser Val Ser Gys Gly Arg

303 GGT AAT GCT GCG CAA TCG AAC TAT GCG TTC GCG AAC TCT ACC ATG GAG GGT ATT TCG GAA
101 Gly Asn Ala Gly Glu Ser Asn Tyr Gly Phe Ala Asn Ser Thr Met Glu Arg Ile Gys Glu

363 CAG CCG CCG CAC GAT GCG CTC CCA GGT CTT GCG GTG CAA TCG GGT GCG ATT GGT GAC TTG
121 Glu Arg Arg His Asp Gly Leu Pro Gly Leu Ala Val Glu Trp Gly Ala Ile Gly Asp Val

423 GCG ATT ATC TTG GAA GCG ATG GGT ACC AAT GAC ACA GTC GTT GCG GCG ACA CTG CCA CAG
141 Gly Ile Ile Leu Glu Ala Met Gly Thr Asn Asp Thr Val Val Gly Gly Thr Leu Pro Glu

483 CCG ATC TCG TCG TCG ATG GAG GTG CTG GAC CTC TTC CTG AAT CAG GCG CAC GCA GTC CTG
161 Arg Ile Ser Ser Gys Met Glu Val Leu Asp Leu Phe Leu Asn Glu Pro His Ala Val Leu

543 AGC AGT TTT GTG CTG GTT GAG AAG AAA GCT GTG GCG CAT GGT GAT GGT GAA GCG CAG AAG
181 Ser Ser Phe Val Leu Val Glu Lys Lys Ala Val Ala His Gly Asp Gly Glu Ala Glu Arg

603 GAT CTG GTG AAA GCA GTC GCA CAG ATC CTA GCG ATG GCG GAC CTG GCA GCG ATT AAC TTG
201 Asp Leu Val Lys Ala Val Ala His Ile Leu Gly Ile Arg Asp Leu Ala Gly Ile Asn Leu

663 GAC AGC TCG CTG GCA GAC CTC GCG CTG GAC TCG CTG ATG GGT GTG GAA GTG CCG CAG ATC
221 Asp Ser Ser Leu Ala Asp Leu Gly Leu Asn Asn Leu Met Gly Val Glu Val Arg Glu Ile

723 CTG GAA GGT GAA CAT GAT CTG GTG CTA GCG ATT GGT GAA GTA GCG CAA CTG ACA CTG TCG
241 Leu Glu Arg Glu His Asp Leu Val Leu Pro Ile Arg Glu Val Arg Glu Leu Thr Leu Arg

783 AAG CTT CAG GAA ATG TCG TCG AAG GCT GCG TCA GAC ACT GAG TTG GCA GCG CCG AAG TCG
261 Lys Leu Glu Glu Met Ser Ser Lys Ala Gly Asp Asp Thr Glu Leu Ala Ala Pro Lys Ser

843 AAG AAT GAT ACA TCG CTG AAG CAG GCG CAG CTG AAT CTG AGT ATC CTG CTG GTG AAC TCG
281 Lys Asn Asp Thr Ser Leu Lys Glu Ala Glu Leu Asn Leu Ser Ile Leu Leu Val Asn Pro

903 GAG GCG GCT ACC TTA ACA GCA CTC AAC TCA GTG CAG AGC TCT GAG GCG GCT CTG TTC TTA
301 Glu Gly Pro Thr Leu Thr Arg Leu Asn Ser Val Glu Ser Ser Glu Arg Pro Leu Phe Leu

963 GTG CAG CCG ATT GAA GGT TCG ATC ACT GTG TTC CAG AGC CCG GCT GCG AAG CTC AGT TTA
321 Val His Pro Ile Glu Gly Ser Ile Thr Val Phe His Ser Leu Ala Ala Lys Leu Thr Val

1023 CCG ACC TAC GGT CTG CAG TCG ACC CAA GCG GCG CCG CTG GAC AGC ATT CCA AAC CTT TTA
341 Pro Thr Tyr Gly Leu Glu Gys Thr Glu Ala Ala Pro Leu Asn Asn Ile Pro Asn Leu Gys

1083 GCG TAC TAC ATT GAT TCG ATC AAG CAG CTG CAG GCT GAG GCG GCG CAC GCA GTG GTG TTA
361 Ala Tyr Tyr Ile Asp Gys Ile Lys Glu Val Glu Pro Glu Gly Pro His Arg Val Ala Gys

1143 TAT TCT TTT GCA GCT TGT GTA GCG TTC GAG ATG TCG TCG CAG CTG CAG GCG CAG CAG TTA
381 Tyr Asn Phe Gly Ala Gys Val Ala Phe Glu Met Gys Ser Glu Leu Glu Ala Glu Glu Gys

