DIFFERENTIAL DISPLAY-POLYMERASE CHAIN REACTION (DD-PCR) ANALYSIS OF ADIPOCYTE GENE EXPRESSION DURING MARBLING IN ANGUS X HEREFORD STEERS

By

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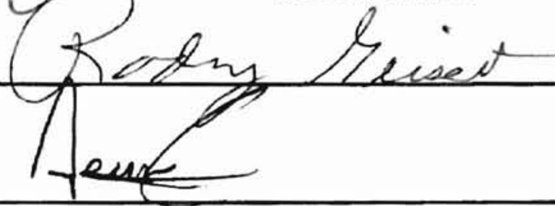

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NOMENCLATURE

bp	base pair
°C	degree Celsius
cDNA	Complementary Deoxyribonucleic Acid
Ci	Curie
Cl	chloride
cm	centimeters
d	days
DD-PCR	Differential Display-Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
g	grams
h	hours
Mg	magnesium
mg	milligrams
min	minutes
ml	milliliters
mm	millimeters
μg	micrograms
μl	microliters
μm	micromoles

%	percent
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
rpm	revolutions per minute
r.t.	room temperature
U	units
V	volts
W	watts
x g	gravity

CHAPTER I

INTRODUCTION

The single most important characteristic of beef quality as viewed by the consumer is meat tenderness (Miller, 1995). It has been demonstrated that consumers are willing to pay more for a steak that is known to be more tender compared to a less tender counterpart (Boleman et al., 1997). However, surveys have documented that consumers are dissatisfied with the eating quality of beef either prepared at home or at a restaurant setting 20% to 25% of the time (Miller, 1995). That being the case, a consumer will have an undesirable eating experience while eating beef one of every four or five opportunities (Smith et al., 1995). Meat from one tough beef carcass has the potential of reaching as many as 542 consumers (Savell et al., 1991). A single bad experience with consumption of a poor quality serving of meat can spread via word of mouth to as many as 950 potential consumers (Smith, 1995). Meat tenderness is of vital importance if beef producers are concerned with continuing to keep consumers as satisfied end-users in the future.

Factors such as breed type, U.S. quality grade, postmortem aging and geographical source of cattle can influence overall palatability (Morgan et al.,

1991). The core of palatability inconsistencies is the relationship the previously described factors have on marbling and skeletal muscle presented for consumption by the consumer. For the beef industry to maintain and increase its market share of protein sources, the consumer needs to have a consistent supply of beef that is uniform and tender (Nelson, 1998).

Attributes of meat quality could be related to gene expression within individual animals. Evaluation of gene expression during the process of adipocyte differentiation within prime muscle groups could provide a method to sort out the mechanisms for not only the initiation of marbling, but also how gene expression may possibly affect overall eating palatability. Since 1992, development of differential display-polymerase chain reaction (DD-PCR) has been utilized as a procedure to identify differentially expressed gene sequences between tissues or cells (Matz and Lukyanov, 1998). DD-PCR allows researchers to take a "snap shot" of cell activity through extraction and comparison of mRNA between immature and mature cells, healthy and diseased cells, or differentiating cells (Liang, 1992). The following review of literature will attempt to provide background information on attributes of palatability, metabolic attributes of adipocytes, and methodology for DD-PCR.

Chapter II

REVIEW OF LITERATURE

Attributes of Palatability

Parameters to quantify meat palatability have been elusive. Traditionally, researchers have stated that the term palatability represents tenderness, juiciness, and flavor of the cooked meat product. These three cooked meat characteristics define what the consumer finds appealing in beef products and what the beef industry is attempting to supply to its consumers on a consistent basis. Of the three components, tenderness has been designated as the most important factor influencing meat palatability as well as also being the most variable (Savell et al., 1989).

One of the problems the beef industry faces is trying to produce a homogeneous end product from a heterogeneous supply of live animals. Variables that influence beef tenderness in live animals range from production traits such as genetics and management style to carcass traits, which include marbling ability along with physiological age of the animal at slaughter (Morgan et al., 1991). According to the 1995 Beef Quality Audit, the aggregate response

of concerns from purveyors, retailers, restaurateurs and packers were: *Low Overall Uniformity and Consistency (1), *Inadequate tenderness (2), *Low overall palatability (3), *Excessive external fat (4), Price of beef is too high for the value received (5), *Insufficient flavor (6), Excessive weights of cuts and boxes of cuts (7), *Inappropriate USDA quality grade mix (8), Incidence of injection-site lesions is too high (9) and *Low overall cutability (10) (Smith, 1995). Seven (indicated by *) out of the ten concerns are related to tenderness inconsistencies. If researchers better understood the genetic potential of individual animals, production of tender beef could become an everyday reality and not just a far away goal.

Marbling. Inferences dating back to Biblical times indicate that fatness was associated with the highest quality of meat.

> "But the father said to his servants, 'Bring the best robe and put it on him. Put a ring on his finger and sandals on his feet. Bring the fattened calf and kill it. Let us have a feast and celebrate." Luke 15:23, NIV

But researchers have not consistently shown that fat is necessarily correlated with quality (Romans et al., 1965; Dolezal et al., 1982a; Wheeler et al., 1994; Wheeler et al., 1997). The current USDA grading system revolves around the amount of marbling found in a cross-section of the *longissimus dorsi* muscle, which is used to determine not only the quality of the product, but also influences product value and carcass yield. In 1916, the United States government decided to develop a set of standards that would categorize carcasses into grades (USDA, 1997). In 1926, these standards were revised and became the Official United States Standards for the Grades of Carcass Beef (USDA, 1997). The

following year the voluntary beef grading and stamping service officially began and continues to be used today (USDA, 1997).

Beef grading is based on a hierarchical ranking system that places carcasses in a certain category based on physiological maturity of the carcass, amount of marbling between the 12th and 13th rib juncture of the *longissimus dorsi*, and meat firmness (USDA, 1997). These requisites are meant to predict the desirability of the cooked product for the consumer. The current grades of beef which are ranked from most desirable to least desirable are: U.S. Prime, U.S. Choice, U.S. Select, U.S. Standard, U.S. Commercial, U.S. Utility, U.S. Cutter, and U.S. Canner.

A meat scientist in the 1950's made the following observation:

> "It is doubtful that fatness by itself is responsible for a marked increase in tenderness and juiciness. It is disconcerting that something which has appeared so obvious to so many for so long should be so extraordinarily difficult to prove in the laboratory" (Cover et al., 1956).

This observation is still true today; marbling has not been consistently shown to correlate very highly with overall palatability or more specifically tenderness. Some researchers have shown that marbling is positively associated with palatability (McBee and Wiles, 1967; Jennings et al., 1978; Tatum et al., 1980; Dolezal et al., 1982a; Berry, 1993; Wheeler et al., 1997). On the other hand, there have been studies that have suggested marbling has a very low effect or no effect on palatability (Tuma, 1962; Romans et al., 1965; Prost et al., 1975; Garcia-de-Siles et al., 1982; Wheeler et al., 1994).

With such varying research results, other research has been conducted to find non-marbling indicators that may contribute to overall palatability. Length of time that cattle have been fed a high-energy diet regardless of marbling score could substitute as a predictor of palatability (Adams, 1977; Harrison et al., 1978; Dolezal et al., 1982b). Texture of the beef product has also been suggested as an indicator of tenderness (Bray, 1964). Amount of connective tissue and solubility of connective tissue has been suggested as a source of toughness. Calcium ion stores and calcium enzymes (calpain and calpistatin) are other possible role players in the palatability of beef (Koohmaraie, 1995). Total percent lipid or chemical fat found in the *longissimus dorsi* has been shown to establish parameters of acceptable palatability (Campion and Crouse, 1975; Savell and Cross, 1989). However, none of these procedures have been the "silver bullet" in completely understanding why some carcasses produce tough while others produce tender beef.

In a landmark publication on adipocytes and its relationship to the palatability of beef, Smith and Carpenter (1974) discuss five theories that may explain the effect marbling has on beef. The bite theory hypothesizes that within a bite-size portion of cooked meat, marbling reduces the overall mass per unit of volume, which in turn lowers bulk density. This theory suggests that within a certain portion of meat some amount of protein is replaced with lipid. Another interpretation of this theory suggests that marbling may dilute the amount of muscle fibers in a given area. Thus, the less dense an area, the easier it is to shear or bite through that piece of meat.

According to the strain theory, as adipose is being formed in the muscle, a portion of that marbling is being deposited inside the walls of the perimysium and endomysium (i.e., connective tissue). As the marbling accumulates within the connective tissue, the rigid structural integrity is weakened (Smith and Carpenter, 1974). By decreasing the width, thickness, and strength of the connective tissue, the muscle increases in tenderness. The looser structure provides an easier route for heat penetration and results in more efficient solubilization of the connective tissue strands.

Another theory that relates to marbling's effect on muscle is the lubrication theory. When heat is applied, the intramuscular fat dissolves into a liquid state. The fat and meat juice blend together and serve as a lubricant during the mastication process. This suggests that marbling relates directly to juiciness and indirectly to tenderness.

The insurance theory states that marbling retards the adverse effect of using high-temperature, dry heat methods of cookery (frying, broiling, etc.). Intramuscular fat provides meat with a safety net against improper methods of preparation such as product that is cooked too rapidly, too extensively, or by the wrong method of cookery. Adipose tissue does not conduct heat as efficiently as muscle. This suggests that meat with a higher degree of marbling can tolerate higher external cooking temperatures without being overcooked internally.

The insulation theory suggests marbling, KPH (kidney, pelvic, and heart) fat, and subcutaneous fat work together to increase the tenderness of meat. Fat depots retard the rate in which postmortem chilling occurs. An increase in

tenderness occurs because the fat insulates the muscle fibers against "cold-shock" during postmortem chilling (Smith, 1976).

Time-on-feed. Traditionally, to increase the amount of marbling the regimen was to increase the days on feed of a high energy concentrate diet. The longer cattle are fed a high-energy grain diet, the greater the probability that animal will produce a carcass that will grade U. S. Choice or higher (Zinn et al., 1970; Campion and Crouse, 1975; Tatum et al., 1980; May et al., 1992; Van Koevering et al., 1995). However, an increase in marbling score has not consistently been proven to increase tenderness. Cattle type has been documented to influence shear force values for cattle that have been fed for the same period of time on a high energy concentrate diet (McKeith et al., 1985a). Burson et al. (1980) summarized that time-on-feed and the amount of marbling did not reflect any significance during sensory evaluation on muscle fiber tenderness, overall tenderness, juiciness, or flavor intensity scores. Although there were no significant palatability differences, Burson did see an increase in USDA quality and yield grade as time-on-feed increased. Warner-Bratzler shear force values and taste panel scores were also found to be unaffected with increasing days on feed (Dinius and Cross, 1978).

A number of studies have supported the correlation between time-on-feed and tenderness (Tatum et al., 1980; Dolezal et al., 1982b; May et al., 1992; Van Koevering et al., 1995). Adams et al. (1977) and Harrison et al. (1978) went as far as to suggest that when feedlot cattle are fed a high energy concentrate feed for a specified period of time, the meat will reach acceptable palatability levels

irregardless of quality grade. Dolezal et al. (1982b) suggested that the minimum number of days on feed (i.e. high concentrate diet) is 90; whereas, Van Koevering et al. (1995) suggested 119 d, and May et al., (1992) indicated that 84 d results in beef carcasses with acceptable palatability traits. Tatum et al. (1980) demonstrated that the number of steaks that would meet the requirements for the sensory panel category "very desirable" was increased from 37.5 percent to 47.7 percent when cattle were fed 160 d versus 100 d, respectively.

Not only does quality grade improve as time-on-feed increases, but external or subcutaneous fat increases as well. Researchers have investigated establishing USDA quality grades fully or at least in part on backfat thickness (Dolezal et al., 1982a; Riley et al., 1983). In 1978, Bowling et al. reported that cattle on high-energy concentrate diet (grain-finished) have significantly greater backfat and improved tenderness scores compared to steaks from forage-finished beef. There was no significant difference in mean marbling score between the grass-finished and grain-finished cattle. Smith et al. (1969) made the observation that cooked beef *longissimus* muscles that were extracted from the most peripheral and lateral locations exhibited the least amount of tenderness. Smith's data demonstrated that an increase in external fat reduced cold shortening, which is a decrease in sarcomere length, when the carcass is exposed to a rapid decline in temperature. In 1971, Smith et al. stated that the reduction in the rate at which postmortem chilling occurs, increases tenderness in beef carcasses. Other research has also documented that subcutaneous fat insulates against a rapid decline in carcass temperature (May et al., 1992). In

1974 and again in 1976, Smith et al. demonstrated that very lean goat and sheep carcasses exhibited extensive shortening of the myofibrils when subjected to cold temperatures during postmortem chilling.