1203 GCA GCG GCG GCG G
401 Pro Ala Pro Ala

```

Figure 9. FAS1 Sequence (Witowski et al, 1987). The first base corresponds to base number 59 on the chromatogram of pSFAS1.

The antisense plasmid, pASFAS1, was unable to be completely sequenced although four separate attempts were made. The orientation was verified with the sequencing although only twenty-five matching base pairs could be detected between the chromatogram and the 3' end of FAS1 (Figure 9). The poor chromatogram may have been due to secondary structure between the insert and the plasmid thereby inhibiting the DNA polymerase from remaining attached to the plasmid. However, secondary structure analysis of the first one hundred base pairs following the primer site in the antisense and sense plasmid revealed that both structures are very similar in shape and energy (Figure 10). Since the sense plasmid was successfully sequenced, the antisense plasmid should have also been able to be sequenced using this method. Ethanol or salt contamination in the DNA preparation may have also caused poor sequencing. Each sample was prepared similarly, therefore it is unknown why pASFAS1 wasn't easily sequenced (personal communication Susan Lootens, Dr. Newman).

## Aim #2: Cell Lines

### Methods of Cell Line Establishment

The adipogenic cell line, TA1, was used in all experiments. Cells were cultured as described by Dickerson et al, 1992. All four vectors were transfected into TA1

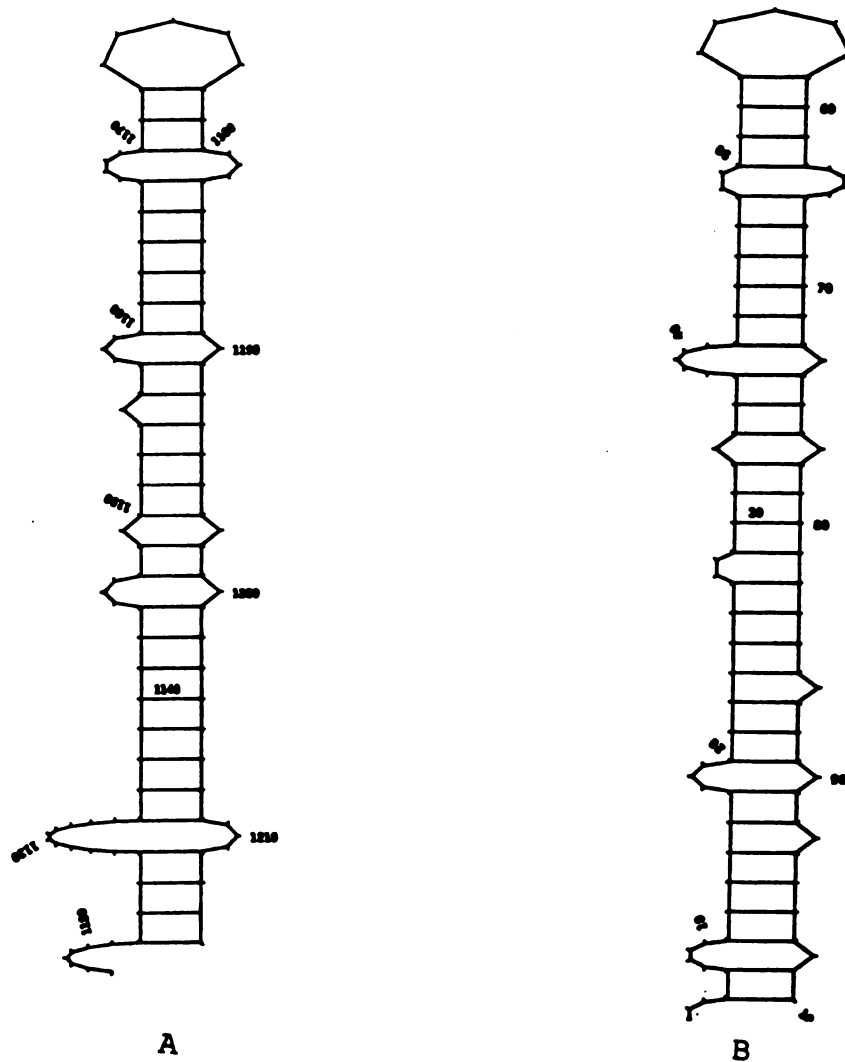


Figure 10. Secondary Structure. The secondary structure of the first 100 nucleotides of the antisense transcript (A) and the sense transcript (B). Both are very similar in energy and appearance.

cells using the lipofection method according to Felgner et al, 1986. Polyclonal (from pooling surviving cells) and monoclonal cell lines (differential dilution) were established in the presence of 0.4 mg/ml geneticin (also known as G418). Once cell lines were established, all transfected cell lines were cultured in the presence of 0.2 mg/ml G418 until induced to differentiate (Felgner et al, 1986, Sambrook et al, 1987). Differentiation was induced with 1  $\mu$ M dexamethasone and 1.25  $\mu$ M indomethacin (Dickerson et al, 1992). Southern blots of genomic DNA were done using an FAS1 random primed or riboprobe in order to detect the presence of the transfected plasmids (Sambrook et al, 1987). Total RNA was isolated using RNA Stat-60 per manufacturer's instructions (Tel-Test "B", Friendswood, Texas), and mRNA was isolated from total RNA using Promega's PolyAtract mRNA Isolation Kit (Promega, Madison, Wisconsin). Detection of antisense mRNA was attempted with Northern blots of both total RNA and mRNA (Sambrook et al, 1987) using riboprobes synthesized with Stratagene's RNA transcription protocol (Stratagene, La Jolla, California). An enzyme linked immunosorbant assay (ELISA) was performed on cells transfected with pCAT and nontransfected cells per manufacturer's instructions (Boehringer Mannheim, Indianapolis, Indiana). Procedures are outlined further in Appendix B.

A regression curve was generated using the standards provided in the ELISA kit. An inverse regression formula



was used to determine amount of CAT protein in CP2 and nontransfected cells. Comparisons of the determined amount of CAT protein from the nontransfected and CP2 cells were made using Student's t-test (Gill, 1978).

## Results and Discussion of Cell Line Establishment

Stable monoclonal and polyclonal cell lines were established. After selection pressure for four weeks, cells of each line were pooled for polyclonal cell lines and diluted for monoclonal cell lines. Viable cells were apparent in each case while nontransfected cells were completely nonviable in only three weeks. A minimum of three monoclonal cell lines were established for each cell line: AM1, AM2, AM3 etc. Polyclonal cell lines were designated AP2 (antisense polyclonal cell line number 2), CP2 (CAT polyclonal), SP2 (sense polyclonal) and 3P2 (pcDNA3 polyclonal). Monoclonal cell lines were similarly designated AM1, CM1, SM2 and 3M1.

Establishment of the cell lines is assured by the antibiotic geneticin (G418). G418 is toxic to normal eucaryotic cells and prevents survival by inhibiting ribosomal function. The four vectors used each contain the neomycin gene that confers resistance to G418 by providing a protein which cleaves this antibiotic in the eucaryotic cell. Any cell not containing a transcribing vector could not survive more than one replication in the presence of 0.4

mg/ml G418. The cells were cultured for four weeks at this level. Remaining cells were furthered cultured in 0.2 mg/ml G418 whenever used until the point of differentiation (at confluency).

Genomic DNA was isolated from the polyclonal cells lines and digested with EcoR1. A Southern blot probed with FAS1 was unable to repetively detect the presence of transfected plasmid. Each probe, whether nonradioactive digoxigenin or radioactive P-32, detected a common large band in every cell line assumed to be the endogenous FAS gene. The digoxigenin FAS1 detected a 1200 base pair fragment in antisense polyclonal cell DNA and in the sense monoclonal cell DNA but not in the other cells with transfected pASFAS1 or pSFAS1. The nontransfected, CAT or pcDNA3 containing cells had a detected 1200 bp fragment in any blot probed. With random primed radioactive FAS1 probes, no bands, except endogenous FAS1, were detected in Southern blots of the polyclonal cells lines. The same blots were stripped and reprobed with a radioactive riboprobe synthesized from pASFAS1. Again, no bands except positive controls were detected. As a final approach, a digoxigenin probe was made to the entire pASFAS1 plasmid. Two Southern blots of genomic DNA from the polyclonal cells lines were probed: uncut genomic DNA and EcoR1 digested genomic DNA (>15 ug in each blot). This probe should detect FAS1 as well as the plasmids used in each cell line. In this case, there was some detection in each cell line,

including the nontransfected control. No distinct bands were detected.

It is unknown why the plasmid could not be detected reproducibly in the transfected cells lines since the geneticin selection insures that the plasmid is present in the cells. If the plasmid was present in only one or two copies per genome, the system used to detect the plasmids may not have been sensitive enough. The riboprobe was used for this reason. Since the synthesis of riboprobes allows for more incorporation of radioactive nucleotides, this probe should have caused more exposure on the film when bound to a complementary fragment of DNA as compared to the random primed probe. However, the riboprobe did not detect anything except positive controls. The digoxigenin plasmid probe had a much wider range of DNA it could detect. By digesting the DNA with EcoR1, the probe should detect the 1200 base pair FAS1 as well as the remainder of the plasmid, 5440 base pairs in length. However only a similar smear of DNA was detected in each cell line.

Antisense FAS mRNA was also unable to be detected by Northern blotting. Northern blots of total RNA (10-20 ug per lane), when probed with either the sense or antisense riboprobe, showed no differences between cell lines. Northern blots of mRNA (0.3 ug per lane) also failed to detect antisense mRNA. Only endogenous FAS was detected in each cell line. This result is not uncommon in antisense work; others have been unable to detect antisense mRNA

(Pepin et al, 1992; Fire et al, 1991; Celano et al, 1992; Kim and Wold, 1985). Poor or no detection of antisense mRNA may be due to instability of antisense mRNA or rapid degradation of the sense:antisense hybrid (Kim and Wold, 1985). Since antisense mRNA really has no purpose in the cell, it is likely that the cell would degrade it quite rapidly making it difficult to detect.

The ELISA assay, a colormetric assay detecting CAT protein, showed a significantly greater amount of CAT protein in CP2 cells as compared to nontransfected cells (Figures 11 and 12). This experiment was repeated and verified this result, however numerically both the CP2 and nontransfected cells had four fold lower activity in the second experiment. This difference between experiments is due to differences between the ELISA kits used. The second kit showed much lower results in other experiments within the laboratory.

The ELISA assay offers further evidence that this plasmid can transcribe RNA usable within the cell. The CP2 cell line produced the active CAT protein in significant amounts as compared to nontransfected cells. Though this can not be used to measure how much antisense or sense mRNA is produced in the other cell lines, it does show that this plasmid can be used to incorporate exogenous genes into the TA1 cell.

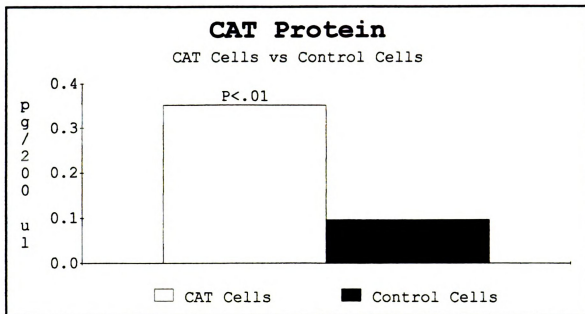


Figure 11. CAT Protein in Experiment #1. Amount of CAT protein in polyclonal cells transfected with pCAT compared to cells not transfected. Amount of protein is determined per 200  $\mu$ l of cell extract. Significance is indicated on the chart itself.

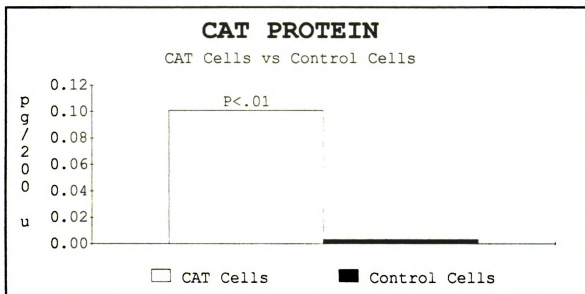


Figure 12. CAT Protein in Experiment #2. Amount of CAT protein in polyclonal cells transfected with pCAT compared to cells not transfected. Amount of protein is determined per 200  $\mu$ l of cell extract. Significance is indicated on the chart itself.

The ELISA assay offers further evidence that this plasmid can transcribe RNA usable within the cell. The CP2 cell line produced the active CAT protein in significant amounts as compared to nontransfected cells. Though this can not be used to measure how much antisense or sense mRNA is produced in the other cell lines, it does show that this plasmid can be used to incorporate exogenous genes into the TA1 cell.

### Aim #3

#### Methods of Measuring FAS Activity

Cells were observed visually after staining with Oil Red O stain (Pollard et al, 1989). Oil Red O specifically stains lipids red and leaves the remainder of the cell colorless. FAS enzyme activity was measured on all cell lines. Cells were rinsed in PBS, incubated in the presence of digitonin to release the protein, then placed in potassium phosphate buffer. After centrifugation, the extract was assayed using the Cary 2200 recording spectrophotometer with the DS-15 enzyme program (Dickerson et al, 1992). Protein was assayed using the Bradford procedure (Bradford et al, 1976). These procedures are written in detail in Appendix C.

Statistical analysis on enzyme assays were performed using Bonferoni t-tests for individual blocks and combined using the chi square test (Gill, 1978).

## Results and Discussion of Staining and FAS Activity

Visually, the cell lines look very similar. Photographs were taken nine days after the cells were induced to differentiate (Figures 13-21). The Oil Red O stain is specific for lipid which appears as dark circular droplets in the photographs.

The result of the staining with Oil Red O Stain is somewhat ambiguous. As photographs can be very subjective, any differences seen between the cell lines may not represent the true differences. Looking at the plates themselves, one may say that the lipid droplets in the monoclonal antisense cell lines look smaller but there is very little differences in amount of lipid droplets. The cells do differ somewhat among cell lines in size. The TA1 cells transfected with pCAT appear more cuboidal and do not generally grow as quickly the first day after plating. As seen in the photographs, the CAT cells have as much lipid accumulation as the others.

A more objective method for determining differences among the cells lines is the FAS enzyme activity assay. One monoclonal and each polyclonal cell line was assayed. Each treatment was assayed at three different times (blocks) and

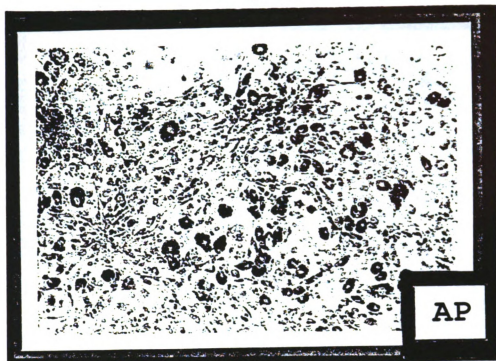


Figure 13. Antisense polyclonal cell line (AP2) stained with Oil Red O stain nine days after induction of differentiation.

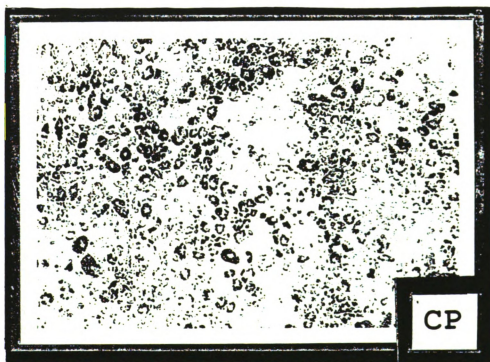


Figure 14. pCAT polyclonal cell line (CP2) stained with Oil Red O stain nine days after induction of differentiation.



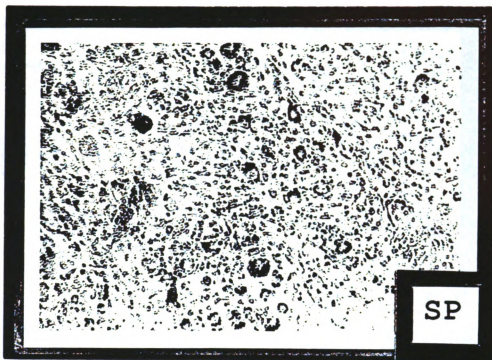


Figure 15. Sense polyclonal cell line (SP2) stained with Oil Red O stain nine days after induction of differentiation.

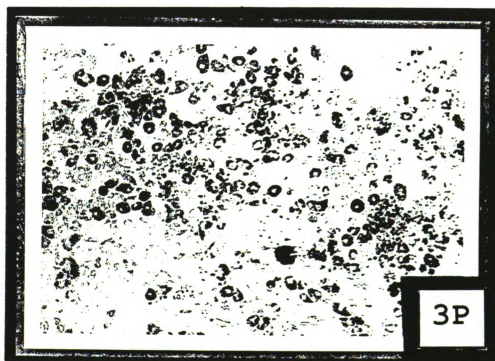


Figure 16. pcDNA3 polyclonal cell line (3P2) stained with Oil Red O stain nine days after induction of differentiation.

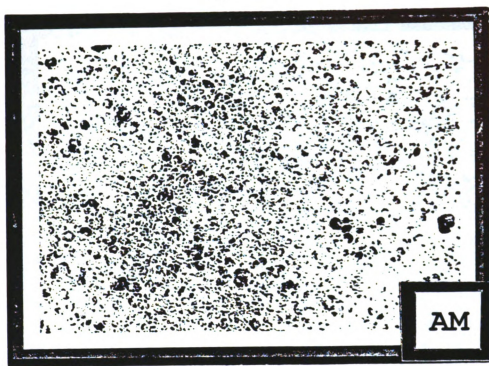


Figure 17. Antisense monoclonal cell line (AM1) stained with Oil Red O stain nine days after induction of differentiation.

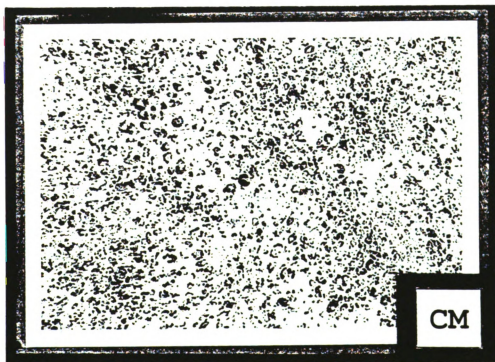


Figure 18. pCAT monoclonal cell line (CM1) stained with Oil Red O stain nine days after induction of differentiation.

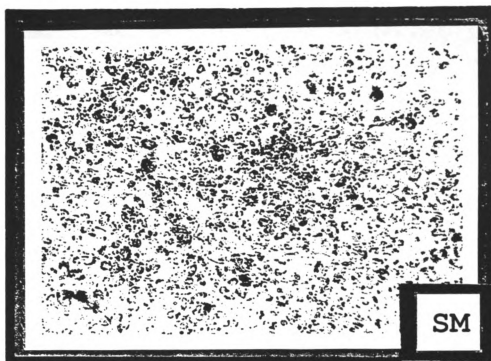


Figure 19. Sense monoclonal cell line (SM2) stained with Oil Red O stain nine days after induction of differentiation.

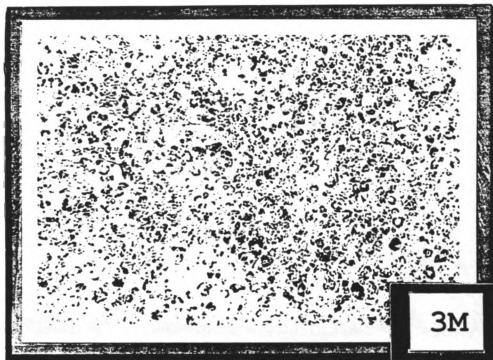


Figure 20. pcDNA3 monoclonal cell line (3M1) stained with Oil Red O stain nine days after induction of differentiation.

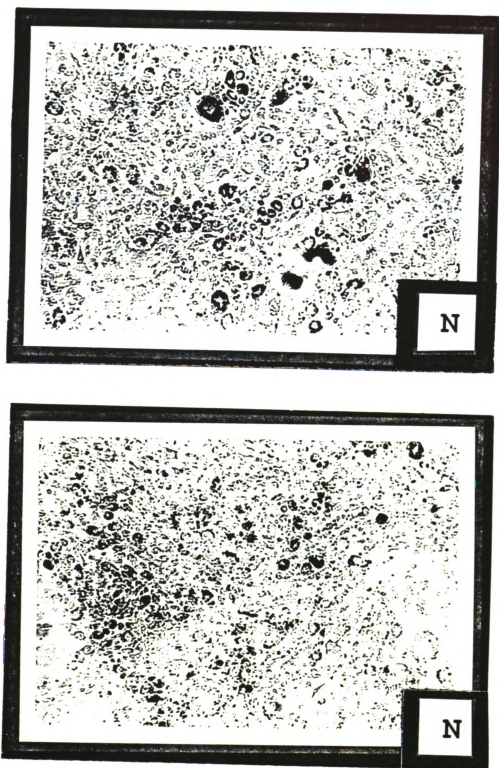


Figure 21. Nontransfected cells (N) stained with Oil Red O stain nine days after induction of differentiation. Both pictures were taken at the same time but from different wells.

statistics performed using a Bonferoni t-test. Individual block statistics are shown in Appendix D. The p-values were pooled and overall differences were determined by combining the p-values using a Chi-square test (Figure 22 and 23).

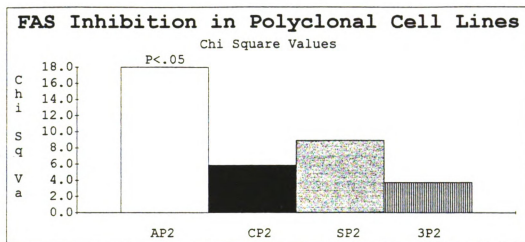


Figure 22. FAS activity in polyclonal cell lines. Activity was measured in nmoles of NADPH disappearance/mg of protein per minute and compared within days to obtain a p-value which was combined among days. A Chi-square test compared inhibition of cell lines to control cells (nontransfected). The greater the chi-square value the more inhibition of FAS activity. Activity is significantly lower in AML, CML and SM2 cells when compared to control cells.

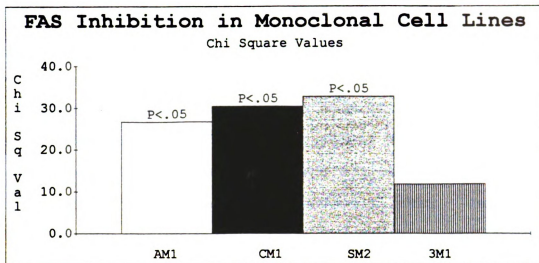


Figure 23. FAS activity in monoclonal cell lines. Activity was measured in nmoles of NADPH disappearance/mg of protein per minute and compared within days to obtain a p-value which was combined among days. A Chi-square test compared inhibition of cell lines to control cells (nontransfected). The greater the chi-square value the more inhibition of FAS activity. Activity is significantly lower in AM1, CM1 and SM2 cells when compared to control cells.

Within the polyclonal cell lines, only AP2 significantly lowered FAS activity ( $P < .05$ ) as compared to nontransfected cells. Within the monoclonal cell lines, the antisense, sense and CAT cell lines all significantly lowered FAS activity ( $P < .05$ ).

The enzyme assays gave a more objective but hardly a conclusive result. Among the polyclonal cell lines, only AP2 (antisense) had a significant lowering effect on FAS enzyme activity ( $P < .05$ ). None of the other polyclonal cell lines depressed activity as compared to control cells. However, the monoclonal cell lines told a different story. The antisense, sense and CAT cell lines all lowered

activity. The CM1 line in particular lowered activity 38% as compared to controls. The antisense and sense cell lines lowered FAS activity by 24% and 21%, respectively.

These confusing results have made it difficult to determine the effect of antisense FAS in TA1 cells. Without a doubt, there was a lowered enzyme activity in some of the cell lines including both antisense cell lines. Using only the data from the polyclonal cell lines, this experiment was successful. The antisense plasmid lowered activity and did so specifically. The three control plasmids, pSFAS1, pCAT and pcDNA3, did not lower activity in polyclonal cells. If the transfection and selection process affected FAS, all the cell lines would have lower FAS activity. AP2 visually also appears similar to the other cell lines. Therefore, pASFAS1 did not effect the cell's ability to differentiate or accumulate fat droplets.

The inconclusiveness is mainly caused by the monoclonal cell lines. Three of the four plasmids lowered FAS activity. The only similar item between these three plasmids is the presence of an insert in the multiple cloning site. Since pcDNA3, the plasmid with no insert, is the only monoclonal cell line that did not lower FAS activity, it can be assumed that the plasmid itself does not inhibit this enzyme. If the insert did cause the FAS inhibition, it seems that it should have also done so in the polyclonal cell lines.

The CAT cells (CM1) had the lowest enzyme activity. These cells may have produced the CAT protein at a level which was inhibitory to the FAS enzyme as well as other cellular processes. These cells did tend to grow slower and be more cuboidal when initially plated.

The required levels of exogenous protein in the cellular environment may have affected the cell's ability to produce active FAS. The plasmid in the TA1 cell genome did need to be active to keep the cell alive in the presence of geneticin. If the cell required the plasmid to be continuously transcribed and the neomycin mRNA continuously translated, it may have reduced the cell's ability to produce normal quantities of FAS and/or other proteins. However, if this was a requirement in the cells, all the monoclonal and pCDNA3 polyclonal cell lines should have lower enzyme activity as well.

The site of integration of the plasmid into the genome may have caused the inhibition of FAS. Each monoclonal cell line originates from a single cell and therefore is expected to have the integrated plasmid in the same location in the genome. A possible integration site effect that could inhibit FAS in this case is that the plasmid could have integrated into the endogenous FAS gene thereby disrupting its transcription. This is a remote chance since FAS is 18,000 base pairs in length and the mouse genome is approximately three billion base pairs in length. Inhibiting FAS in this manner is also unlikely since



disrupting this gene may also be fatal to the cell. If the plasmid had integrated into any gene required for growth or lipogenesis, this may have also caused FAS inhibition. The polyclonal cells lines represent a population of cells each with a different integration site. While one or more of these cells may have the plasmid integrated into a site that lowers activity by a separate gene interruption, this effect will be diluted by other cells with sites of integration with no effect. Polyclonal cells would have integrated the plasmid into different locations thereby creating a collection of cells each with a differently ordered genome. Even if the plasmid did integrate into the FAS gene, it could not have done this in every cell within a polyclonal population. In each of the polyclonal cell lines as well as in the monoclonal cell lines, integration location of the plasmid was not determined. In the monoclonal cell lines, the site of integration plays a more important role since the entire population is the same. Therefore, FAS inhibition may be due to antisense in the polyclonal cell lines but may be due to site of integration in the monoclonal cell lines.

Antisense targeted to the DNA has also inhibited some gene products (Cooney et al, 1988; Celano et al, 1992 and Postel et al, 1991). These examples all involved oligodeoxynucleotides, very short fragments in length (27 nucleotides). The antisense message used here is not only RNA but is over 1200 nucleotides in length. If the