Data suggest that 6 to 10 mm of external fat thickness is sufficient to reduce the postmortem chilling rate, thus decreasing the risk of tenderness reduction resulting from cold shortening (Dutson et al., 1975; Meyer et al., 1977; Lochner et al., 1980; Tatum et al., 1982). Backfat has been suggested to effect steaks during the cooking process the same way that subcutaneous fat insulates the carcass during postmortem chilling (Berry, 1993). Steaks that were low Choice exhibited higher tenderness scores when the external fat remained on the meat than when it was removed from the meat.

Metabolic Attributes of Adipocytes

Metabolic aspects of marbling. White adipose tissues consist mainly of adipocytes that are responsible for the bulk of stored energy in the body. Energy is stored in the form of triglycerides, which are composed of a glycerol backbone and three fatty acids. Triglycerides form into droplets that eventually become large globules, which occupy a majority of the space inside a fat cell. Due to the nonpolar nature of triglycerides, they can be stacked very tightly together and contain very little water (Stryer, 1995).

Variability of the triglyceride arises from the attached fatty acids, which can vary in the number of carbons (C) and number of double bonds in the fatty

acid chain. The number of double bonds in the carbon chain determines the classification of a triglyceride. If there are double bonds present in the carbon chain, the fatty acid is referred to as unsaturated; whereas, a fatty acid lacking double bonds is referred to as saturated. Most fatty acids contain an even number of carbon atoms with the majority falling in the range of 12 to 20. Palmitic acid, stearic acid and oleic acid, which contain 16 C and no double bonds, 18 C and no double bonds, and 18 C and 1 double bond, respectively, are the most commonly found fatty acids in nature (Stryer, 1995).

Triacylglycerol storage. One of the key enzymes involved in triacylglycerol storage is lipoprotein lipase (LPL). Adipose tissue is one of the main producers of this enzyme (Abate and Garg, 1995). Once LPL is synthesized and secreted from the adipocyte, it migrates to the luminal surface of capillaries where it is bound to the plasma membrane (Abate and Garg, 1995). From this vantage point, LPL can hydrolyze circulating triacylglycerols and release free fatty acids. Adipocytes may then accumulate and utilize the free fatty acids for lipid storage. Once intracellular transport of the free fatty acids into the adipocytes has occurred, they are re-esterfied with glycerol-3-phosphate and assembled back into triacylglycerol (Abate and Garg, 1995). Since triacylglycerols still accumulate in the adipocytes of patients that have a deficiency in LPL, LPL is not essential for triacylglycerol to be disassembled in the bloodstream and reassembled in the adipocytes (Abate, 1995). However, normally LPL does play a vital role in the triacylglycerol pathway.

Acylation stimulating protein (ASP) is composed of 76 amino acids with a MW $\approx$ 9 KDa (Baldo et al.,1993). Triacylglycerol storage in the adipocyte is partly regulated by ASP. As ASP increases, triacylglycerol storage also increases (Cianflone et al., 1989). Although the exact mechanisms are not fully understood, ASP needs three precursors for formation: factors B, C3 and the enzyme adipsin (ADN), also known as compliment factor D. All three precursors are synthesized in the adipocyte (Cianflone, 1997a). Glucose transport into the adipocyte appears to be increased by ASP. This occurs in an indirect manner because the adipocyte is stimulated by ASP to increase the number of glucose transporters on the cell surface of the adipocyte (Cianflone, 1997b). Thus, ASP works through a second messenger system that involves protein kinase C and diacylglycerol (DAG) (Baldo et al., 1995). As ASP increases within the adipocyte, the amount of glucose in turn increases which results in the re-esterification of free fatty acids into the storage depot (triacylglycerols).

Another indirect activator of triacylglycerol storage is insulin. Again, glucose, the glycolytic precursor, is the actual fuel that drives lipid storage. Insulin acts only as a stimulant for glucose transport (Abate and Garg, 1995). Equally as important is the fact that insulin is an antagonistic regulator of hormone-sensitive lipase (HSL), the major enzyme responsible for triacylglycerol breakdown. Other hormones that inhibit lipolysis in adipose tissue and promote lipogenesis are glucocorticoids, estrogens and progesterone (Abate and Garg,

1995). More research is required to better understand the exact endocrine mechanism for promotion of triglyceride accumulation inside the adipocyte.

Lipolysis. Degradation of the triglyceride and the rebuilding of the energy depot are continuous cycles. Inside the fat cell, the major enzyme responsible for degrading the triglyceride into free fatty acids is HSL. Externally, the major regulators of HSL are the catecholamine hormones: epinephrine and norepinephrine. The receptors responsible for the adipocyte sensitivity to catecholamines are beta-adrenergic receptors (Abate and Garg, 1995). The location of the adipocyte determines the amount of beta-adrenergic receptors found on the cell's surface, but more information is needed to verify this observation (Abate and Garg, 1995).

Adipose Endocrinology

The discovery of leptin in 1994 by Zhang and co-workers brought new light upon adipocyte regulation. Adipocytes would no longer be thought of as only a reservoir for stored energy. With Zhang's discovery, the adipocyte is now considered to be a part of the endocrine system. Leptin (167 amino acids) is a protein hormone, which is secreted by adipocytes and is regulated by the hypothalamus (Zhang et al., 1994). In the early 1950's, researchers postulated that mammals had a feedback loop in which the hypothalamus could sense and adjust feed intake and the rate in which the body expended energy to maintain body weights (Zhang et al., 1994). Several hypotheses have been discussed

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since the 1950's that would explain the mechanism in which this could occur. One such hypothesis, the lipostatic theory, appears to be supported by the discovery of leptin. The lipostatic theory states that the size of the body fat deposits are regulated by the central nervous system (CNS), and a "messenger" is produced by fat metabolism which circulates in the bloodstream (Zhang et al., 1994). Once the fat metabolism product is in the bloodstream, this "messenger" can influence the energy balance by interacting with the hypothalamus. The messenger was unidentified until the discovery of leptin in 1994 (Zhang et al., 1994).

Although research with leptin has been limited to mainly rat and mouse studies (Zhang et al., 1994; Halaas et al., 1997), the implications from understanding the regulation of leptin physiology could be very beneficial for the beef industry as well. Since finishing beef cattle are on feed for so long, understanding mechanisms that control feed intake would have useful implications for the finishing period.

Leptin research has taken many different routes since its discovery nearly 6 years ago. One such study examines how leptin may be the regulator in the "set point" theory for body weight (Zhang et al., 1994). Obese individuals, whether we are referring to mice, cattle or humans, have a higher "set point" for optimal body weight compared to lean subjects. Unfortunately, obesity usually is associated with hypertension, hyperlipidemia, and non-insulin-dependant diabetes mellitus (Halaas et al., 1997). Leptin has been identified as a negative-feedback loop regulating body weight, and plasma leptin levels have been

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positively correlated with the size of adipocytes (Halaas et al., 1997). Treatment of rats and mice with recombinant leptin reduces their food intake and body fat levels (Zhang et al., 1994; Halaas et al., 1997). This would suggest that one of the principal roles of leptin is to signal starvation, and that was the early hypothesis in leptin research (Lee, 1996). However, it was found that obese individuals have high plasma levels of leptin, which would suggest leptin insensitivity (Halaas et al., 1997). It must be considered that leptin insensitivity could result from defective leptin receptors or a lower number of leptin receptors (Gura, 2000). More research is needed on leptin receptors to determine the exact magnitude of their role in leptin insensitivity. It was hypothesized that leptin insensitivity would be associated with a positive energy balance and increase size in adipocytes for a stable balance at an increased weight size. Once an animal reaches obesity, the body becomes insensitive to leptin. Halaas et al. (1997) utilized ob/ob (mice that are obese due to a mutation of the leptin locus) and wild-type mice to demonstrate that there are different degrees of leptin insensitivity or resistance. This research indicated that leptin can act to reduce the energy expenditure that is usually seen when caloric intake is reduced. The interpretation of this statement suggests that although leptin can suppress starvation, increased leptin levels in lean animals act to return an animal to its "set point" weight. The statements made by Halaas seem logical being that early studies with leptin were demonstrated on mice and rats with mutations where his studies were demonstrated on wild type mice. Once an animal becomes obese, the body tissues become insensitive to leptin. To

understand how and why leptin insensitivity occurs, the feedback loop interaction between the hypothalamus and leptin must be revisited.

Stephens et al. (1995) suggested that after 30 days of leptin treatment the number of neurons expressing neuropeptide Y were significantly decreased. Not only is neuropeptide Y located in the hypothalamus (arcuate nucleus), but it is also associated with food intake, reduction in thermogenesis, and an increase in plasma insulin and corticosterone levels. Stephens' results demonstrated that treatment with leptin is consistent with a reduction of neuropeptide Y synthesis and release. Reduction in NPY results in a decrease in food intake, a decrease in plasma insulin, and lower corticosterone levels which could explain leptin's effect of metabolism during starvation (Stephens et al., 1995).

As research continues to discover new roles for leptin, its action in the regulation of body weight becomes clearer. Sahu (1998) found other targets of leptin in the hypothalamus. Besides neuropeptide Y, leptin affects galanin (GAL), melanin-concentrating hormone (MCH), neurotensin (NT), and proopiomelanocortin (POMC) (Sahu, 1998). In Sahu's study, rats were fed *ad libitum* water and rat chow. His results agreed with Stephens' study that neuropeptide Y (NPY) is inhibited by leptin treatment. Furthermore, leptin decreases gene expression of neurons that have an increased effect on feed intake such as NPY, MCH, GAL, POMC. However, gene expression is increased in neurons that have a negative effect on feed intake (Sahu, 1998). Another hypothalamic messenger that has been shown to be influenced by leptin

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is corticotropin-releasing hormone (CRH) which also decreases feed intake (Sahu, 1998).

A study by Shimabukuro (1997) that could possibly have implications for understanding leptin's role in food intake, concentrated on non-neural mechanisms of leptin. *In vitro* studies indicated that leptin reduced triglyceride (TG) levels in cultured pancreatic cells. Leptin reduced formation of TG from free fatty acids (FFA) and increased oxidation of FFA (Shimabukuro, 1997). Shimabukuro (1997) demonstrated that an increase in leptin expression and secretion induces hyperleptinemia in rats which exhausts levels of TG in the liver, skeletal muscle and pancreas. Even though TG levels were depleted, the amount of FFA or ketones in the blood did not increase, which suggests breakdown of TG in the cell (Shimabukuro, 1997). This research illustrates another role in which leptin could regulate body weight, and ultimately feed intake, through decreasing the stored amount of TG in cells.

Although the majority of studies on leptin have been conducted with the mouse, rat, and human subjects, there are some studies that have investigated the affects of leptin on domestic animals. One such study conducted by Barb and coworkers (1998) demonstrated the influence of leptin upon growth hormone (GH) in swine. When leptin was administered (directly into the intracerebroventricular region of the hypothalamus) to prepuberal gilts, there was an increase in GH release. This increase was detected between 15-30 minutes after the injection (Barb et al., 1998). Thyroxine and insulin growth like factor 1 (IGF-1) levels were not affected by leptin treatment. The relationship between

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GH and leptin is not fully understood but could offer some hope of increased profitability in swine as well as beef due to GH ability to increase protein accretion while decreasing fat synthesis. GH treatment may provide a way to moderate fat deposition while still allowing for an increase in muscle mass.

Another study involving leptin monitored leptin levels in bovine subcutaneous adipose tissue by fasting mature cows (Tsuchiya et al., 1998). Cattle were maintained on a concentrate and roughage diet when samples of subcutaneous fat were excised between the seventh and tenth rib and snap frozen until leptin levels were analyzed. After a 48 h fast samples were taken from the same area on the other side of the animal. After the 48 h, animals were allowed to consume feed for three h before adipose samples were again harvested. Leptin levels were analyzed using a lysate protection assay developed by Tsuchiya et al. (1998). Levels of leptin decreased 47% from when the animals were on feed, and after feeding for three hr animals still exhibited a 33% decrease in leptin. This bovine study supports the findings in mouse and rat studies that leptin gene expression is regulated by feeding (Zhang et al., 1994; Tsuchiya et al., 1998). Further research of leptin will allow further insight into the physiological role leptin plays in adipose endocrinology.