antisense FAS did somehow inhibit transcription by binding the DNA, the sense or antisense mRNA could bind to its complementary DNA strand and form a triple helix structure. Jorgensen (1990) incorporated an extra sense gene for the purple color in petunias into its genome only to have it act like antisense and cause the flower to lose its normal purple color to white. This sense gene acted as antisense and inhibited normal production of the purple pigment. Even with these examples, it is unlikely that the RNA produced by these plasmids (if there is RNA produced) interacted and blocked transcription of FAS at the DNA level.

Most antisense experiments sited in this thesis use an antisense message against the 5' end of the gene, not the 3' end as used here. Most messages are shorter than 1200 base pairs as well. A 5' target, including the AUG translation start site, may have been more inhibiting to FAS since it is such a large message. If hybridization between the antisense and sense messages occur, the duplex formed would be formed at a location far from the ribosomal attachment and start of translation. The hybrid may not have stopped translation if the ribosome was moving along. Blocking the site of initiation would prevent the ribosome from even binding. The long messages here may have sites or a string of sites that could still hybridize to the endogenous messages, inhibiting them or slowing down, the normal translation process. This may also be a reason that the

sense FAS and CAT mRNA produced by this plasmid lowered enzyme activity in the monoclonal cells.

### Overall Conclusions

The goal of this project was to inhibit FAS with an antisense transcript in TA1 cells. The enzyme was successfully inhibited in both the monoclonal and polyclonal antisense cell lines.

Separate questions did arise from this project. For example, why was antisense FAS mRNA not detected? Why was the plasmid not conclusively detected in Southern blots? In both of these cases, perhaps the assay wasn't sensitive enough. The alternatives, that the antisense RNA or the plasmid was not present, are very unlikely considering the cells live in the continuous presence of geneticin. However, any future work done to inhibit FAS using this method should determine why the RNA and DNA couldn't be detected.

Another incongruity involves the sense and CAT monoclonal cell lines. Why did these two have lower FAS activity? Did these extra genes somehow interfere with the normal cellular system? In order for antisense inhibition to be utilized in the future, nonspecific inhibition must be eliminated. The site of integration may need to be determined in order to show that it was the antisense

message rather than the site of integration that caused the enzyme inhibition.

This project of FAS inhibition provides an excellent example of the power and problems associated with antisense technology. Antisense has provided methods to create mutants, treat diseases or inhibit gene products. The mechanism of action needs to be delineated before antisense can be used to its full potential. Further research will provide the needed answers for understanding and using antisense.

## APPENDIX A

## Appendix A

## Methods for Aim #1

A. Ligation  
Sambrook et al, 1987

1. Digestion and dephosphorylation of pcDNA3
  - a. Mix the following in order:
    1. 0.5  $\mu$ g (1  $\mu$ l of stock) of pcDNA3
    2. 2  $\mu$ l of buffer H (buffer for EcoR1)
    3. 2  $\mu$ l dephosphatase buffer
    4. 14  $\mu$ l sterile Milli-Q water
    5. 1  $\mu$ l Calf intestinal phosphatase
    4. 1  $\mu$ l of EcoR1 (20 U/ $\mu$ l)
  - b. Digest at 37° C for 1.5 hours.
2. Digestion of pFAS1
  - a. Mix the following in order:
    1. 1  $\mu$ l pFAS1 (5  $\mu$ g)
    2. 2  $\mu$ l buffer H
    3. 16  $\mu$ l sterile Milli-Q water
    4. 1  $\mu$ l EcoR1 (20 U/ $\mu$ l)
  - b. Digest at 37° C for 1.5 hours
3. Gel Electrophoresis of fragments
  - a. Prepare 0.7% gel (40 ml gel) by weighing out .28 g of agarose.
  - b. Put into a 250 ml Erlenmeyer, add 42 ml 0.5X TBE.
  - c. Put in microwave for 1 minute or until dissolve (do not let it boil over).
  - d. Allow it to cool to 50° C on bench top (it will feel warm to the touch).
  - e. Add 2  $\mu$ l of Ethidium Bromide (USE GLOVES).  
Final concentration of Ethidium Bromide is 0.5  $\mu$ g/ml.
  - f. Set up gel box per manufacturer instructions.
  - g. Remove air bubbles with pipet tip.
  - h. Allow gel to harden (usually 20 minutes).
  - i. Remove ends and comb.
  - j. Place in gel box.

- i. Add 0.5X TBE until it just covers gel (about 260 mls).
  4. Prepare samples in microfuge tubes:
    - a. Add 1  $\mu$ l of dye for each 5  $\mu$ l of sample.
    - b. Prepare a standard marker with dye.
    - c. Prepare other controls needed for gel:uncut pcDNA3, add dye.
    - d. Heat microfuge tubes for 10 minutes at 55-60° C.
    - e. Briefly pipet sample up and down before loading into well.
    - f. Add sample to well (volume depends on well size used), keeping track of the order added.
    - g. Put on gel box lid.
    - h. Plug electrodes into power supply.
    - i. Turn on power supply.
    - j. Adjust voltage to 50 volts.
    - k. Run for 2 hours then check it.
    - l. When leading dye has run 3/4 of the length of the gel, turn off power supply and remove gel. Put gel on UV box.
    - m. Wear safety shield or eye protection before turning on UV box.
    - n. Visualize and take photograph.
  5. Elution of FAS1 and pcDNA3
    - a. Use USBioclean MP Kit per manufacturer's instructions. (US Biochemical Corporation Cleveland, Ohio)
    - b. Resuspend FAS1 in 10  $\mu$ l of TE-8
    - c. Resuspend pcDNA3 in 20  $\mu$ l of TE-8
  6. Ligation of FAS1 and pcDNA3
    - a. Add in order the following:
      1. 2  $\mu$ l FAS1
      2. 2  $\mu$ l pcDNA3
      3. 1  $\mu$ l T4 ligase buffer
      4. 4  $\mu$ l sterile Milli-Q water
      5. 1  $\mu$ l T4 ligase
    - b. Incubate overnight at 16° C.
- B. Preparation of competent bacteria  
A modification of Sambrook et al, 1987
1. Day 1 (day before ligation is set up)
    - a. Streak DH5a onto 100 mm SOB agar plate.
    - b. Wait 5 minutes (bacteria soaks into plate).

- c. Invert plate and incubate at 37° C overnight.
2. Day 2 (day ligation is set up)
  - a. Inoculate 25 ml of SOB in a sterile orange capped tube with a single colony from plate.
  - b. Shake at 37° C overnight. (OD is approx .4-.6)
3. Day 3 (ligation complete)
  - a. Chill culture on ice, keeping it on ice through-out remainder of the procedure.
  - b. Transfer cells to sterile chilled 30 ml Corex tube.
  - c. Centrifuge at 7000 x g for 5 minutes at 4° C to pellet cells.
  - d. Remove supernatant and resuspend pellet in 5 ml (1/5 volume) of ice cold sterile 0.1 M MgCl<sub>2</sub>.
  - e. Keep on ice for 15 minutes
  - f. Repeat step c.
  - g. Remove supernatant and resuspend pellet in 0.5 ml (1/50 volume) of ice cold sterile 0.1 M CaCl<sub>2</sub>.
  - h. Keep on ice for 60 minutes.
  - i. Immediately use for transformation or store by aliquoting 50 µl to sterile microfuge tubes and adding 12.5 µl of sterile 75% glycerol. Quick freeze the tubes and store at -80° C.
- C. Transformation of DH5a with plasmid  
Sambrook et al, 1987
  1. Chill pipet tips and sterile microtubes on ice or in freezer for this procedure.
  2. Place competent bacteria (50 µl aliquots) on ice for 10 minutes.
  3. Add DNA (2.5 µl or less) to each bacterial tube keeping them on ice. Add ligation mixture to one tube, a known concentration of another plasmid for a positive control and no plasmid to one tube for a negative control.
  4. Swirl tubes gently to mix the DNA.
  5. Store on ice for 30 minutes exactly.
  6. Heat shock at 42° C for 90 seconds exactly with out shaking tubes.



7. Rapidly transfer bacteria back to the ice for 1-2 minutes.
  8. Add 800  $\mu$ l SOC to each tube and warm to 37° C.
  9. Incubate tubes at 37° C for 45 minutes to allow bacteria to recover.
  10. Transfer 100  $\mu$ l of each bacterial culture to an ampicillin plate and 100  $\mu$ l to a non-ampicillin plate.
  11. Spread culture with sterile glass rod (dip in 70% ethanol, flame, cool on plate then use).
  12. Allow 5-10 minutes for bacteria to soak into the plate.
  13. Invert and incubate at 37° C overnight. Colonies appear in 20-24 hours.
  14. To store, seal with parafilm and keep at 4° C.
- D. Mini-prep of plasmids with Magic Prep Kit  
Per manufacturer's protocol  
Promega, Madison, Wisconsin
1. Day 1
    - a. Pick a colony from a transformed plate and inoculate 3 ml of SOB media containing 50  $\mu$ g/ml of ampicillin.
    - b. Shake culture at 37° C overnight.
  2. Day 2 - follow kit's instructions  
Resuspend all miniprep plasmids in 20  $\mu$ l TE-8.
- E. Restriction enzyme digests  
Sambrook et al, 1987
1. Set up digest as follows from minipreps:
    - a. 5  $\mu$ l of plasmid DNA  
1.5  $\mu$ l enzyme buffer  
7.5  $\mu$ l sterile Milli-Q water  
1  $\mu$ l enzyme
    - b. Incubate at 37° C for 1 hour
    - c. Run on gel (see above)
  2. Digests for other gel checks
    - a. 1-10  $\mu$ g of DNA  
1X final volume of buffer  
1  $\mu$ l of 20 U/ $\mu$ l of enzyme  
water to volume depending on well size in gel and volume of DNA used.
- F. Large Scale Plasmid Preparation  
Modification of Sambrook et al, 1987

1. Day 1
  - a. Pick a colony from a transformed plate and inoculate 3 ml of SOB media containing 50 µg/ml of ampicillin.
  - b. Shake culture at 37° C overnight.
2. Day 2
  - a. Prepare 1000 ml of SOB media containing 50 µg/ml of ampicillin in sterile Erlenmeyer flask. Warm to 37° C.
  - b. Inoculate flask with 1 ml of the culture using sterile technique.
  - c. Shake culture at 37° C overnight.
3. Day 3
  - a. Set centrifuge to 4° C and sterilize all needed glassware by autoclaving.
  - b. Divide flask into 4 sterile polypropylene bottles.
  - c. Centrifuge at 5000 x g for 15 min at 4° C.
  - d. Pour off supernatant (save to dispose of properly).
  - e. Resuspend pellet with 25 ml of 10 mM NaCl (25 ml of NaCl per 250 ml of culture) in each polypropylene bottle.
  - f. Centrifuge at 5000 x g for 15 min at 4° C.