Adipose Differentiation

Not only have great strides been made in adipose endocrinology but also in the understanding of the stages of differentiation from a preadipocyte to a

mature adipocyte. White adipose tissue develops from an embryonic stem cell precursor that can differentiate into adipocytes, chondrocytes, osteoblasts, or myocytes (Figure 1). Transformation of an embryonic precursor cell into the final terminal cell of a mature adipocyte involves a whole series of reprogramming gene expression during differentiation. As the cell reaches each different developmental stage, different genes need to either increase or decrease in expression for the adipocyte to reach terminal differentiation or maturity. Although research has yet to discover the actual steps in this process, adipocyte research has focused on the point of growth arrest in preadipocytes.

One of the main research tools that has allowed researchers to gain insight into when the preadipocytes begin to differentiate from the growth arrest state is the utilization of preadipocyte cell cultures that are arrested at different stages of development. Multipotential stem cell lines (10T1/2 and Balbc/3T3) are cell lines not committed into differentiating into an adipocyte; and preadipocyte cell lines (3T3-L1, 3T3-F442A and Ob1771) that have committed but not fully begun to differentiate into an adipocyte are ideal for *in vitro* investigation of differentiation (MacDougald and Lane, 1995). These cell lines have been the basis for detecting markers that serve as checkpoints for the adipocyte differentiation process. Studies using these *in vitro* preadipocyte models have demonstrated that transcription factors play in adipocyte differentiation (MacDougald and Lane, 1995). The two main families that researchers believe play a major function in transcriptional adipocyte differentiation are the

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CAAT/enhancer binding protein (C/EBP) and peroxisome proliferator-activated receptor (PPAR) families (See Figure 1).

The PPAR family is a subgroup of nuclear hormone receptors that consist of PPARγ, PPARα and PPARδ (Gregiore et al., 1998). PPAR's form heterodimers with retinoic acid X receptors (RXR) (Altiok et al., 1997). After dimerization and ligand activation, the PPAR/RXR complex appears to regulate transcription through response elements (PPRE) (Altiok et al., 1997). PPREs are known to transcriptionally activate genes associated with adipose fatty acid-binding protein, phosphoenolpyruvate carboxykinase, lipoprotein lipase, and stearoyl-CoA desaturase (Mandrup and Lane, 1997). Ligands that activate the PPAR family are long chain fatty acids, fatty acid metabolites, thiazolidinediones (new class of drugs that increase insulin sensitivity), and 15 deoxy 12, 14-prostaglandin J_2 (Gregoire et al., 1998). In adipose tissue and adipocyte cell lines, PPARγ is expressed at its highest level prior to the transcriptional activation of most adipocyte genes (Figure 1) (Altiok et al., 1997). The role of PPARα and PPARδ in differentiation is much less defined in adipose differentiation. PPARα may play more of a role in brown adipose tissue than white adipose, since there are low amounts found in white adipose tissue compared to much higher concentrations in brown adipose (Chawla and Lazar, 1994). Disruption of the PPARα gene indicates that it may play a more integral role in beta-oxidation than adipose development (Lee et al., 1996). More

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research is needed to determine the exact roles the PPAR family mediates adipocyte differentiation.

The C/EBP family of transcription factors consists of C/EBPα, C/EBPβ and C/EBPδ. This family contains a C-terminal basic region/leucine zipper domain which allows the C/EBP to bind DNA or to homodimerize or heterodimerize with itself or another family member (McDougald and Lane, 1995). Of the three, C/EBPα is most understood. It appears that C/EBPα is involved in the termination of mitotic clonal expansion that coincides with the onset of expression of adipocyte genes such as leptin and insulin receptor (Figure 1) (MacDougald and Lane, 1995). The other role that C/EBPα may play is its ability to autoactivate itself so that the gene continues expression in the terminally differentiated state of a mature adipocyte (Mandrup and Lane, 1997).

Although C/EBPα appears to mainly play a role at the end of mitotic division and maintenance of the terminally differentiated adipocyte, C/EBPβ and C/EBPδ appear to function mainly during preadipocyte proliferation (Figure 1) (MacDougald and Lane, 1995). Levels of C/EBPβ and C/EBPδ are reduced when the preadipocyte reaches growth arrest (MacDougald and Lane, 1995). More research is needed to fully understand the role in which C/EBPβ and C/EBPδ play in the maturation of the adipocyte. Once the roles of the PPAR and CEBP families or more defined in adipose biology they have the potential to serve as markers for cattle that will signal when the animal should be harvested.

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Differential Display-Polymerase Chain Reaction.

Humans, cattle, mice and other higher organisms have approximately 100,000 different genes of which about 15% are actively expressed in any one cell at a set point in time (Liang and Pardee, 1992). Qualitative and quantitative differences in gene expression dictate the physiological status of a specific cell. To better understand cell biology, researchers compare gene expression of cells during the physiological changes of its life such as growth, disease, aging, cell cycle regulation, programmed cell death, etc. (Matz and Lukyanov, 1998). Subtractive hybridization has been used in the past to determine differences in messenger RNA (mRNA) expression (Sager et al., 1993). However, this method is expensive, time consuming, contains many technical obstacles and yields few positive results. Another method that has recently been utilized to efficiently generate candidates of mRNAs that are differentially expressed is differential display-polymerase chain reaction (DD-PCR) (Liang and Pardee, 1992). This method is based on the principles of polymerase chain reaction and utilizes random primer pairs designed to amplify over 90% of all expressed mRNAs in a given sample. The net result is a cross-section of the population of expressed (mRNA) gene sequences in the tissue.

Genomic DNA located in the nucleus of a cell (diploid cell) contains a copy of each gene found in an individual. In the nucleus, DNA is transcribed into mRNA, which carries a copy of the gene from the nucleus to the ribosomes where it is translated into a protein. Regions of mRNA can be amplified by using

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a combination of random primers identified through DD-PCR. The power of DD-PCR is that it allows a researcher to screen mRNA for quantitative expression differences among treatment groups. Templates of mRNA can be detected by DD-PCR whether they are upregulated or downregulated. To accomplish DD-PCR, samples of single stranded mRNA are synthesized into double stranded complimentary DNA (cDNA) through PCR. The first phase of preparing a cDNA fragment from mRNA is anchoring the 3' end of the new product by using a 26 base pair sequence (XXTTTTTTTTTCGGGATATCACTCAGCA) to anneal to the mRNA sequence (Figure 2). The underlined portion of the sequence represents the T-7 promotor site. The XX represents a combination of two different nucleic acid pairs (reading 5' to 3'): UC, GC CC, AC, UG, GG, CG, UU, GU and CU. Each doublet should match 8% of the mRNA pool, and produce approximately 800 to 1,200 different first-strand cDNAs (Genomyx, 1997). A second PCR using twenty different arbitrary primers (Table 1) are attached with a fluorescent tag to anchor the 5' end (Figure 2). The sequences that are generated from the random annealing sites will generate different amplicon products of varying sizes across the genes at which the primers bind. These amplicons are then loaded on an acrylamide sequencing gel, with the smaller fragments migrating to the bottom of the gel while the slower, larger transcripts are at the top of the gel. The fluorescent tag will illuminate when scanned by a GenomyxSc™ Fluorescent Imaging Scanner (Genomyx Corporation, Foster City, CA). Because samples are run in adjacent lanes, differential expression of the amplified product can be readily detected and compared between treatment groups.

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Figure 1. Development of an adipocyte and the known genetic markers during the different stages of maturity. Adapted from MacDougle and Lane, 1995.

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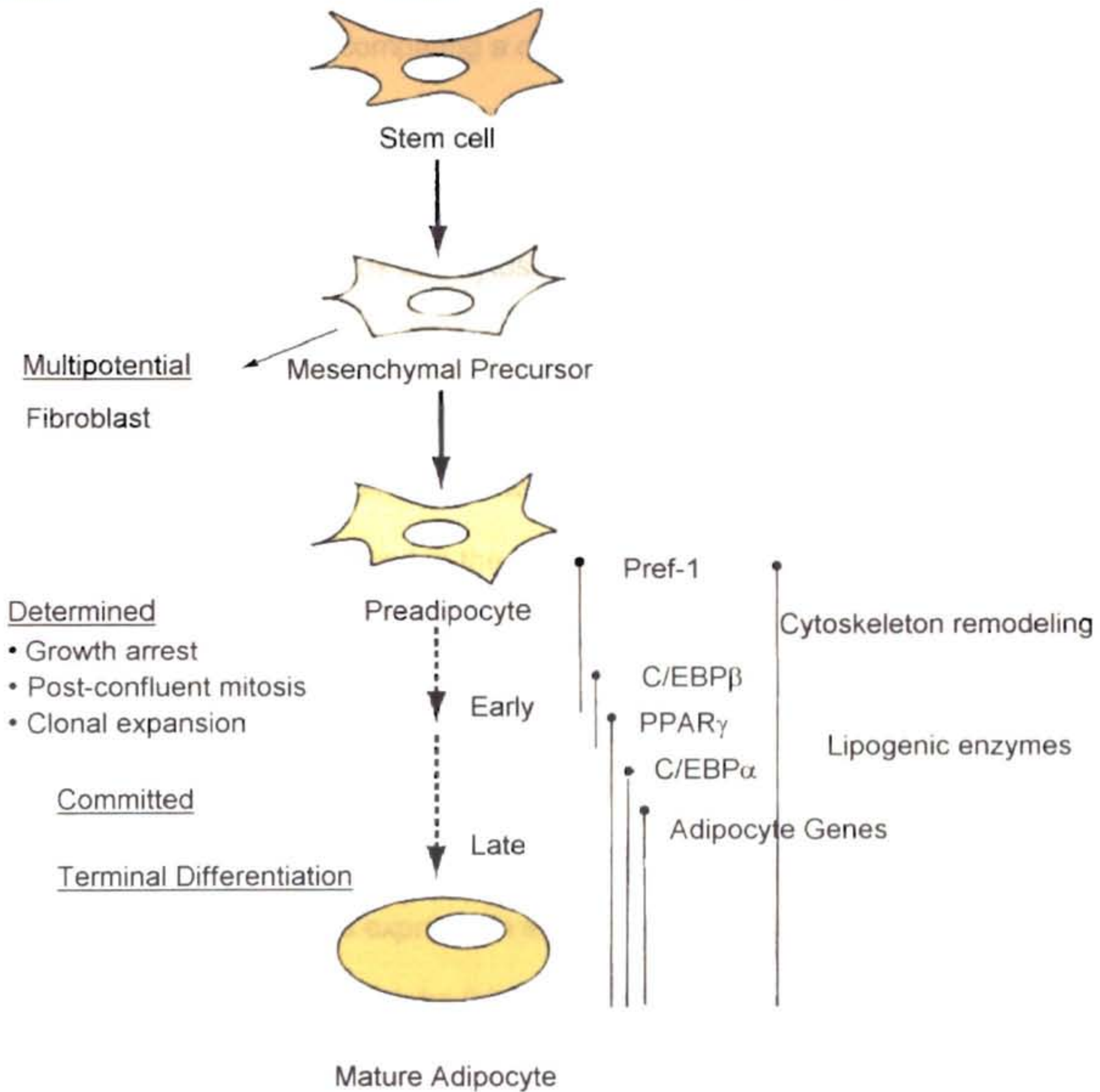
Cell Differentiation



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Once a gene product is identified as exhibiting a difference between the treatments, the band can be excised from the gel.

One example of successful study utilizing DD-PCR includes identification of alpha 6 integrin as a possible tumor repressor (Sager et al., 1993). Alpha 6 integrin was identified by comparing a cell line derived from a benign tumor to a cell line from and extremely malignant tumor in mammary cells (Sager et al., 1993). Integrins are a family of receptors that form trans-membrane bridges between extracellular ligands and cytoskeletal components of the cytoplasm and which are localized in the basal plasma membrane of epithelial cells (Sager et al., 1993). Thirty bands were observed in DD-PCR gels to exhibit a gene expression difference; all differences were down regulated or lost in the malignant tumor line compared to the benign tumor cell line. One band that exhibited a difference was cloned, sequenced and identified as alpha 6 integrin. In the Northern blot, a control sample of normal mammary epithelial cells was compared with the cells from a benign and malignant tumor. Northern blot results revealed a reduction of alpha 6 integrin expression in the benign tumor and complete loss of gene expression in the malignant tumor cells (Sager et al., 1993). Further studies with alpha 6 integrin will further determine the role it may play in suppressing breast cancer.

Another study using DD-PCR was one of the first to report that expression of aldose reductase appeared to be regulated by polypeptide growth factors using DD-PCR (Donohue et al., 1994). Cells from NIH 3T3 cells were either serum starved or stimulated by fibroblast growth factor-1 (FGF-1) so that genes

induced by FGF-1 could be isolated and identified. After DD-PCR analysis, cloning and sequencing an amplicon that had high homology with the aldo-keto reductase superfamily was identified as fibroblast growth factor regulated-1 (FR-1). Donohue et al. (1994) suggests that FR-1 elevated gene expression could be involved in increasing the rate of glycolysis or membrane phospholipid synthesis during the cell cycle.

Many studies involving oncology research have involved DD-PCR as well (Okamato et al., 1994; Sun et al., 1994; McCarthy et al., 1995). Studies evaluating differentiating genes involved with tissue transplant rejection have also utilized DD-PCR (Utans et al., 1994; Russell et al., 1994). Resolving gene expression differences during brain development have also benefited from DD-PCR (Watson and Margulies, 1993; Joseph et al., 1994). Differential display-PCR has been showed as a powerful tool in locating gene expression differences in the progression of disease as well as growth and development of cells in many different fields.

The feedlot stage of cattle production provides a model for observing adipocyte differentiation with the technology of DD-PCR and could provide insights into regulation of gene expression. The objective of the present study was to evaluate gene expression during intramuscular adipocyte differentiation and development of the various phases of marbling in feedlot steers utilizing DD-PCR to determine differential gene expression.

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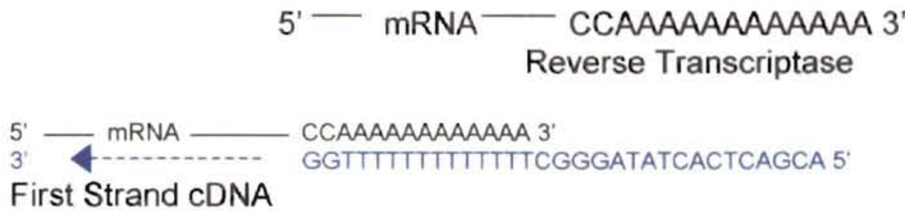
Oligonucleotide anchored primer (31 bases)

3' GGTTTTTTTTTTTTCGGGATATCACTCAGCA 5'

Anchor Primer T7 (17 bases) promotor sequence

First-strand cDNA synthesis.



Arbitrary 5' primer (26 bases) with a fluorescent tag

5' ACAATTTCACACAGGAXXXXXXXXXX 3'

M13 (16 bases) reverse primer sequence 10-base arbitrary sequence

Synthesis of double-stranded cDNA fragments

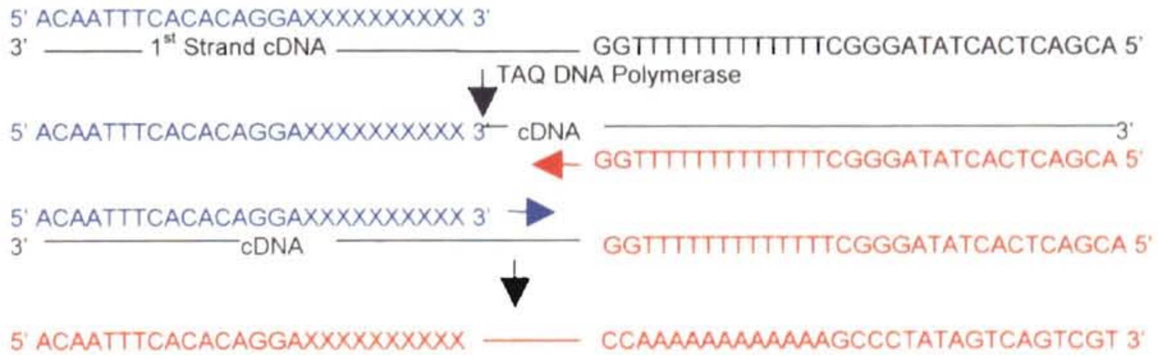


Figure 2. Overview of primers used in polymerase chain reactions.

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Table 1. 3'-arbitrary primers used in DD-PCR.

M13r-ARP1	ACAATTTCACACAGGACGACTCCAAG
M13r-ARP2	ACAATTTCACACAGGAGCTAGCATGG
M13r-ARP3	ACAATTTCACACAGGAGACCATTGCA
M13r-ARP4	ACAATTTCACACAGGAGCTAGCAGAC
M13r-ARP5	ACAATTTCACACAGGAATGGTAGTCT
M13r-ARP6	ACAATTTCACACAGGATACAACGAGG
M13r-ARP7	ACAATTTCACACAGGATGGATTGGTC
M13r-ARP8	ACAATTTCACACAGGATGGTAAAGGG
M13r-ARP9	ACAATTTCACACAGGATAAGACTAGC
M13r-ARP10	ACAATTTCACACAGGAGATCTCAGAC
M13r-ARP11	ACAATTTCACACAGGAACGCTAGTGT
M13r-ARP12	ACAATTTCACACAGGAGGTACTAAGG
M13r-ARP13	ACAATTTCACACAGGAGTTGCACCAT
M13r-ARP14	ACAATTTCACACAGGATCCATGACTC
M13r-ARP15	ACAATTTCACACAGGACTTTCTACCC
M13r-ARP16	ACAATTTCACACAGGATCGGTCATAG
M13r-ARP17	ACAATTTCACACAGGACTGCTAGGTA
M13r-ARP18	ACAATTTCACACAGGATGATGCTACC
M13r-ARP19	ACAATTTCACACAGGATTTTGGCTCC
M13r-ARP20	ACAATTTCACACAGGATCGATACAGG

Sequences are 5' to 3'. Underlined sequences are M-13 promotor regions.

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Chapter III

DIFFERENTIAL DISPLAY-POLYMERASE CHAIN REACTION (DD-PCR) ANALYSIS OF ADIPOCYTE GENE EXPRESSION DURING MARBLING IN ANGUS X HEREFORD STEERS

INTRODUCTION

The single most important characteristic of beef quality, from the side of the consumer, is meat tenderness (Miller et al., 1995). It has been demonstrated that consumers are willing to pay more for steak that is known to be tender than a tough counterpart (Boleman et al., 1997). However, surveys have documented that consumers are dissatisfied with the eating quality of beef either prepared at home or at a restaurant setting 20% to 25% of the time (Miller, 1995). That being the case, a consumer will have an undesirable eating experience while eating beef one of every four or five settings (Smith et al., 1995). Meat from one tough beef carcass has the potential of reaching as many as 542 consumers (Savell et al., 1991). A single bad experience with consumption of a poor quality serving of meat can spread via word of mouth to as many as 950 potential consumers (Smith, 1995). Meat tenderness is of vital importance if beef producers are concerned with continuing to keep consumers as satisfied end-users in the future.

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Due to the many variables that contribute to the overall palatability of beef, tenderness is a difficult equation to understand. Cattle that appear phenotypically able to produce product that is acceptable for consumers do not always do so. The Beef Quality Audit of 1995 described problems associated with low overall tenderness and low overall palatability. The Quality Audit stated that one of the best strategies for improving the quality and consistency of beef was to continue to discover, develop, and apply technology to enhance the quality of beef (Smith et al., 1995).

Humans, cattle, mice and other higher organisms have approximately 100,000 different genes of which about 15% are expressed in one cell at a particular point in time (Liang and Pardee, 1992). Differences in which genes are expressed, and level of its expression drives the physiological status of cell function. To better understand cell biology, researchers compare gene expression of cells during growth, disease, aging, cell cycle regulation and programmed (Matz and Lukyanov, 1998). Differential display-polymerase chain reaction (DD-PCR) can be utilized as a technique to distinguish differences between gene expression by comparison of the amount of gene transcription between cells in various physiological states (Liang and Pardee, 1992; Sager et al., 1993). Little is known concerning gene regulation during the stages of marbling in feedlot steers. Identifying genes that turn on or turn off during marbling would provide a better understanding of adipose development. Differentiation of adipocytes in the intramuscular tissue during the process of marbling provides an excellent model to utilize DD-PCR to search for differential

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expression of genes. The current research was conducted to evaluate changes in mRNA expression associated with the marbling or adipocyte differentiation of feedlot steers.

MATERIALS AND METHODS

Animals

Angus x Hereford steers (n=50) of known origin and genetics were selected from a ranch in western Oklahoma. The ancestries of these animals were well established in that animals had historically been selected with the propensity to deposit high amounts of intramuscular fat (i.e., marbling) and produce carcasses which graded U.S. Choice or higher. Animals were raised, weaned and preconditioned using standard commercial practices. At 12 months of age, steers were randomly blocked by weight, assigned to 10 pens of 5, and fed an *ad libitum* diet (Table 2) at the Willard Sparks Beef Research Facility in Stillwater, Oklahoma. Animals were fed for either 20, 86, 121 or 146 days. Days on feed were designed to yield cattle at different stages of adipocyte deposition. Harvest groups (days on feed) were targeted to have either 0, 0.7, 1.3 or 1.8 cm of subcutaneous fat at the $12^{th}/13^{th}$ rib interface at the time of slaughter. Animals were routinely weighed, and ultrasound was used to monitor the subcutaneous fat deposition of the steers during the feeding period. After the group average of steers (15 head- 3 pens of 5) reached target subcutaneous fat levels, the

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Table 2. Composition of diet fed to steers during the 20, 86, 121, or 146 days of the feedlot period (%, dry matter basis (DMB)).

Ingredient	Ration
Dry corn	86.50
Cotton seed hulls	5.09
Supplement**	8.39
NEm Mcal/cwt	99.00
NEg, Mcal/cwt	60.00
Crude protein	13.00

*All animals were stepped up to a 95% concentrate corn based diet within 21 d.
**Supplement %,DMB: cottonseed meal 60.75, Calcium carbonate 13.35, Wheat midds 11.8, Urea 5.29, Potassium Chloride 4.01, Salt 4.01, Rumensin™ 80 0.25, Tylan™ 40 0.15, Vitamin A-30 0.14, Selenium 600 0.06, Magnesium oxide 0.05, Zinc sulfate 0.04, Copper sulfate 0.01.

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animals were humanely harvested at the Food and Agricultural Products Center in Stillwater, Oklahoma. Following a 24 or 36 h chilling period (0-1°C), trained Oklahoma State University personnel collected carcass yield and quality grade factors consisting of hot carcass weight, *longissimus* muscle area, subcutaneous fat thickness, kidney, pelvic and heart fat percentage, skeletal and lean maturity and marbling amount.

Tissue collection

Prior to dissection, work surfaces and lab equipment were sterilized with a 10% Clorox™ solution. *Longissimus dorsi* sections (approximately 250 g) were collected from the left side of each carcass approximately 10 min following exsanguination. Each *longissimus* section was removed at the 13th rib using a sterile scalpel and immediately transferred to a 4°C processing room. Immediately upon removal, muscle cores were weighed and rinsed in sterile 0.9% saline to remove any foreign material. Intramuscular adipose tissue (marbling) was dissected from the muscle core, and the surrounding muscle fibers were separated from the intramuscular fat. The adipose and muscle fiber samples were placed on ice during dissection, placed in aluminum foil, snap frozen in liquid nitrogen and stored in an -80°C freezer until extraction of cellular RNA. All samples were snap frozen between 30 and 40 minutes of exsanguination to reduce RNA degradation.

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RNA extraction

Approximately, 0.3 to 1 g of adipose tissue was extracted for RNA with 4 ml of TRIzol™ reagent (Gibco, Grand Island, NY). Tissue samples were homogenized with a VirTishear™ homogenizer (The Virtus Company, Inc., Gardiner, NY) for 45 seconds and allowed to incubate at r.t. for 5 min. After shaking gently, 1 ml of chloroform was added to the homogenized sample. Samples were vortexed for 15 seconds and incubated at r.t. for 3 min. Following incubation, samples were centrifuged at 4750 x g for 30 min at 4°C. The upper aqueous phase was transferred into a fresh tube, 2.5 ml of isopropyl alcohol was mixed with the sample, and the solution incubated at r.t. for 10 min. The sample was centrifuged at 4750 x g for 10 min, the supernatant discarded. The resulting RNA pellet was washed once with 3 ml of 75% ethanol, vortexed and centrifuged at 4750 x g for 5 min. After the ethanol was removed, the RNA pellet was air dried for 5 min. and the RNA pellet was dissolved in 200 μl of TE (Tris-chloride, ethylenediaminetetraacetic acid) buffer for storage at -80°C.

Quantification of RNA. The concentration of RNA in the samples was measured by combining 5 μl of RNA with 995 μl of Ribogreen™ RNA quantification reagent (Molecular Probes, Inc., Eugene, OR). Fluorescence was measured in a TD-700 fluorometer (Turner Designs, Sunnyvale, CA).