  - g. Pour off supernatant. Store at -80° C if desired.
  - h. Resuspend pellet in 4 ml of Solution A and put on ice.
  - i. Prepare the following: add 144 mg of lysozyme to 8 ml of Solution A. Sterile by filtering through a sterile .22 µm filter. Add 2 ml of this to each polypropylene bottle with resuspended pellet.
  - j. Lyse cells for 10-15 minutes at room temperature. Allow it to clear before continuing.
  - k. Remove all clumps by pipetting/inverting.
  - l. Add 12 ml of Solution B to cell lysate.
  - m. Mix thoroughly and GENTLY. Do not vortex. Solution becomes snotty looking, allow this to clear before continuing. This takes at least 20 minutes at room temperature.
  - n. Add 7.5 ml of 3 M Na-acetate, pH 5.0. Mix by inversion.

- o. Chill on ice for 10 minutes then centrifuge for 10 minutes at 5000 x g at 4° C.
- p. Recover supernatant with sterile pipet and put in sterile 150 ml glass Corex bottle. Do not fill Corex bottles more than 1/3 full.
- q. Add RNase A to 20 ug/ml and let sit at 37° C for 15-30 minutes.
- r. Add 1 volume of P:C:I and shake vigorously for 5 minutes. Handle with care.
- s. Centrifuge 10 minutes at 3000 x g at 4° C.
- t. Layers will separate. Remove top layer (aqueous) to clean sterile Corex bottle. Do not remove white interface layer.
- u. Add 1 volume C:I. Shake vigorously for 5 minutes then centrifuge as in step s.
- v. Remove top (aqueous) layer into sterile 30 ml Corex tubes. Do not fill more than 1/4 full.
- w. Add 2 volumes of absolute ethanol to precipitate DNA.
- x. Place tubes at -20° C for 1 hour to overnight.
- y. Centrifuge tubes for 15 minutes at 4° C at 5000 x g.
- z. Carefully pour off ethanol, leaving white pellet stuck to tube.
- aa. Rinse pellet with 70% ethanol. If pellet become dislodged, centrifuge at 5000 x g for 10 minutes at 4° C.
- bb. Remove 70% ethanol, drain and dry thoroughly by inverting on benchtop and air drying.
- cc. Dissolve pellet in 1.6 ml sterile water and transfer to a sterile 15 ml Corex tube.
- dd. Add 0.4 ml 4 M NaCl and mix by swirling tube.
- ee. Add 2 ml 13% PEG.
- ff. Incubate on ice for 1 hour or overnight at 4° C.
- gg. Centrifuge DNA for 10 minutes at 5000 x g at 4° C.
- hh. Drain carefully without dislodging pellet and rinse it with 2.5 ml of 70% ethanol.
- ii. Centrifuge 10 minutes at 5000 x g at 4° C.
- jj. Resuspend pellet in TE-8. The amount depends on size of pellet or desired concentration.

- kk. Determine concentration with spectrophotometer at 260 and 280 nm. DNA concentration in  $\mu\text{g}/\mu\text{l}$  =  
 $\text{Abs at 260 nm} \times \text{dilution factor} / 20$ .  
 Ratio of 260/280 should be greater than 1.8 and less than 2.0.

## Solutions and Reagents for Goal #1

### 1. Restriction Enzymes and Buffers

Purchased from Gibco and BMB  
 Store at  $-20^{\circ}\text{C}$ .

### 2. DNA T4 Ligase and Buffer

Purchased from BMB, no. 481 220  
 Boehringer Manneheim, Indianapolis, Indiana  
 Store at  $-20^{\circ}\text{C}$

### 3. Calf Intestinal Phosphatase

Purchased from Gibco, no. 8009SA  
 Gibco, Gaithersburg, Maryland  
 Store at  $4^{\circ}\text{C}$ .

### 4. Agarose

HT Agarose from Integrated Separation Systems  
 no. SE141057B.  
 IST, Nattick, Massachusetts

### 5. 5X TBE (0.45 M Tris, 0.45 M Boric acid, 10 mM EDTA)

	<u>2 liters</u>
Tris Base (MW=157.6)	141.84 g
Boric acid (MW=61.83)	55.60 g
EDTA, sodium salt (MW=372.2)	7.44 g
Milli-Q water	1700.00 ml

Dissolve above in 1800 ml of Milli-Q water.  
 Adjust volume to 2 liters with Milli-Q water.  
 No need to pH. Autoclave to sterilize in glass bottles for 45 minutes at 15 psi.

## 6. 0.5X TBE (working solution)

Add 40 ml of 5X TBE to 360 ml Milli-Q water.  
Mix and store at room temperature.

## 7. Ethidium Bromide (10 mg/ml)

Purchased from Sigma, no. E-1510  
Sigma, St. Louis, Missouri  
Stored at 4° C in protective container.

## 8. Gel loading dye

Purchased from Sigma, no. G-2526  
Sigma, St. Louis, Missouri  
Store in a dark area at room temperature.

## 9. USBioclean MP Kit

Purchased from US Biochemical Corporation  
No. 74200  
USB, Cleveland, Ohio  
Store at 4° C, Rinse buffer at -20° C.

10. 0.1 M  $\text{MgCl}_2$ 

Add 5 ml of sterile 2 M  $\text{MgCl}_2$  to 95 ml of sterile  
Milli-Q water in a sterile glass bottle.

11. 2.5 M  $\text{CaCl}_2$ 

	<u>20 ml</u>
$\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ (MW=219.0)	10.95 g
Milli-Q water	10.0 ml

Dissolve reagent in Milli-Q water. Bring to  
volume with Milli-Q water. Sterilize by filtering  
through a sterile .22  $\mu\text{m}$  filter. Store in 1.0 ml  
aliquots in sterile microfuge tubes at -20° C.

12. 0.1 M  $\text{CaCl}_2$ 

Add 4 ml of sterile 2.5 M  $\text{CaCl}_2$  to 96 ml of  
sterile Milli-Q water in a sterile glass bottle.  
Store on ice for this procedure.

## 13. 75% glycerol

	<u>100 ml</u>
Glycerol (glycerin)	75 ml
Milli-Q water	25 ml

Place in glass bottle and sterilize by autoclaving for 30 minutes at 20 psi. Store at room temperature.

## 14. SOB Media

	<u>1 liter</u>
Bacto-tryptone	20.0 g
Bacto yeast extract	5.0 g
NaCl	0.5 g
250 mM KCl	10.0 ml
2 M MgCl <sub>2</sub>	5.0 ml
2 M MgSO <sub>4</sub>	10.0 ml
Milli-Q water	900.0 ml

Dissolve Bacto-tryptone, Bacto yeast extract and NaCl in 900 ml of Milli-Q water. Add 10 ml of 250 mM KCl. Adjust pH to 7.6 with 5 N NaOH (About .2 ml). Adjust volume to 1 liter with Milli-Q water. Autoclave in glass bottle to sterilize for 45 minutes at 15 psi. Before use at 5 ml of 2 M MgCl<sub>2</sub> and 10 ml of 2 M MgSO<sub>4</sub>. Store at room temperature.

## 15. 250 mM KCl

	<u>100 ml</u>
KCl (MW=74.55)	1.86 g
Milli-Q water	90.0 ml

Dissolve KCl in Milli-Q water. Bring to 100 ml with Milli-Q water. This does not need to be sterilized for use in SOB since SOB is sterilized after its addition.

16. 2 M MgCl<sub>2</sub>

	<u>100 ml</u>
MgCl <sub>2</sub> -6HOH (MW=203.3)	40.66 g
Milli-Q water	50.0 ml

Dissolve reagent in Milli-Q water. Adjust volume to 100 ml with Milli-Q water. Sterilize by autoclaving in a glass bottle for 30 minutes. Store at room temperature.

17. 2 M  $\text{MgSO}_4$ 

	<u>100 ml</u>
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (MW=246.48)	49.3 g
Milli-Q water	50.0 ml

Dissolve reagent in Milli-Q water. Bring to volume with Milli-Q water. Sterilize by autoclaving in a glass bottle for 30 minutes at 15 psi. Store at room temperature.

## 18. SOB Agar Plates

Add 1.5 g of agar to 100 ml of SOB (this makes 5 plates). Autoclave to sterilize in glass bottles for 30 minutes at 20 psi. Remove bottle from autoclave and cool in 55° C water bath. Add  $\text{MgSO}_4$  and  $\text{MgCl}_2$  as in SOB and ampicillin if desired. Quickly pour plates in 100 mm petri dishes. Allow agar to harden in plates. Store in 4° C wrapped in plastic. Agar plates with ampicillin are only good for 2 weeks, others can be kept up to 30 days.

## 19. 1 M glucose

	<u>100 ml</u>
glucose (MW=180.16)	18.0 g
Milli-Q water	80.0 ml

Dissolve glucose in Milli-Q water. Adjust volume to 100 ml with Milli-Q water. Sterilize by filtering through a sterile 0.22  $\mu\text{m}$  filter. Store at 4° C in a sterile glass bottle or plastic tubes.

## 20. SOC Media

	<u>10 ml</u>
1 M glucose (sterile)	0.2 ml
SOB (sterile)	9.8 ml

Add reagents to sterile 15 ml orange capped tube. Store at 4° C. Use sterile technique when handling this solution, it contaminates easily.

## 21. 50 mg/ml Ampicillin stock solution

	<u>1 ml</u>
Ampicillin	50 mg
Milli-Q water	1.0 ml

Dissolve ampicillin in 1 ml of Milli-Q water. Sterilize by filtering through a sterile 0.22  $\mu$ m filter. Store at -20° C. Solution is stable for only 2 weeks.

## 22. Magic Miniprep Systems

Purchased from Promega, no. A7100  
Promega, Madison, Wisconsin  
Store at room temperature.

## 23. Solution A

	<u>1000 ml</u>
1 M Tris-Cl, pH 7.5	25.0 ml
0.5 M EDTA, pH 8.0	20.0 ml
sucrose	150.0 g
Milli-Q water	800.0 ml

Dissolve reagents in Milli-Q water. Adjust volume to 1 liter. Sterilize by filtering through a sterile 0.22  $\mu$ m filter. Store at 4° C in a glass bottle.

## 24. Solution B

	<u>100 ml</u>
NaOH (MW=40.0)	8.0 g
10% SDS	10.0 ml
Milli-Q water	80.0 ml

Dissolve NaOH in Milli-Q water. Add 10 ml of SDS and adjust volume to 100 ml with Milli-Q water. Store at room temperature in glass bottle.

## 25. 13% Polyethylene glycol (PEG)

	<u>200 ml</u>
PEG	26.0 g
Milli-Q water	150.0 ml

Dissolve PEG in Milli-Q water. Bring to volume with Milli-Q water. Filter through a sterile .22  $\mu$ m filter to sterilize and store at 4° C in a sterile glass bottle.

## 26. Phenol:Chloroform:Isoamyl (P:C:I)

Purchase from Sigma, no.P-3803.  
Sigma, St. Louis, Missouri  
Store at 4° C in light protected container.



## 27. Chloroform:Isoamyl (C:I)

Mix under the hood 240 ml chloroform with 10 ml isoamyl alcohol. Store at room temperature under the hood in a dark or foil wrapped glass bottle.

## 28. 10 mg/ml RNase A

Dissolve pancreatic RNase (RNase A) to a 10 mg/ml concentration in 10 mM Tris-Cl, pH 7.5, 15 mM NaCl. Heat to 80° C for 10 minutes. Allow solution to cool slowly to room temperature. Aliquot into sterile microtubes and store at -20° C.

## 29. 4 M NaCl

	<u>100 ml</u>
NaCl (MW=58.44)	23.38 g
Milli-Q water	70.0 ml

Dissolve NaCl in Milli-Q water. Adjust volume to 100 ml with Milli-Q water. Sterilize by autoclaving in a glass bottle for 30 minutes at 15 psi then store at room temperature.

## 30. 10 mM NaCl

Add 1.25 ml of 4 M NaCl to 498.75 ml of sterile Milli-Q water in a sterile glass bottle. Store at room temperature.

## 31. Lysozyme

Purchased from BMB no. 837 059  
Boehringer Manneheim, Indianapolis, Indiana  
Store at 4° C in dessicator.

## 32. 3 M Na-acetate, pH 5.0

	<u>100 ml</u>
Na-acetate (MW=82.03)	24.61 g
DEPC-treated Milli-Q H <sub>2</sub> O	50.00 ml

Dissolve Na-acetate in DEPC-treated Milli-Q water. Adjust pH to 5.0 with glacial acetic acid. Bring to volume with Milli-Q water. Filter through a sterile .22 µm filter unit. Store in a sterile glass bottle at 4° C.