RNA pools. RNA samples from each harvest group were pooled to reduce false positives. Two replicates of each group were used for the DD-PCR

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analysis. A replicate consisted of selecting RNA from five animals closest to the average mean marbling score in each harvest group. A total of 5 μg RNA from each of the five animals in a replicate group were mixed together. Due to freezer failure, RNA from only one animal was available for the 0 backfat group.

DNAse I treatment. For removal of DNA contamination, each pooled group of RNA sample was treated with DNAse I. A master mix containing DEPC water, 10X DNAse I 1 U (Gibco, Grand Island, NY), and RNAsin 10 U (Promega, Madison, WI) was utilized. After equal volumes of RNA and master mix were combined, tubes were incubated at 37° C for 15 min. To deactivate the DNAse I, the mixture was brought to 200 μl with the addition of diethyl-polycarbonate (DEPC) treated water and an equal volume of phenol acid (pH 4.5)/chloroform (1:1). The tube was centrifuged at 4000 x g for 5 min at 4°C. After centrifugation, the supernatant was removed and placed in a new sterile microfuge tube. An equal volume of chloroform was added, mixed and centrifuged at 4000 x g for 5 min at 4°C. The upper phase was removed and pippetted into a new tube, 2 volumes of isopropanol was supplemented to the sample which was then mixed for 3 min and centrifuged at 10,000 x g 10 min at 4°C. The resulting RNA pellet was rinsed with 70% ethanol and allowed to air dry for 10-15 min. Pellets were suspended in 50 μl of DEPC water, incubated for 10 min at 60°C, and stored at -80°C.

Complimentary DNA preparation. The pooled samples of total RNA were reverse transcribed by combining 200 U of Moloney murine leukemia virus

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reverse transcriptase (M-MLV-RT), 1.0 μg of oligo (dT) primer, 0.5 mM each of dATP, dCTP, dGTP, dTTP, 50 mM Tris-HCL (pH 8.3), 75 mM KCL, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 20 U of RNAsin, 1.0 μg total RNA and bringing to a total volume of 26 μl with DEPC treated water. The M-MLV-RT, reaction buffer, RNAsin and oligo$(dT)_{15}$ primer were purchased from Promega Corporation (Madison, WI). The sample preparation was incubated at 22°C for 15 min, followed by a 30 min incubation at 42°C using a Perkin Elmer Cetus DNA Thermal cycler Model 480 (Norwalk, CT). The reaction was terminated by heating at 95°C for 5 min and quickly cooling to 4°C.

RNA quantification and quality. RNA replicates were evaluated to determine that equal amounts of RNA were brought to volume with DEPC water. Complimentary DNA (cDNA) made from RNA replicates were amplified with primers for glyceraldehyde-3-phosphate dehydrogenase (G3PDH). Primer design and conditions for G3PDH amplification have been previously described by Yelich et al. (1997). Total volume for the PCR reaction was 26 μl containing 2 μl of cDNA product, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM G3PDH primer, 1 U Taq polymerase (Promega, Madison, WI). PCR products were electrophoresed on a 1% agarose gel at 96 V for 30 min with ethidium bromide. To validate removal of genomic DNA contamination in the RNA samples, cDNA was amplified with primers to leptin from porcine. The 5' (5'-GTCACCAGGATCAAT-GACAT-3') and 3' (5-'AGCCCAGGAATGAAGTCCAA-3') primers span an intron between the second and third exon. Primers were designed based on the ob

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gene sequences from mouse human and rat (Sasaki et al., 1996). In porcine, the leptin primer sequence amplified a product of ~2200 bp (Sasaki et al., 1996). Dr. Archie Clutter, Oklahoma State University, generously provided Leptin primers. The PCR reaction using the leptin genomic primer consisted of: 2 μl of cDNA product, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM leptin genomic primer, 1 U Taq polymerase (Promega, Madison, WI) brought to final volume of 26 μl with DEPC treated water. Conditions for the PCR were one cycle of 95°C for 2 min, 60°C for 1 min, 72°C for 2 min and 45 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min with tubes maintained at 4°C until products were electrophoresed on a 1% agarose gel.

To establish adipose tissue was not contaminated with adjacent skeletal muscle tissue following the initial dissection procedure, cDNA from the RNA pooled samples of adipose and muscle tissue were amplified by primers designed to amplify porcine leptin receptors. The leptin receptor primer consisted of 5'-CCAAACCTCGAGGAAAGTTTACC-3' and 5'-AGGCTGCTCC-TATGATACCTCAA-3' (Ernst et al., 1997). The primers are designed to amplify a product of 380 bp (Ernst et al., 1997). The PCR reaction consisted of 1 μl of cDNA product, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM leptin receptor primer, and 1 U Taq polymerase (Promega, Madison, WI). Conditions for the PCR were one cycle of 95°C for 2 min, 60°C for 1 min, 72°C for 2 min and 45 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min with tubes maintained at 4°C until products were electrophoresed on a 1% agarose gel.

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Differential display-PCR. The sequences for the ten 3' anchor primers used for synthesizing complementary DNA are (XXTTTTTTTTTCGGGATA-TCACTCAGCA). The underlined portion of the sequence represents the T-7 promotor site. The XX represents a combination of two different nucleic acid pairs (reading 5' to 3'): UC, GC CC, AC, UG, GG, CG, UU, GU and CU (Operon Technologies, Alameda, CA). The cDNA was generated by mixing 0.2 μg of RNA with 1 μg of a 15-mer oligo (dT) primer and DEPC water to a final volume of 10 μl. Samples were incubated at 65°C for 5 minutes, and then placed on ice. Following incubation, 10 μl of master mix containing 1X Superscript buffer, 10 mM DTT, 40 U SuperScript Reverse Transcriptase II (Gibco, Grand Island, NY), 5 U RNAsin (Promega, Madison, WI) and 0.5 mM each of dATP, dCTP, dGTP, dTTP was added to the 10 μl of RNA mixture. Sample was then placed in a MJ Research PTC-225 Peltier Thermal Cycler (MJ Research, Inc., Waltham, MA) and cycled through 10 minutes at 25°C; 42°C for 60 minutes; 70° for 15 minutes. The resulting product was held at 4°C in an MJ Research PTC-225 Peltier Thermal Cycler (MJ Research, Inc., Waltham, MA).

A fluorescently tagged 5' anchor primer containing one of 20 different arbitrary primers (Table 1) was utilized in a second PCR amplification. A master mix of 10 μl of 3.75 mM $MgCl_2$, 0.25 mM dNTP, 0.5 U Taq polymerase with 1X PCR buffer (Sigma-Aldrich, St. Louis, MO), 0.35 μM of respective 3' anchor primer, and 0.35 μM of respective 5' arbitrary primer. PCR conditions for DD-PCR reactions were: 1 cycle of 95°C for 2 min; 4 cycles of 92°C for 15 seconds,

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50°C for 30 seconds and 72°C for 2 min; 25 cycles of 92°C for 15 seconds, 60°C for 30 seconds and 72°C for 2 min; one cycle of 72°C for 7 minutes with samples held at 4°C at termination of amplification.

Four-microliters of fluoro DD-PCR sample were mixed with 1.5 μl of fluoro DD-PCR loading dye, and the mixture was held at 95° C for 2 minutes. Samples were loaded on a 5.8% polyamide denaturing gel and ran at 3000V, 100W, 55°C for 3 h on Genomyx LR DNA sequencer (Genomyx Corporation, Foster City, CA).

A GenomyxSc™ Fluorescent Imaging Scanner (Genomyx Corporation, Foster City, CA) was utilized to analyze gels. Images were transferred to Adobe Photoshop 4.0 (Adobe Systems, Incorporated, San Jose, CA) for comparison of changes in gene expression between treatment groups. Amplicons (bands) were scored on a scale of 0 to 3 (Table 3). For a gene expression difference to be scored, the two replicates from the harvest group must duplicate with the same intensity. A gene receiving a score of 3 (Figure 3) exhibited the most distinct difference in gene expression between the leaner animals (groups 1 and 2) and the fatter animals (groups 3 and 4); whereas, a score below 3 showed lower degrees of differences in gene expression between treatment groups. After the gel was analyzed, the selected bands were excised from the gel, and incubated for 1 hour at 65°C in a 1.5 ml microfuge tube containing 50 μl of sterile nuclease-free TE solution (10mM Tris-Cl pH 7.4). Tubes were stored at -80°C until utilized for reamplification.

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Reamplification of DD-PCR product. Samples were reamplified for sequencing. The PCR reaction combined 4 μl DD-PCR product, 1.5 mM $MgCl_2$, 0.25 mM dNTP, 2 U Taq polymerase, and 10X PCR buffer (Promega, Madison, WI), 0.2 μM anchor primer M-13 and 0.2 μM arbitrary primer T-7 to a total volume of 40 μl Operon Technology, Inc (Alameda, CA). PCR parameters were 4 cycles of 95°C for 2 min, 92°C for 15 sec, 50°C for 30 sec, 72°C for 2 min, 25 cycles of 92°C for 15 sec, 60°C for 30 sec, 72°C for 2 min, and 1 cycle of 72°C for 7 min, and maintained at 4°C until product was electrophoresed.

PCR products from the reamplification reaction were run on a 2% agarose gel, bands excised and then purified with a Qiagen purification kit (Qiagen, Santa Clarita, CA). Purified PCR product was sequenced by a 373 DNA Sequencer (Applied Biosystems, Foster City, CA) at the Oklahoma State University DNA Sequencing Core Facility (Stillwater, OK) using M-13 universal primer (Promega, Madison, WI). Sequences were submitted to GenBank at the National Center for Biotechnology website (http: www.ncbi.nlm.nih.gov) to check for sequence homology with known genes.

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Table 3. Scale is representative of the ranking system assigned to bands from DD-PCR gels. If a band exhibited no gene expression difference a band would receive a score of 0 and not excised. If band did exhibit a gene expression difference it was given a score between 1 to 3 and excised for further analysis.

Score	Intensity of gene expression difference.
0	No difference in gene expression between groups 1 and 2 versus 3 and 4.
1	Slight difference between treatment groups 1 and 2 versus 3 and 4.
2	Moderate difference between treatment groups 1 and 2 versus 3 and 4.
3	Distinct difference between treatment groups 1 and 2 versus 3 and 4.

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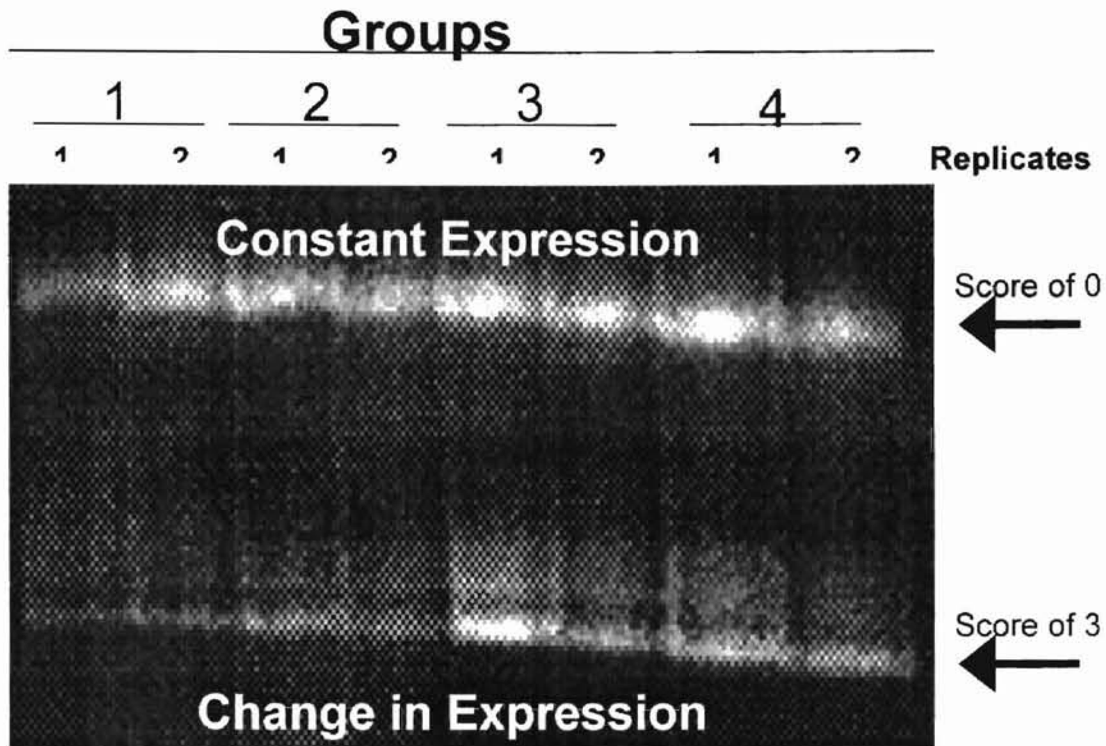

Figure 3. Magnified section of DD-PCR gel representing a gene with constant expression across treatment groups (score 0) and a gene exhibiting differential expression (score 3). Each group has two replicates of a pool of 5 animals except group 1 which is represented with one animal in each lane.

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Northern hybridization. Select cDNA products were sub-cloned into TOPO™ 2.0 plasmids using TOPO™ cloning kit (Invitrogen, Carlsbad, CA). Plasmids were harvested by a Wizard Plus Miniprep™ (Promega, Madison, WI) and sequenced to confirm the identity and orientation of the inserted cDNA.

To compare and contrast gene expression in other tissues with adipose; kidney, heart, liver, skeletal muscle tissue and subcutaneous fat were collected for controls during Northern blot analysis using methods previously described. Samples of RNA (20 μg) extracted from kidney, heart, liver, skeletal muscle tissue, subcutaneous fat and intramuscular adipose as well as individual RNA from four animals representing low marbling and five animals representing high marbling groups were prepared for analysis by adding 6.6 μl of 10 X MAE (0.4 M 3-[N-morpholino] propane sulfonic acid), 0.1M sodium acetate, 0.5 M EDTA, 14.4 μl of formaldehyde/formamide mix and 4.5 μl of loading buffer. The samples were loaded into a 1% formaldehyde gel, and electrophoresed for 8 h at 40V in 1 X MAE buffer. The gel was washed 10 minutes in DEPC treated water and RNA transferred (2 h) onto a positively charged nylon membrane (Boehringer Mannheim, Indianapolis, IN) with 10X SSC using a 785 Biorad vacuum blotter (Biorad, Hercules, CA). The RNA was cross-linked onto the nylon membrane by a Stratagene 2400 UV Stratalinker (Stratagene, La Jolla, CA).

Complimentary DNA for NAT1 (novel apolipoproteinB editing catalytic sub-unit 1 APOBEC-1 target 1) (Yamanaka et al., 1997) and myopodin were linearized and probes generated by mixing 6 μg of the product with 5X

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Transcription buffer, 100 mM DTT, 2.5 mM d A, C, GTP, 1 mM UTP, RNAsin (Promega, Madison, WI), RNA polymerase primer (SP6 or T7) and radiolabeled [^{32}P]-uridine triphosphate ([^{32}P]-UTP) (3000 ci/mmole). The mixture was incubated at 37° C for 2 h, following which 3 μl of RQ1- DNAse I (Promega, Madison, WI) and .5 μl of RNAsin were added and sample incubated at 37° C for an additional 30 min. Yeast tRNA (8μl – 25 mg/ml) and 40 μl of phenol/chloroform/Isoamyl alcohol mix (1:0.5:0.5) were added and sample centrifuged (13,000 X g) at 4°C for 5 min. Radiolabeled probe was isolated with a G-25 Sephadex Quickspin column (Boehringer Mannheim, Indianapolis, IN), and probe was ethanol precipitated and resuspended in 100 μl of DTT. Specific activity of each probe was quantified in an LS 5000 Beta counter (Beckman Instruments, Inc., Fullerton, CA).

Pre-hybridization and hybridization solutions consisted of 20X SSPE, 100X Denhart's solution, 10% SDS, formamide, 50 mM sodium phosphate, 200 μg of herring sperm, and DEPC treated water. Membranes were pre-hybridized at 42° C in a hybridization incubator (Lab-Line Instruments, Inc., Melrose Park, IL). After at least 1 hr, the pre-hybridization solution was removed and the hybridization solution with the ^{32}P-labeled probe (10,000 Ci/ml) was added and incubated over night in the hybridization incubator.

Membranes were removed from the glass cylinders and washed with 1X SSC, 0.1% SDS two times for 30 min at 50°C and once with 0.2X SSC, 0.1% SDS for 15 min at 65°C. An image was obtained by immediately laying the

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membrane on a Biorad GS-525 Molecular Imager System (Biorad, Hercules, CA). Following image analysis the membrane was then exposed to X-ray film for 3 days.

Statistical Analysis. Growth and carcass data were analyzed with least squares methods using the General Linear Models procedure of SAS (SAS, 1990). The model included affects of days fed group. Group mean comparisons were compared by using Duncan's multiple range test.

Results

Growth and carcass traits. Performance data is presented in Table 4. Steers were stratified by days on feed and will be referred to as time-on-feed 20 d (Group 1), 86 d (Group 2), 121 d (Group 3) and 146 d (Group 4). Steers from Group 1 were heavier ($P > .05$) when compared to steers from Groups 2, 3, and 4 when the animals were placed on feed, but there were no significant differences in beginning weight among Groups 2, 3 and 4. Mean end-weights, which correspond with preselected treatment endpoint targets, were different ($P < .05$). Group 1 gained 0.94 kg for the twenty d on feed, which was lower ($P < .05$) compared to groups 2, 3 and 4. Average daily gain was similar between groups 2,3 and 4.

Table 4. Performance traits of feedlot steers stratified by days on feed.

Trait	Days On Feed				SEM
	20	86	121	146	
Number	5	15	15	15	
Beginning Weight, kg	366.8[a]	350.3[b]	350.7[b]	346.7[b]	14.3
Ending Weight, kg	385.6[a]	460.3[b]	496.6[c]	539.4[d]	26.3
Average Daily Gain, kg	0.94[a]	1.50[b]	1.38[b]	1.47[b]	0.4

[a,b]Information having different superscripts, within the same row, are statistically different (P<.05).

Data for the carcass traits is presented in Table 5. Dressing percent was lower ($P < .05$) in group 1 compared to groups 3 and 4 with group 2 intermediate. Ribeye area was smaller ($P< .05$) in treatment groups 1 and 2 compared to 3 and 4. Hot carcass weight, fat thickness, KPH (kidney, pelvic, heart) percent fat, yield grade and marbling mean average scores were different ($P < .05$) among all groups. Days on feed resulted in 0, 6, 67 and 87% of the steers grading Choice in groups 1, 2, 3, and 4, respectively (Table 5).

RNA quantity and quality for DD-PCR. The uniformity of RNA quantity evaluated through PCR amplification of cDNA from the pooled samples using G3PDH primers is presented in Figure 4. A 430 bp product, which is the expected size of G3PDH, was equally amplified for each RNA pooled replicate from group 1, 2, 3 and 4. Group 1 contained RNA from only one animal. Purity of RNA from genomic contamination was verified with PCR of cDNA samples with leptin primers, which spanned a region from exon two to exon three. No product was detected in the cDNA from the DNAse treated RNA pools used in DD-PCR (Figure 5). However, a range of small amplification products was evident in the positive genomic bovine sample. Adipose purity from contamination by adjacent skeletal muscle tissue was confirmed through amplification of cDNA using a leptin receptor primer that amplified a 398 bp product in cDNA from skeletal muscle but not in adipose samples (Figure 6). These data confirmed that adipose RNA sample pools used in DD-PCR were similar in concentration, not contaminated with free skeletal muscle RNA and did not contain genomic DNA.

Table 5. Carcass traits of feedlot steers stratified by days on feed.

Trait	Days on Feed				SEM
	20	86	121	146	
Number	5	15	15	15	
Dressing Percentage	57.1^{a}	59.3^{ab}	61.4^{b}	61.1^{b}	1.1
Hot Carcass Weight, kg	218.6^{a}	273.0^{b}	305.0^{c}	327.5^{d}	16.9
Fat Thickness, cm	0.46^{a}	0.74^{b}	1.09^{c}	1.35^{d}	0.04
Ribeye Area, cm^2	61.9^{a}	67.1^{a}	74.8^{b}	73.6^{b}	0.5
KPH Fat, %	0.8^{a}	1.7^{b}	2.1^{c}	2.7^{d}	0.2
Yield Grade	2.0^{a}	2.6^{b}	2.9^{c}	3.6^{d}	0.2
Marbling Score[1]	220^{a}	326^{b}	426^{c}	480^{d}	26.5
Choice, %	0	6	67	87	
Select, %	0	67	33	13	
Standard, %	100	27	0	0	

[a,b]Information having different superscripts, within the same row, are statistically different ($P < .05$).

[1]Marbling Score: 200 to 299 = Traces; 300 to 399 = Slight; 400 to 499 = Low Choice.

Figure 4. Representative of ethidium bromide stained agarose gel from a PCR using glycerol-3-phosphate dehydrogenase (G3PDH) primers to express a 430 bp product. Note the similar concentrations of PCR product across groups. Each group was represented by two replicate pools of 5 animals. (-) indicates negative control (no cDNA added) and M represents marker.

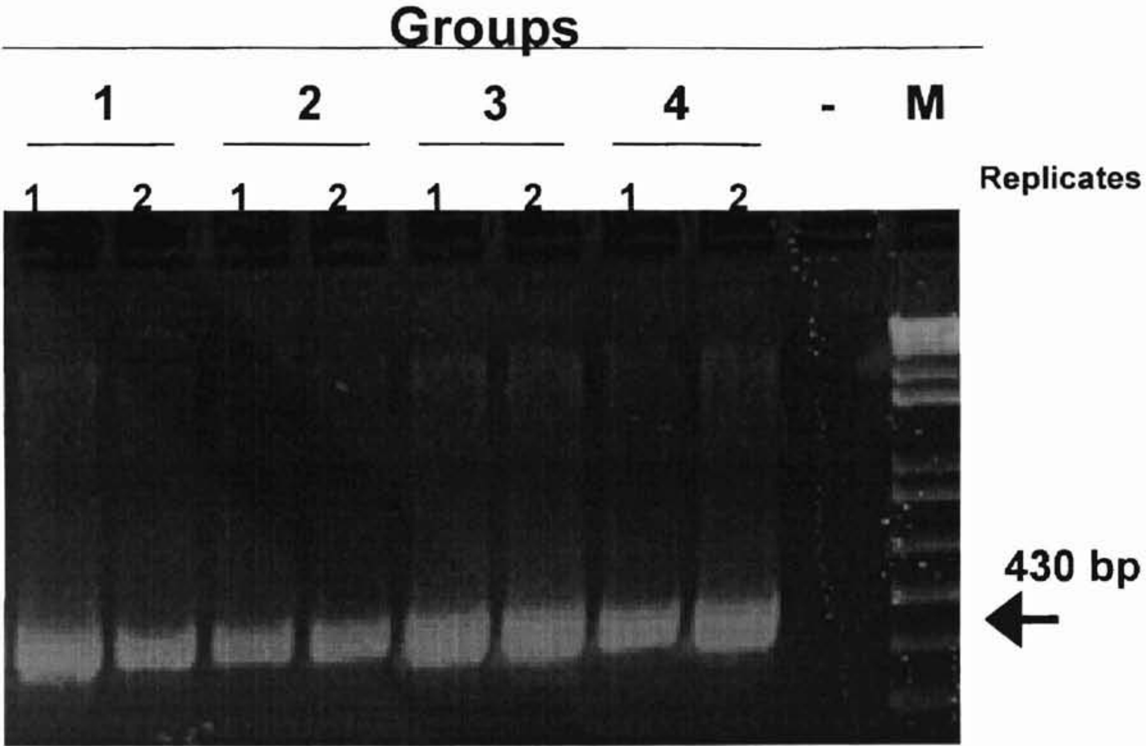


Figure 5. Representative of ethidium bromide stained agarose gel from a PCR using leptin primers, between exons 2 and 3, to amplify genomic DNA. Each group represented two replicate pools of 5 animals. Note lack of genomic DNA expression in cDNA of pools. (-) indicates negative control (no cDNA added), (+) indicates positive control containing bovine genomic DNA and M represents marker.