33. 10% SDS (sodium dodecyl sulfate)

	<u>100 ml</u>
SDS	10.0 g
Milli-Q water	80.0 ml

Dissolve SDS in Milli-Q water. Bring to volume with Milli-Q water. Filter through a sterile .22  $\mu$ m filter. Transfer to a sterile glass bottle and store at room temperature.

34. 0.5 M EDTA, pH 8.0

	<u>1000 ml</u>
EDTA sodium salt (MW=372.2)	186.1 g
Milli-Q water	800 ml

Dissolve EDTA in Milli-Q water. Adjust pH to 8.0 with 1 N NaOH. Adjust volume to 1 liter with Milli-Q water. Sterilize by autoclaving in glass bottles for 45 minutes at 15 psi.

35. 50 mM NaOH

	<u>500 ml</u>
NaOH (MW=40.0)	1.0 g
Milli-Q water	450 ml

Dissolve NaOH in Milli-Q water. Bring to volume with Milli-Q water. Store at room temperature.

36. 10 mM Tris-Cl, 1 mM EDTA, pH 8.0 (TE-8)

	<u>1 liter</u>
Tris-HCl (MW=157.6)	1.58 g
EDTA sodium salt (MW=372.2)	0.372 g
Milli-Q water	900 ml

Dissolve reagents in Milli-Q water. Adjust pH to 8.0 with 5 N NaOH. Adjust volume to 1 liter with Milli-Q water. Autoclave to sterilize in glass bottles for 45 minutes at 15 psi. Store at room temperature.

37. 100% glycerol (100 ml)

Sterilize in glass bottle by autoclaving for 30 minutes at 15 psi.

## APPENDIX B

## APPENDIX B

## Methods for Aim #2

A. Growth and Maintenance of TA1 Cells  
Dickerson et al, 1992

1. Media and Maintenance of Cells
  - a. Dulbecco's Modified Eagle Medium was used for all cell culture work. DMEM, at pH 7.3, was filter sterilized before use.
  - b. 10% Fetal Bovine Serum, .005% Antibiotic/Antimycotic and .001% gentamicin was added to DMEM before use.
  - c. All transfected cell lines were selected with 0.4 mg/ml of geneticin and upon completion of selection, cultured in 0.2 mg/ml of geneticin.
  - d. All TA1 cells were grown in a sterile controlled environment kept at 37° C, 5% CO<sub>2</sub>, 95% O<sub>2</sub>.
2. Plating cells from frozen stocks
  - a. Remove cells from liquid nitrogen.
  - b. Place vial in room temperature water until just thawed.
  - c. Remove cells from vial into a sterile plastic 50 ml tube containing 5 ml of 10% FBS in DMEM.
  - d. Centrifuge cells for 4 minutes at speed #3 on table top centrifuge.
  - e. Pour off liquid and resuspend cells in 10% FBS in DMEM.
  - f. Plate into 75 sq cm flasks.
3. Trypsinization and counting of cells
  - a. Remove media from the cells.
  - b. Rinse twice with PBS.
  - c. Add 1X (10X trypsin is diluted in PBS) to flask. Use 5 ml for 75 sq cm plate.
  - d. Swirl and/or gently slap plate to help cells come off plate.

- e. Check for complete removal of cells from plate with microscope.
  - f. Quickly remove cell-trypsin solution into a plastic tube containing twice the volume of 10% FBS in DMEM.
  - g. Centrifuge for 4 minutes at speed #3 on the table top centrifuge.
  - h. Resuspend cells in 10% FBS in DMEM (the volume depends on desired volume or number of flasks combined in step f).
  - i. Using a sterile pipet tip w/ pipetman or sterile Pasteur pipet, remove a small amount of the cell suspension and place on hemacytometer until the solution is pulled under cover slip. Repeat with opposite end of the hemacytometer.
  - j. Using the microscope on lowest power (6.3), count the four corner squares on each end. Average the eight totals then multiply by 10,000. This is number of cells per ml.
4. Plating cells
- a. Plate cells into desired size plates or flasks with at least 50,000 cells/ml. All TA1 cells are plated and cultured in 10% FBS in DMEM.
  - b. Feed cells every third day unless otherwise noted.
5. Induction of Fat Synthesis
- a. Plate cells and culture until complete confluence is reached.
  - b. Remove media and replace with growth media containing 1  $\mu$ M dexamethasone and 1.25  $\mu$ M Indomethacin.
  - c. Remove inducing media after 48 hours. Replace with growth media.
- B. Transfection by Lipofection for 100 mm plate  
Felgner et al, 1987
- 1. Dilute DNA (7  $\mu$ g) into 1.5 ml of HBS.
  - 2. Dilute lipofection (33  $\mu$ g) into 1.5 ml HBS.
  - 3. Mix the above two dilutions.
  - 4. Remove media from cells.
  - 5. Wash cells twice with 5 ml of HBS.
  - 6. Add the 3 ml mix of DNA-lipofection to plate.

7. Incubate at 37° C, 5% CO<sub>2</sub> for 3-5 hours.
  8. Remove HBS mixture and add 10 ml of DMEM containing 10% Fetal Bovine Serum (FBS).
  9. After 16 hours at 37° C, 5% CO<sub>2</sub>, remove media and replace with fresh DMEM/10%FBS.
  10. Cells can be harvested 2-3 days later or replated in selective media for stable transfections 2 days after transfection.
  11. Selection continued for 4 weeks with media containing .4 mg/ml of G418 replaced every two or three days.
  12. Cells remaining after the selection period were pooled for the establishment of polyclonal cell lines. Monoclonal cell lines were established by dilution and plating into 96 well plates. Monoclonal cell lines were plated as outlined above with .4mg/ml G418.
- C. Genomic DNA Isolation with PureGene Kit  
Per Manufacturer's protocol  
Gentra Systems, Inc. Minneapolis, Minnesota
- D. Genomic DNA digestion and electrophoresis  
Sambrook et al, 1987
1. Digestion
    - a. Digest 3-7 µg of genomic DNA with EcoR1 for 1 hour at 37° C as above.
    - b. Electrophorese on .7% gel for 3 hours at 45 volts as described above.
- E. Southern Blotting  
Sambrook et al, 1987
1. Preparation of Gel
    - a. Trim excess from gel.
    - b. Mark bottom right corner of gel.
    - c. Denature DNA by soaking gel in 1.5 M NaCl and .5 N NaOH for 45 minutes with gentle agitation at room temperature.
    - d. Rinse gel in Milli-Q water.
    - e. Neutralize by soaking in 1 M Tris, pH 7.4 and 1.5 M NaCl for 30 minutes at room temperature with gentle agitation.
    - f. Change neutralization solution and replace with fresh and continue soaking for 15 more minutes.

2. Preparation of paper, wick and nylon membrane
  - a. Place Whatman 3MM paper over a glass plate so it hangs into a container filled with 10X SSC. The SSC should come up almost to glass plate.
  - b. Clear all bubbles between 3MM paper and glass with glass rod.
  - c. Cut a piece of nylon membrane the size of the gel. Wet in Milli-Q water then soak in 10X SSC for at least 5 minutes.
  - d. Cut two pieces of 3MM paper 1mm larger than gel and soak in 2X SSC to wet.
3. Set up of Southern Transfer
  - a. Place gel face down on 3MM paper over the glass.
  - b. Remove air bubbles as before.
  - c. Place nylon on gel and remove air bubbles. Mark nylon to orient it to gel.
  - d. Place the two 3MM papers (wet) on membrane and remove air.
  - e. Place at least 2 inches of cut-to-size paper towels on membrane.
  - f. Cover with another glass plate and 500 g of weight.
  - g. Surround gel with parafilm or plastic wrap to prevent other routes for liquid transfer.
  - h. Allow transfer to occur overnight.
  - i. Remove paper towels and 3MM paper. Mark wells of the gel onto membrane by flipping over the gel/membrane carefully. Use a dull pencil or pen.
  - j. Soak nylon in 6X SSC for 5 minutes at room temperature to remove gel pieces.
  - k. Air dry on a paper towel.
  - l. UV link DNA to membrane by exposing to UV light for 2 minutes.
  - m. Store membrane in air tight bag or use immediately.
4. Southern Hybridization  
Sambrook et al, 1987
  - a. Prehybridize membrane in Southern prehybridization solution (1 ml/cm squared of membrane) for two hours at 42° C. If using digoxigenin labelled probes, follow Boehringer Manneheim's protocol for

hybridization and probe synthesis.  
Carefully remove all bubbles before sealing.

- b. Prepare Random Prime Radioactive Probe using Random Primed Labelling Kit, BMB, per manufacturer's instructions or ribo-probe using Stratagene's RNA transcription kit per manufacturer's protocol.
- c. Denature probe for 10 minutes at 100° C and immediately chill on ice (only random primed probe).
- d. Remove prehybridization solution from bag and replace with Southern hybridization solution. Seal bag with Seal-a-Meal™.
- e. Using a syringe w/ needle, pull up probe into syringe barrel then carefully poke through bag in a corner containing the membrane. Push probe solution into bag. Take care to avoid the addition of air bubbles. Reseal corner.
- f. Hybridize overnight at 42° C.
- g. Remove membrane (properly dispose of radioactive hybridization solution) and place in container with 2X SSC, .1% SDS. Incubate 15 minutes at room temperature with gentle shaking.
- h. Remove solution and replace with .1X SSC, .5% SDS. Incubate 30 minutes at 42° C.
- i. Check blot with counter, if very hot, repeat washing step h. If under 1000 cpm, seal blot (air tight!) in Seal-a-Meal™ bag and place in cassette.
- j. Expose to X-ray film overnight at -70° C. Use two pieces of film to allow two separate exposure times.
- k. Develop film. Re-expose if necessary.

F. RNA Extraction from cultured cells w/ RNA STAT-60  
Per Manufacturer's protocol  
Tel-Test "B", Friendswood, Texas

G. mRNA Isolation with PolyAtract mRNA Isolation System (Promega)  
Isolation was completed using total RNA as a starting point per manufacturer's instructions.  
Promega, Madison, Wisconsin



H. Northern Blot Protocol  
Jump et al, 1984

1. Sample Preparation:
  - a. Samples in sterile microtubes should contain at least 3  $\mu$ g total RNA in a volume of 5.5  $\mu$ l of TE-8.0 or DEPC-treated water. Generally, one will run 10  $\mu$ g total RNA or 3  $\mu$ g of mRNA per lane.
  - b. To the RNA sample in 5.5  $\mu$ l volume, add 14.5  $\mu$ l Denaturing mix and cap the microfuge tube. Heat denature sample at 60° C for 5 minutes.
  - c. Add 5.0  $\mu$ l 4X-dye, mix and apply this 25  $\mu$ l sample to the gel well.
2. Running gel:
  - a. Run gel at constant voltage (45 volts) for approximately 3 h.