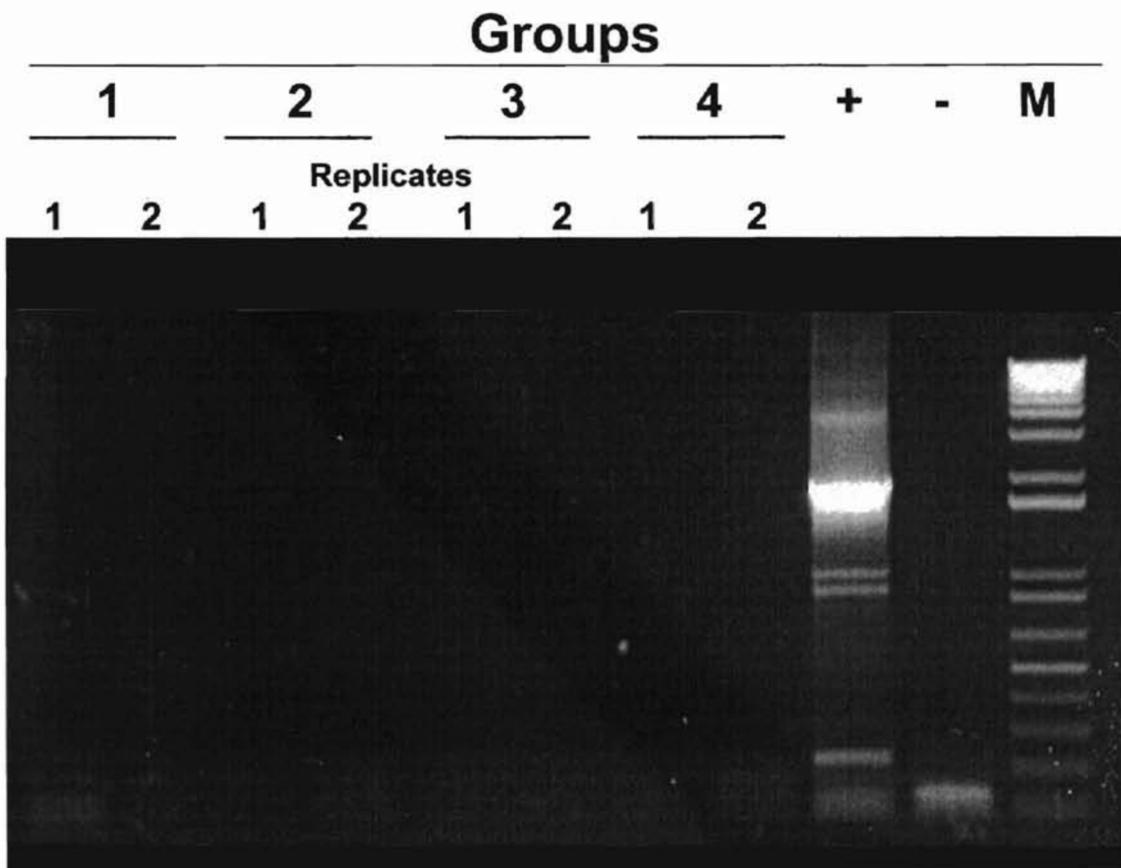
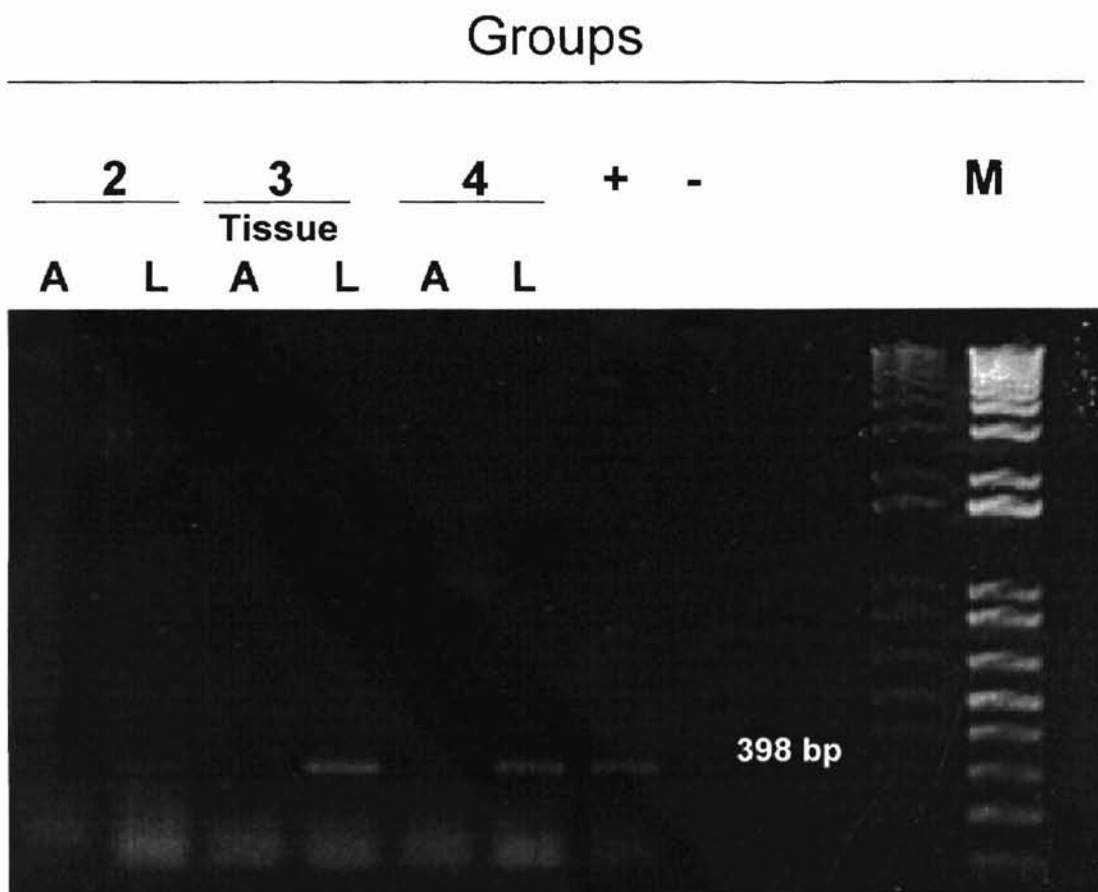


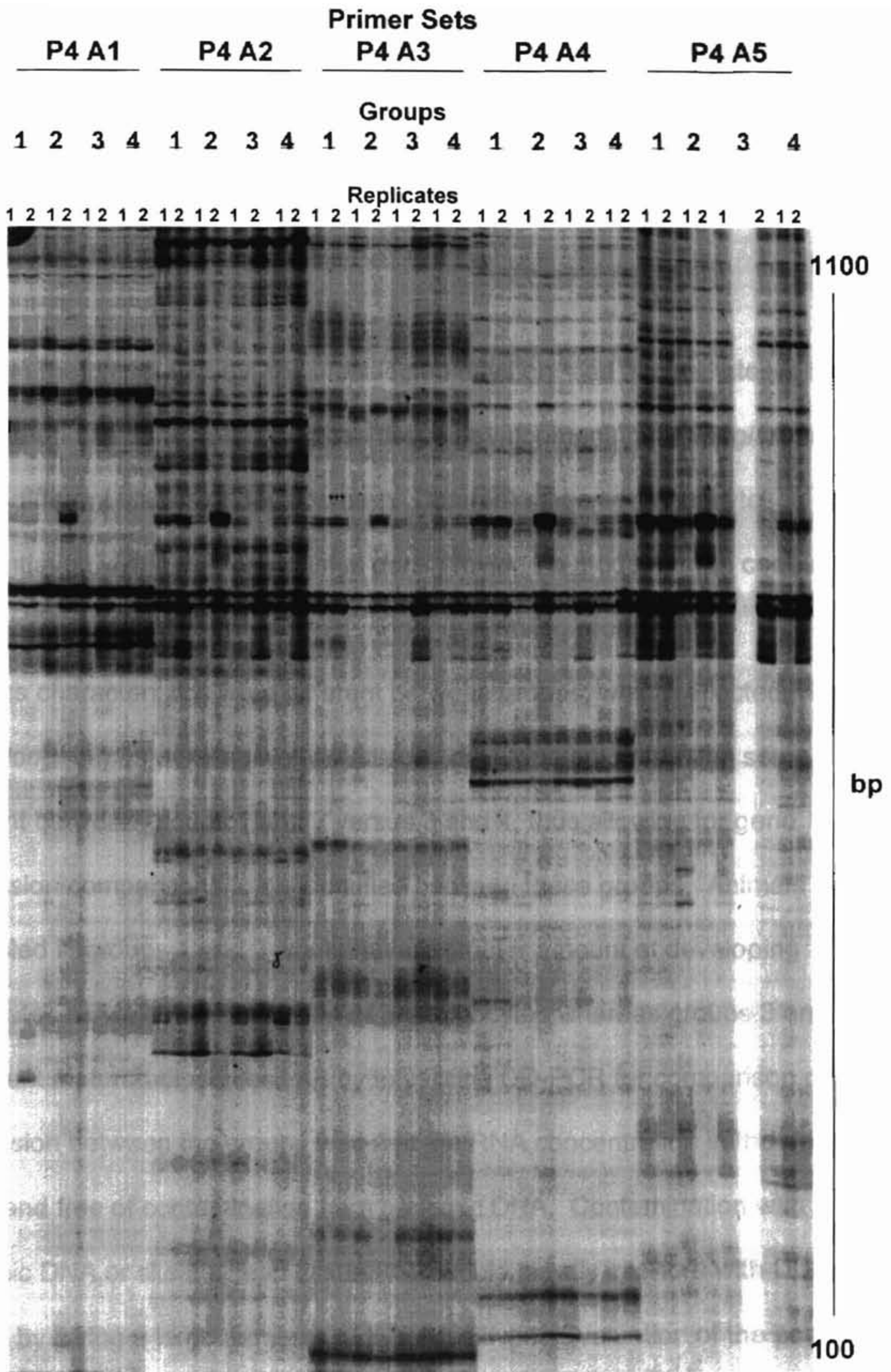
Figure 6. Representative of ethidium bromide stained agarose gel from a PCR using leptin receptor primers to express cDNA from (A) adipose and (L) lean. Note that the expected 398bp PCR product was only present in lean cDNA. (-) indicates negative control (no cDNA added), (+) indicates positive control containing skeletal lean cDNA and M represents marker.



DD-PCR. Fluorograph of a representative DD-PCR gel is presented in Figure 7. Upon visual evaluation, 300 bands from 40 DD-PCR gels scored 1 to 3 for differential gene expression. Of the 300 bands excised, 70 exhibiting the greatest amount of difference (score 3) between groups 1 and 2 versus 3 and 4 were reamplified and sequenced. Thirty-nine of the bands were either contaminated with a second band or contain two separate genes that did not allow clean sequencing (Table 6). These genes will need to be cloned to separate genes for sequencing and identification of bands. Sequence was obtained from 31 of the remaining excised bands, which were submitted to GenBank of the National Center for Biotechnology to match homology with known genes. The DD-PCR for the 31sequences is presented in Appendix 1. Ten sequences did not have significant homology to entries presently available in GenBank (Table 6). Homology to known genes was detected for 21 of the excised bands (Table 6 and 7). To further characterize and confirm DD-PCR results, NAT1 and myopodin were selected for Northern Blot analysis because of their high potential of playing a role in differentiation of adipose tissue. A number of homology matches were metabolic enzymes and structural proteins that are consistent with the cellular activity of developing adipose.

Northern hybridization. RNA from adipose tissue was hybridized with labeled cDNA from NAT1 and myopodin. Myopodin cDNA hybridized with a gene product of 2.0 Kb on the Northern blot, which is very similar to the size of 18 S ribosomal protein (1.9 Kb) (Figure 8). Hybridization of RNA with Nat1 cDNA

Figure 7. Representative of a scanned image of a fluorescently tagged fluorograph of a DD-PCR gel. Five combinations of 5' (P) and 3' (A)primers are represented.


Primer Sets
P4 A1
P4 A2
P4 A3
P4 A4
P4 A5
Groups
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
Replicates
1100
bp
100

resulted in a hybridization product of the expected size of 3.7 Kb. Expression of NAT1 RNA was greater in low marbling compared to high marbling individual cattle (Figure 9).

Discussion

In the present study, the preselected days on feed allowed steers to reach targeted endpoints to obtain adipose tissue development in the *longissimus dorsi* during different stages of development. Different stages of adipocyte differentiation within the *longissimus dorsi* provided a model for the comparison of differences in gene expression during the marbling process.
Carcass characteristics were different between groups, which reflected maturity of the *longissimus dorsi* as well as adipose development. Marbling score was different between groups 1 and 2 versus 3 and 4, thus allowing for gene expression comparisons to be identified between these groups. Animals harvested in groups 1 and 2 would have a greater amount of developing adipocytes as well as a population of preadipocytes whereas groups 3 and 4 represent mature adipocytes. A key to utilizing DD-PCR for comparison of gene expression between the groups was that the RNA concentration in the pools was equal and free of contamination from genomic DNA. Contamination with genomic DNA or skeletal lean tissue RNA would greatly interfere with DD-PCR results by giving a large numbers of false positives. Evaluation of the pooled RNA samples with leptin primers that amplify genomic DNA, but not cDNA

Table 6. Sequence results from DD-PCR amplicons.