  - b. Remove gel from electrophoresis unit. Cut off standards and one lane of RNA to stain in Ethidium Bromide. Transfer standards and RNA lane to a glass baking dish containing 50  $\mu$ l Ethidium Bromide (stock=10  $\mu$ g/ml) in running buffer. Incubate at room temperature with gentle shaking for 30 minutes. Transfer gel to another glass baking dish containing Milli-Q water and incubate at room temperature for 30 minutes. Transfer gel to UV-transilluminator and photograph.
  - c. Transfer the remaining piece of the gel to a glass baking dish containing 500 ml of 50 mM NaOH and shake gently for 15 minutes at room temperature.
  - d. Cut a piece of nylon membrane to fit the gel and soak it in Milli-Q water for 10 minutes to equilibrate. Then equilibrate for 10 minutes in 10X SSC (10X SSC=250 ml 20X SSC + 250 Milli-Q water).
3. Assemble the blotting apparatus:
  - a. Pour 500 ml 10X SSC into a glass baking dish. Set a glass plate to straddle the dish.
  - b. Prepare a wick of 1 sheet of 3 MM (Whatman) paper, saturate with 10X SSC, and lay over glass plate with the ends

submerged into the 10X SSC. Remove any bubbles between the glass plate and the wick by rolling a sterile pipet over the wick.

- c. Place the gel on the wick face down and remove bubbles as described above.
  - d. Lay plastic wrap on wick to form a frame for the gel.
  - e. Layer the equilibrated nylon membrane on top of the gel and remove air bubbles as described above.
  - f. Cut 2 pieces of 3 MM (Whatman) paper the size of the gel and prewet in 10X SSC. Layer the pre-wetted sheets on top of the nylon and remove any bubbles as described before.
  - g. Cut a stack of paper towels to the size of the gel. Generally the stack should be at least 3 to 4 inches thick. Place the stack of paper towels on top of the pre-wetted 3 MM paper and place a glass plate and a medium sized book on top for weight (weight should equal approximately 500 g).
  - h. Allow the transfer to go for 12 h.
4. Disassemble blotting unit:
- a. Remove the paper towels and 3 MM paper.
  - b. Mark the top of the gel and the wells on the nylon membrane using a dull pencil.
  - c. Transfer the blot to 200 ml 2X SSC (2X SSC=20 ml 20 X SSC + 180 ml Milli-Q water) and gently rub the blot with gloved finger to remove any agarose.
  - d. Transfer to a dry sheet of 3 MM paper and allow membrane to completely dry.
  - e. Place nylon membrane face down on UV box, turn on light for 2 minutes to fix RNA to membrane. Blot is ready for prehybridization and hybridization.
5. Prehybridization and Hybridization
- a. Prehybridize for 2 hours at 42° C in Northern Prehybridization solution in a Seal-a-Meal™ bag.
  - b. Prepare riboprobe according to Stratagene protocol.
  - c. To add probe, poke through the bag in one corner with syringe and inject probe

- carefully. Reseal bag.  
 d. Hybridize overnight at 42° C.

6. Washing Blot:  
 NEVER ALLOW THE BLOT TO DRY DURING WASHING  
 PROCEDURE.

- a. Prepare 2X SSC, 0.1% SDS

Milli-Q water	445 ml
20X SSC	50 ml
10% SDS	5 ml

- b. Carefully remove blot from the bag.  
 c. Immediately place blot into 500 ml of 2X  
 SSC, 0.1 % SDS and wash at room  
 temperature for 10 minutes.  
 d. Prepare 0.1X SSC, 0.1% SDS

Milli-Q water	1970 ml
20X SSC	10 ml
10% SDS	20 ml

- e. Transfer blot to 500 ml of 0.1X SSC, 0.1%  
 SDS and wash at 65° C for 45 minutes.  
 f. Wash the blot at least 2 more times as  
 described above in 0.1X SSC, 0.1% SDS.  
 g. Place blot onto 3 MM paper and dry under  
 heat lamp.  
 h. Wrap blot in cellophane and tape to 3 MM  
 paper cut to the size of the  
 autoradiograph cassette. Place 3 MM  
 paper into cassette. In the dark room,  
 place 2 sheets of X-OMAT film on top of  
 blot and place at -80° C overnight.  
 i. Remove cassette from -80° C and allow it  
 to come to room temperature. Develop  
 top film. If exposure was not long  
 enough, place cassette back at  
 -80° C for another 12-24 h. Develop  
 remaining film as described above.

I. ELISA Assay per manufacturer's instructions  
 Boehringer Mannheim, Indianapolis, Indiana

## Solutions and Reagents for Goal #2

## 1. Dulbecco's Modified Eagle Medium (DMEM)

Purchased from Gibco, no. 12100-046  
Gibco, Gaithersburg, MD  
Store at 4° C.

Add 1 package of DMEM to 800 ml of Milli-Q water stirring continuously. Rinse package with Milli-Q water. Add 3.7 g of sodium bicarbonate solution. Stir for 20 minutes to dissolve completely then pH to 7.2 to 7.4 with HCl. Fill to a volume of 1 liter. Filter sterilize within the sterile hood into sterile glass bottles. Store at 4° C for up to 30 days.

## 2. Fetal Bovine Serum

Purchased from Gibco, no. 16000-028  
Gibco, Gaithersburg, MD  
Lot number 40KA4241  
Filter sterilize into sterile glass bottles.  
Store at -20° C.

## 3. Antibiotic/Antimycotic

Purchased from Gibco, no. 15240-013  
Gibco, Gaithersburg, Maryland  
Aliquot into sterile 15 ml orange capped tubes.  
Store at -20° C.

## 4. Gentamicin

Purchased from Gibco, no. 15710-015  
Gibco, Gaithersburg, Maryland  
Store at 4° C.

## 5. 10X Trypsin-EDTA

Purchased from Sigma, no. T-9395  
Sigma, St. Louis, Missouri  
Store at -20° C.

## 6. Phosphate buffered saline (PBS)

	<u>1000 ml</u>
NaCl (MW=58.44)	8.00 g
KCL (MW=74.55)	0.20 g
KH <sub>2</sub> PO <sub>4</sub> (MW=136.09)	0.24 g
Milli-Q water	800.00 ml

Dissolve all above reagents in Milli-Q water and pH to 7.4 with NaOH/HCl. Bring to volume with Milli-Q water. Sterilize in glass bottle by autoclaving for 45 minutes at 20 psi. Store at room temperature or at 4° C.

## 7. Dexamethasone

Purchased from Sigma, no. D-4902  
Sigma, St. Louis, Missouri  
Store at 4° C.

## 8. Indomethacin

Purchased from Sigma, no. I-7378  
Sigma, St. Louis, Missouri  
Store at room temperature.

## 9. 20 mM HEPES, 150 mM NaCl, pH 7.4 (HEPES buffered saline: HBS)

	<u>100 ml</u>
NaCl (MW=58.44)	0.877 g
HEPES (MW=238.3)	0.477 g
Milli-Q water	90.0 g

Dissolve reagents in Milli-Q water and pH to 7.4 with 1 N HCL or 1 N NaOH. Bring to volume with Milli-Q water. Filter sterilize with a sterile 0.22 µm filter unit. Store in a sterile glass bottle at room temperature.

## 10. Lipofectin

Purchase from Gibco, no. 18292-011  
Gibco, Gaithersburg, Maryland  
Store at 4° C. (DO NOT FREEZE)

## 11. Geneticin

Purchased from Gibco, no. 860-1811 II  
 Gibco, Gaithersburg, Maryland  
 Stock Solution 100 mg/ml.

	<u>20 ml</u>
Geneticin	2.0 g
15 ml HBS, pH 7.4	15 ml

Dissolve geneticin in 15 ml HBS. Fill to volume of 20 ml with HBS. Filter sterilize with .22  $\mu$ m sterile filter. Aliquot in 1 ml portions into sterile microtubes. Store at -20° C.

## 12. Puregene DNA Isolation Kit

Purchased from Gentra Systems, Inc., no D-5500A  
 Gentra Systems Inc., Minneapolis, Minnesota  
 Store at room temperature, enzymes at 4° C.

## 13. Nylon Membranes (used in all blotting)

Purchased from BMB, no 1209-299  
 Boehringer Mannheim, Indianapolis, Indiana  
 Store at room temperature, away from light.

## 14. Southern Prehybridization and Hybridization Solution

6X SSC, 5X Denhardt's, 0.5% SDS, 100 ug/ml tRNA

	<u>25 ml</u>
20X SSC	7.5 ml
100X Denhardt's	1.25 ml
10% SDS	1.25 ml
Milli-Q water	14.75 ml
10 mg/ml tRNA	250 $\mu$ l

Add the first four solutions to a 50 ml sterile orange-capped tube. Immediately before adding this solution to Seal-a-Meal™ bag, boil tRNA for 10 minutes at 100° C. Add tRNA to bag directly after boiling.

## 15. 20X SSC (3 M NaCl, 0.3 M Na-Citrate, pH 7.0)

	<u>1000 ml</u>
NaCl (MW=58.44)	175.3 g
Sodium Citrate (MW=294.1)	88.2 g
Milli-Q HOH	700.0 ml

Dissolve NaCl and sodium citrate in Milli-Q water.

Adjust pH to 7.0 with 1 N NaOH and bring to volume with Milli-Q water. Transfer to glass bottle and autoclave at 15 psi for 45 minutes. Store at room temperature.

16. 100X Denharts solution

	<u>300 ml</u>
BSA	6.0 g
Ficol 400	6.0 g
Polyvinyl pyrrolidine-40	6.0 g

Dissolve each reagent separately in 100 ml Milli-Q water. Once dissolved, combine the three solutions. Prepare 10 ml aliquots in 15 ml orange capped centrifuge tubes. Label and store at -20° C

17. RNA Stat-60

Purchased from Tel-Test"B", no. CS-111  
Tel-Test"B", Friendswood, Texas  
Store at 4° C, in light protected container.

18. 75% Ethanol

Add absolute ethanol to 25 ml of DEPC treated Milli-Q water in a sterile glass bottle until a volume of 100 ml is reached. Store at room temperature.

19. PolyAtract mRNA Isolation System

Purchased from Promega, no. A7100  
Promega, Madison, Wisconsin  
Store at 4° C.

20. 0.4 M 3-[n-Morpholino] propane-sulfonic acid (MOPS), pH 7.0, 100 mM Na-acetate, 10 mM EDTA, pH 8.0 (10X MAE)

	<u>200 ml</u>
MOPS (MW=209.3)	16.74 g
3M Na-acetate pH 5.0 (MW=82.03	6.64 ml
0.5 M EDTA pH 8.0	8.00 ml
DEPC-treated Milli-Q H2O	180.00 ml

Dissolve MOPS in DEPC-treated Milli-Q water and adjust pH to 7.0 with 1 N NaOH (RNase free). Add Na-acetate and EDTA and bring to volume with DEPC-treated Milli-Q water. Filter through a sterile 0.22 µm filter unit and store in a

sterile glass bottle (wrapped in aluminum foil) at room temperature.

21. 1.2% Agarose (make fresh)

	<u>25 ml</u>
Agarose	0.30 g
DEPC-treated Milli-Q water	21.75 ml
10X MAE	2.50 ml
Deionized formaldehyde	0.75 ml

Dissolve agarose in DEPC-treated Milli-Q water and 10X MAE in microwave (approximately 1.5 to 2.0 min), allow to cool to approximately 60° C. Transfer to fume hood and slowly add formaldehyde while mixing. Pour gel in hood.

22. 1X MAE (running buffer: make fresh)

	<u>250 ml</u>
10X MAE, pH 7.0	25.0 ml
DEPC-treated Milli-Q water	225 ml

Make solution in sterile graduated cylinder. Store at room temperature.

23. Denaturing Mix (make fresh)

10X MAE pH 7.0	20.0 $\mu$ l
Deionized formaldehyde	70.0 $\mu$ l
Deionized formamide	200.0 $\mu$ l

Make solution in sterile microfuge tube. Store at room temperature.

24. 50% glycerol, 0.4% bromophenol blue, 0.4% zylene cyanol, 1 mM EDTA (4X-dye)

	<u>100 ml</u>
Glycerol	50.0 ml
Bromophenol Blue	0.4 g
Zylene cyanol	0.4 g
0.5 M EDTA, pH 8.0	200.0 $\mu$ l
DEPC-treated Milli-Q water	40.0 ml

Dissolve reagents in DEPC-treated Milli-Q water. Bring to volume with DEPC-treated Milli-Q water. Filter through a sterile 0.22  $\mu$ m filter unit. Aliquot into sterile 15 ml orange capped centrifuge tubes and store at room temperature.



## 25. 0.5 M EDTA, pH 8.0

	<u>100 ml</u>
EDTA (MW=372.2)	18.61 g
DEPC-treated Milli-Q water	80.00 ml

Dissolve EDTA in DEPC-treated Milli-Q water. Adjust pH to 8.0 with 10 N NaOH (RNase free) (Note: EDTA will not completely dissolve until the pH is approximately 7.0). Bring to volume with DEPC-treated Milli-Q water. Filter through a sterile .22  $\mu$ m filter and store in sterile glass bottle at room temperature.

## 26. 0.2 M EDTA, pH 8.0

	<u>100 ml</u>
EDTA (MW=372.2)	7.44 g
DEPC-treated Milli-Q water	80.0 ml

Dissolve EDTA in DEPC treated Milli-Q water. Adjust pH to 8.0 with 5 N NaOH. Bring to volume with DEPC treated Milli-Q water. Transfer to a glass bottle and autoclave at 15 psi for 45 minutes. Store at room temperature.

## 27. 1.0 M Tris-HCl, pH 8.0

	<u>100 ml</u>
Tris-HCl (MW=157.6)	15.76 g
DEPC treated Milli-Q water	80.0 ml

Dissolve Tris-HCl in DEPC treated Milli-Q water. Adjust pH to 8.0 (or desired pH) with 5 N NaOH. Bring to volume with DEPC treated Milli-Q water. Place in glass bottle and autoclave at 15 psi for 45 minutes. Store at room temperature.

28. 1.0 M Na<sub>2</sub>HPO<sub>4</sub>, pH 6.5

	<u>500 ml</u>
Na <sub>2</sub> HPO <sub>4</sub> (MW=141.96)	70.98 g
Milli-Q water	350.0 ml

Dissolve Na<sub>2</sub>HPO<sub>4</sub> in Milli-Q water. Adjust pH to 6.5 with 1 N NaOH. Bring to volume with Milli-Q water and transfer to a glass bottle. Autoclave at 15 psi for 45 minutes and store at room temperature.

29. Northern Prehybridization/Hybridization Buffer  
 50% formamide, 6X SSC, 5X Denhardts, .1% SDS,  
 .05 M Na<sub>2</sub>HPO<sub>4</sub>, 150 ug/ml tRNA

	<u>25 ml</u>
Formamide	12.5 ml
20X SSC	7.5 ml
100X Denhardts	1.25 ml
1 M Na <sub>2</sub> HPO <sub>4</sub> , pH 6.5	1.25 ml
10% SDS	.25 ml
10 mg/ml tRNA	.375 ml
DEPC treated water	1.875 ml

Add the first four solutions and the DEPC treated water to a sterile 50 ml orange capped tube. Add the SDS. Pour liquid into bag containing blot. Boil tRNA for 10 minutes at 100° C then add it directly to the bag containing the blot and solution.

30. RNA Transcription Kit for Riboprobe synthesis

Purchased from Stratagene, no. 200340  
 Stratagene, La Jolla, California  
 Store at -20° C.

31. Genius 1 DNA Labeling and Detection Kit

Purchased from BMB, no.1093 657  
 Boehringer Mannheim, Indianapolis, Indiana  
 Store at -20° C, antibody at 4° C.

32. ELISA Kit

Purchased from BMB  
 Boehringer Mannheim, Indianapolis, Indiana  
 Store at conditions stated

## APPENDIX C

## APPENDIX C

## Methods for Aim #3

A. Oil Red O Staining  
Pollard et al, 1989

1. Rinse cells gently with PBS three times.
2. Add 0.5 ml formalin to each well in a six well plate.
3. Incubate at room temperature for 30 minutes.
4. Remove formalin and replace with 0.5 ml of Oil Red O.
5. Incubate at room temperature for 10 minutes.
6. Remove Oil Red O and rinse plate with water at the sink.
7. Cover each well just barely with 75% glycerol then replace plate top and tape to seal.
8. View cells under microscope/photograph.

B. FAS Enzyme Activity

1. Extraction of FAS Enzyme  
Dickerson et al, 1992 with modifications by Student et al, 1980.
  - a. Place digitonin solution on stir plate and mix well.
  - b. Remove medium from 2 wells of the 6-well plate.
  - c. Rinse with 0.5 ml PBS, pH 7.4.
  - d. Remove PBS.
  - e. Add 0.5 ml digitonin solution to each well. Incubate at room temperature for ten minutes.
  - f. Before the ten minutes are up, remove medium and rinse with PBS the next 2 wells.
  - g. Transfer the digitonin solution from previous well into the next well and incubate as before.
  - h. Repeat this procedure for the last 2 wells.
  - i. Transfer digitonin solution into a cortex tube containing 1 ml of .4 M  $\text{KPO}_4$ .
  - j. Place the tube on ice.

- k. Centrifuge tubes at 10,000 rpm for 15 minutes at 4° C.
- l. Remove supernatant into a new disposable test tube. Place on ice and run assay.

## 2. Enzyme Assay

Dickerson et al, 1992

- a. Prepare cuvettes, Cary 2200 spectrometer and DS-15.
- b. Add the following in order given to each of four cuvettes:
  - 1. 300 µl EDTA/β-mercaptoethanol/phosphate buffer.
  - 2. 100 µl NADPH
  - 3. 50 µl Acetyl CoA
  - 4. 500 µl, 400 µl, 300 µl, 0 µl of sample
  - 5. 0 µl, 100 µl, 200 µl, 500 µl Milli-Q water
  - 6. After preparing DS-15 and Cary 2200 for assay, 50 µl Malonyl CoA
- c. Parafilm and invert cuvettes.
- d. Load into Cary 2200.
- e. Run assay.

## 3. Protein Determination

Bradford et al, 1976

- a. Prepare dye solution by filtering on day of use. Filter through a .45 µm filter into glass container.
- b. Label 5 ml glass disposable tubes.
- c. Add 100 µl and 50 µl aliquots of extracts to each tube.
- d. Add 50 µl Milli-Q water to tubes with 50 µl aliquots. (Final volume in each tube must be 100 µl).
- e. Turn on spectrophotometer twenty to thirty minutes before use.
- f. Prepare duplicate standards of BSA: 0, .1, .2, .4, .8 and 1.0 mg/ml.
- g. Add 5 ml of dye solution to each test tube.
- h. Parafilm and invert 3 times to mix.
- i. Wait five minutes at room temperature.
- j. Read with spectrometer at A<sub>595</sub> nm.
- k. Concentrations are in mg/ml.

4. Determination of FAS activity.  
Dickerson et al, 1992

1. Activity = nmoles/minute/mg of protein
2. Extinction Coefficient = 6220 Abs<sub>340</sub>
3. Absorbance/minute = slope (loss of NADPH)
4. Reaction volume = 1 ml
5. Sample volume = .5, .4 or .3 ml

$$\begin{aligned}
 \text{Activity} &= \text{abs/min} \quad * \quad \frac{1 \text{ mole NADPH}}{6220 * \text{Abs}_{340} * 1 \text{ L}} \\
 &\quad * \frac{1 \text{ L}}{1000 \text{ ml}} \quad * \quad \frac{1000 \text{ } \mu\text{l rx volume}}{\text{x } \mu\text{l in sample}} \\
 &\quad * \frac{1 \text{ mole FAS}}{1 \text{ mole NADPH}} \quad * \frac{10^9 \text{ nmoles}}{1 \text{ mole}} \\
 &\div \text{mg/ml protein}
 \end{aligned}$$

## Solutions and Reagents

1. Formalin = 4% formaldehyde

	<u>100 ml</u>
Formaldehyde	10 ml
Milli-Q water	90 ml

Add above to sterile glass bottle. Store at room temperature.

2. Oil Red O

	<u>333 ml</u>
Oil Red O stain	0.7 g
Isopropanol	200 ml
Milli-Q water	133.3 ml

Dissolve Oil Red O in isopropanol by stirring at 4° C overnight. Filter through Whatman 3MM paper and place in new clean beaker. Add 133.3 ml Milli-Q water and let stand overnight at 4° C. Filter again. Store in glass bottle at 4° C

## 3. 75% glycerol

	<u>100 ml</u>
Glycerol	75 ml
Milli-Q water	25 ml

Add above to glass bottle. Sterilize by autoclaving for 30 minutes at 20 psi. Store at room temperature.

## 4. Phosphate buffered saline, pH 7.4 (PBS)

	<u>1000 ml</u>
NaCl (MW=58.44)	8.00 g
KCL (MW=74.55)	0.20 g
KH <sub>2</sub> PO <sub>4</sub> (MW=136.09)	0.24 g
Milli-Q water	800.00 ml

Dissolve all above reagents in Milli-Q water and pH to 7.4 with NaOH/HCl. Bring to volume with Milli-Q water. Sterilize in glass bottle by autoclaving for 45 minutes at 20 psi. Store at room temperature or at 4° C.

## 5. Digitonin Solution (.25 M Sucrose, 17 mM MOPS, 2.5 mM EDTA, pH 7.4)

	<u>500 ml</u>
Sucrose (MW=342.4)	42.8 g
MOPS (MW=209.3)	1.8 g
EDTA sodium salt (MW=372.72)	.46 g
Milli-Q water	400 ml

Dissolve in Milli-Q water and pH to 7.4 with NaOH. Fill to volume with Milli-Q water. Store at room temperature in a glass bottle.

## 6. Digitonin

Dissolve .8 mg/ml in digitonin solution. Store in foil wrapped glass bottle at room temperature.

## 7. 4M Potassium Phosphate buffer, pH 6.5

	<u>125 ml</u>
Potassium phosphate (MW=174.18)	8.7 g
Milli-Q water	100 ml

Dissolve potassium phosphate in Milli-Q water and pH to 6.5 with HCl. Store at room temperature in a glass bottle.

## 8. Malonyl CoA

Dissolve 8.5 mg into 10 ml Milli-Q water on day of assay in scintillation vial. Store at  $-20^{\circ}\text{C}$ .

## 9. Acetyl CoA

Dissolve 2.7 mg into 10 ml Mill-Q water on day of assay in scintillation vial. Store at  $-20^{\circ}\text{C}$ .

## 10. NADPH

Dissolve 8.9 mg into 10 ml Mill-Q water on day of assay in scintillation vial. Store at  $-20^{\circ}\text{C}$ .

## 11. 1 M Potassium Phosphate, pH 7.0

	<u>100 ml</u>
Potassium phosphate (MW=174.17)	17.42 g
Milli-Q water	80 ml

Dissolve in Milli-Q water and pH to 7.0 with NaOH. Store at  $4^{\circ}\text{C}$  in a glass bottle.

## 12. .02 M EDTA, pH 8.0

	<u>50 ml</u>
EDTA sodium salt (MW=372.72)	186.2 mg
Milli-Q water	40 ml

Dissolve EDTA in Milli-Q water and pH to 8.0 with NaOH. Fill to volume with Milli-Q water. Store at  $4^{\circ}\text{C}$  in a glass bottle.

14.  $\beta$ -mercaptoethanol

	<u>50 ml</u>
98% $\beta$ -mercaptoethanol	35 $\mu\text{l}$
Milli-Q water	50 ml

Store in glass bottle at  $4^{\circ}\text{C}$ .

15. Potassium Phosphate/EDTA/ $\beta$ -mercaptoethanol buffer

	<u>30 ml</u>
1 M Potassium Phosphate, pH 7.0	15 ml
.02 M EDTA, pH 8.0	7.5 ml
$\beta$ -mercaptoethanol	7.5 ml

Combine above into a sterile glass bottle on day of assay. Can be stored at  $4^{\circ}\text{C}$ .



## 16. Bradford Dye Solution

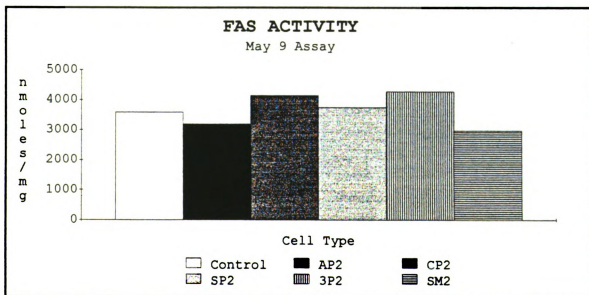
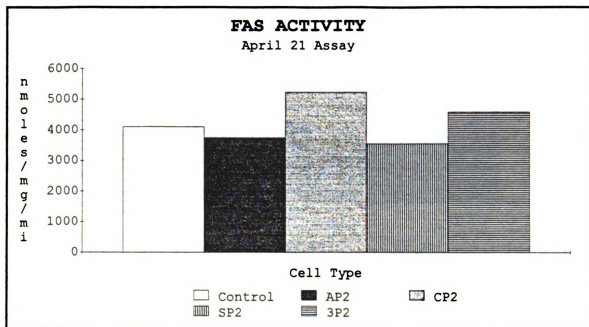
	<u>1 liter</u>
Coomassie G-250 Brilliant Blue	100 mg
95% Ethanol	50 ml
85% Phosphoric Acid	100 ml
Milli-Q water	800 ml

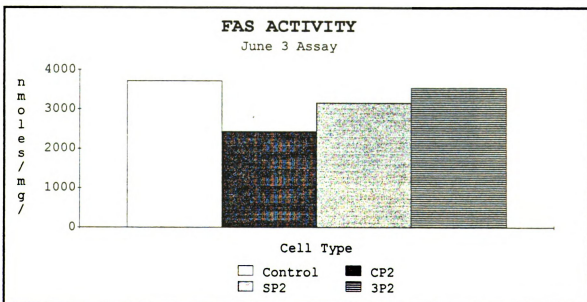
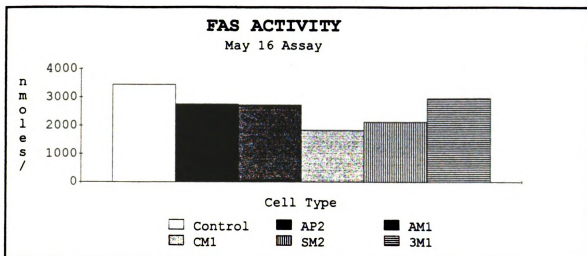
Dissolve G-250 brilliant blue in 95% ethanol by stirring at room temperature for at least 30 minutes. Add phosphoric acid carefully and continue stirring for another 10 minutes. Add Milli-Q water stirring gently. Fill to volume. Filter through Whatman 3MM paper before use. Repeat filtering each time before use if stored. Store in dark or foil wrapped glass bottle at 4° C.

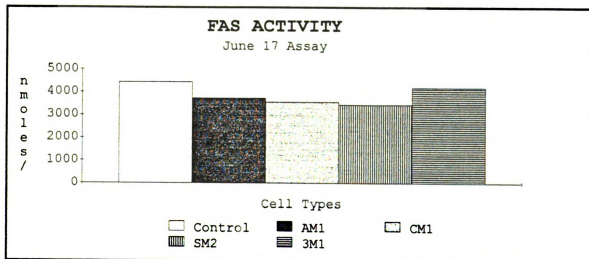
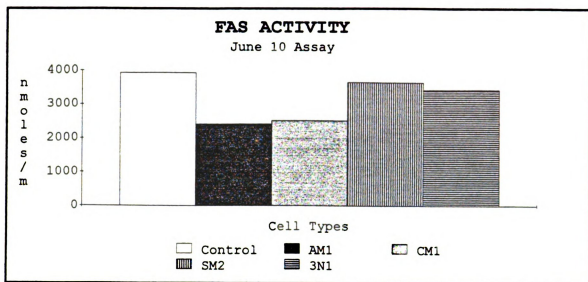
APPENDIX D

## Appendix D

## Individual Day results of FAS Assays







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