Band #	Primer Set	≈ Band Size	Boldest in Treatment Groups	GenBank Match	Sequence Obtained	GenBank Homology
1	2-1(2)	850	1,2,3		No	
2	2-2(8)	850	1,2	Insulin-Regulated SH3 Binding Protein	Yes	74/82 (90%)
3	2-2(10)	500	1,2	LIM protein SLIMMER	Yes	448/485 (92%)
4	2-2(11)	500	1,2		No	
5	2-2(13)	350	1,2		No	
6	2-2(14)	300	1,2		No	
7	2-2(17)	150	3,4	Ribosomal protein S3a	Yes	135/145 (93%)
8	3-2(9)	375	3,4		No	
9	3-2(10)	300	1,2	Heat shock cognate protein	Yes	46/47 (97%)
10	3-4(12)	450	1	Titin	Yes	226/249 (90%)
11	3-6(1)	400	3,4	Ribosomal protein L31	Yes	305/336 (90%)
12	3-7(7)	200	3,4		No	
13	3-8(12)	350	3,4		No	
14	4-1(1)	500	1,2	Mitochondrial genome	Yes	398/437 (91%)
15	4-1(3)	300	1,2		No	
16	4-1(5)	250	3,4		No	
17	4-2(7)	275	1,2		No	
18	4-3(12)	375	1,2		No	
19	4-4(15)	250	1,2		No	
20	4-4(16)	450	1	Nebulin	Yes	77/87 (88%)

21	4-5(18)	350	1,2		No	
22	4-6(1)	750	1,2		No	
23	4-6(3)	350	1,2	Mitochondrial genome	Yes	183/186 (98%)
24	4-7(5)	750	1		No	
25	4-8(8)	750	1,2		No	
26	4-9(9)	775	1	6-phosphofructo-2-kinase	Yes	489/498 (98%)
27	4-10(12)	500	1		No	
28	4-11(13)	650	1		No	
29	4-11(14)	300	1,2		No	
30	4-12(15)	550	1,2		No	
31	4-13(16)	650	1,2		No	
32	4-14(19)	675	1,2		No	
33	4-14(20)	300	1,2		No	
34	4-15(21)	600	1,2		No	
35	5-3(26)	575	1		No	
36	5-6(29)	700	3,4		No	
37	5-7(34)	775	1		No	
38	5-7(38)	350	2,3		No	
39	5-7(39)	175	2,3,4	NAT 1	Yes	107/109 (98%)
40	5-9(44)	650	1	6-phosphofructo-2-kinase	Yes	338/360 (93%)
41	5-10(47)	700	1	Myosin light chain 2, type I	Yes	313/349 (89%)
42	5-11(67)	200	1,2		No	
43	5-12(64)	500	3,4		No	
44	5-13(58)	725	1		No	

45	5-13(60)	250	3,4		No	
46	5-14(57)	150	2,4		No	
47	6-1(49)	500	3,4		No	
48	6-3(53)	175	3,4	Type III pro-collagen	Yes	96/119 (80%)
49	6-7(56)	500	3,4	ATP Citrate-lyase	Yes	90/103 (87%)
50	6-11(62)	250	3,4	Calcium channel alpha-1D subunit	Yes	119/127 (93%)
51	6-14(64)	625	1		No	
52	7-1(66)	475	1		No	
53	7-1(67)	350	1,2		No	
54	7-1(68)	250	3,4	Myopodin	Yes	138/150 (92%)
55	7-2(69)	500	4		No	
56	7-3(73)	325	3,4	ATP Citrate-lyase	Yes	87/95 (91%)
57	7-3(74)	325	3,4	ATP Citrate-lyase	Yes	89/95 (93%)
58	7-6(87)	250	1		No	
59	7-8(91)	400	1		No	
60	7-12(78)	400	1,2		No	
61	7-13(81)	300	1,2	Type III Pro-collagen	Yes	202/218 (92%)
62	7-13(82)	250	1		No	
63	7-14(83)	400	1		No	
64	8-1(96)	200	3,4		No	
65	8-2(100)	400	3,4		No	
66	8-2(101)	175	3,4		No	
67	9-1(114)	475	1,2,3		No	
68	9-1(115)	525	3,4		No	

69	9-7(119)	400	4		No	
70	9-15(131)	700	1,2	Mitochondrial genome	Yes	138/166 (83%)
71	10-13(141)	475	3,4		No	

Table 7. Amplicons that matched significant homology with GenBank entries.

Identity of Homology Match	Boldest in Treatment Groups
Insulin-regulated SH3 binding protein	1,2
LIM protein SLIMMER	1,2
Ribosomal protein S3a	3,4
Heat shock cognate protein	1,2
Titin	1
Ribosomal protein L31	3,4
Mitochondrial genome	1,2
Nebulin	1
Mitochondrial genome	1,2
6-phosphofructo-2-kinase	1
NAT1	2,3,4
6-phosphofructo-2-kinase	1
Myosin light chain 2, type I	1
Type III pro-collagen	3,4
ATP citrate-lyase	3,4
Calcium channel alpha-1D subunit	3,4
Myopodin	3,4
ATP citrate-lyase	3,4
ATP citrate-lyase	3,4
Type III procollagen	1,2
Mitochondrial genome	1,2

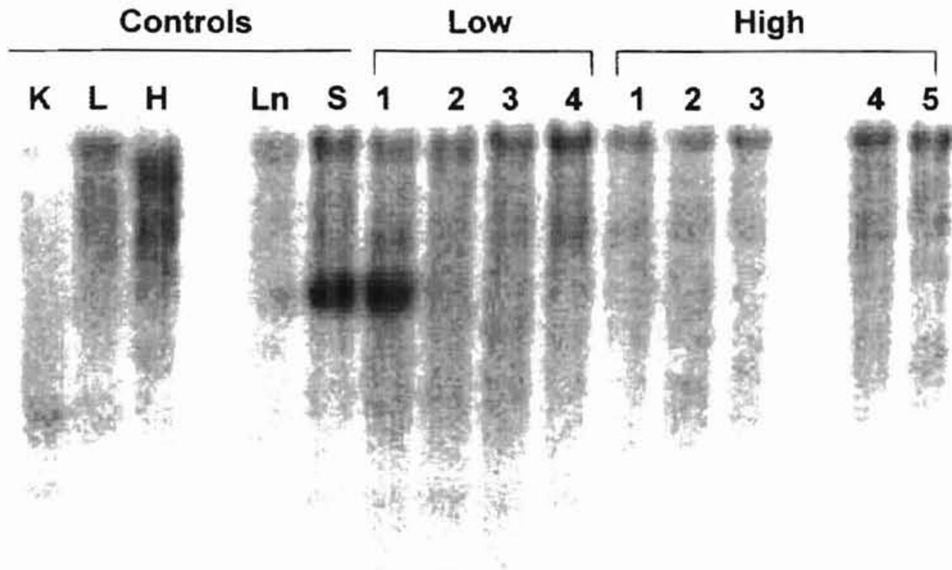


Figure 8. Image of Northern blot hybridization using cDNA labeled to myopodin as a probe to analyze RNA from kidney (K), liver (L), heart (H), skeletal lean (Ln) and subcutaneous adipose (S). Low represents adipose from low marbling steers utilized in the trial. High represents adipose from high marbling steers utilized in the trial.

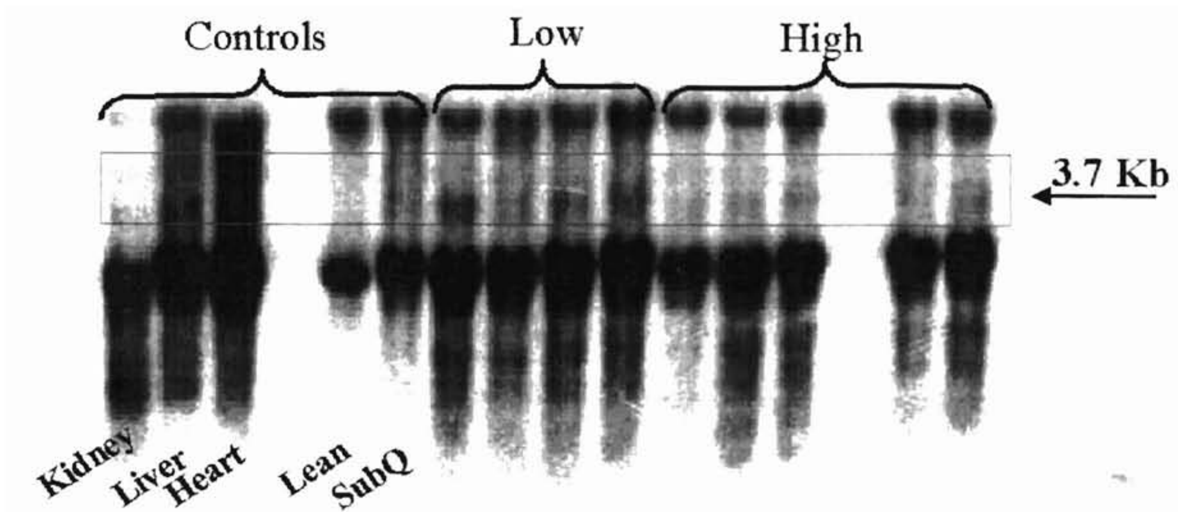


Figure 9. Northern blot hybridization utilizing cDNA to NAT1 as a probe to RNA from kidney, liver, heart, skeletal lean and subcutaneous adipose. Low represents adipose from low marbling steers on trial. High represents adipose from high marbling steers on trial.

constructed from RNA, demonstrated that the RNA samples used in this study had been successfully DNAse treated. Purity of the adipose tissue from the surrounding muscle fibers following dissection was confirmed by the absence of a PCR product when primers for leptin receptor were utilized. A clear 380 bp band was amplified in muscle but not adipose tissue. Therefore, the quantity and purity of the adipose RNA pools provided products in the DD-PCR that reflected changes in gene amplification during marbling. Although pools of RNA from the groups contained 5 animals/ 1 replicate, a freezer breakdown made RNA available for only one animal in group 1. Only having RNA from one animal in the group limited interpretation of the bands. However, gene expression in group 2 animals was similar to group 1 and allowed us to still select bands in comparison to groups 3 and 4. The bands provided sequence results from numerous genes that were upregulated or downregulated in the adipocytes during the course of the finishing period.

In our present study ATP-citrate lyase expression was observed to be greater in groups that are at the latter stages of adipocyte maturity (3 and 4). ATP-citrate lyase is the primary enzyme responsible for the production of cytosolic acetyl Coenzyme A (CoA) (Church, 1995). Acetyl CoA is an important intermediate in the metabolism of pyruvate and fatty acids such as acetate. Acetate is one of the major substrates for energy storage in beef cattle (Church, 1988). Increased expression of citrate lyase would be expected in adipose that is terminally differentiated and that is storing energy (Ailhaud et al., 1992). Other studies in mice (Bernlohr et al., 1985) have also indicated citrate lyase as a

marker in terminally differentiated adipocytes (Martin et al., 1999). Another amplicon from the present study that is consistent with current known adipocyte markers is type 3 pro-collagen. Ailhaud et al. (1992) states that collagen VI is a marker in mouse preadipocytes. In the present study pro-collagen type 3 amplicons were identified and may serve a similar role in cattle.

The amplicon product that was similar to novel apolipoproteinB editing catalytic sub-unit 1 target 1 (NAT1) is of particular interest because it is a translational repressor (Yamanaka et al., 1997). Messenger RNA of NAT1 is similar to the carboxyl-terminal portion of the translational initiation factor eukaryotic initiation factor4G (eIF4G) (Yamanaka et al., 1997). For ribosomes to translate mRNA into proteins, eukaryotic initiation factors are required to unwind mRNA and bind the ribosomal complex to the mRNA (Lewin, 1994). Once the ribosomal complex is attached to the mRNA proteins can then be synthesized. Some of the known initiation factors (Yamanaka et al., 1997) are eIF4A, eIF4E and eIF4G, which are referred to as eIF4F (Figure 10). Of particular interest to the present study is the relationship of eIF4G and eIF4A, because NAT1 competes with eIF4A for binding with eIF4G. If eIF4G does not bind with eIF4A the mRNA will not attach to the ribosome subunits, thus translation does not occur (Yamanaka et al., 1997). Studies utilizing 3T3 cells (derived from mouse preadipocytes and adipocytes) have demonstrated that genes like PPAR (peroxisome proliferator-activated receptor) (C/EBP) and CEBP (CAAT/enhancer binding protein) families are repressed during different stages of adipose development (Altiok et al., 1997; MacDougald et al., 1995). Greater gene

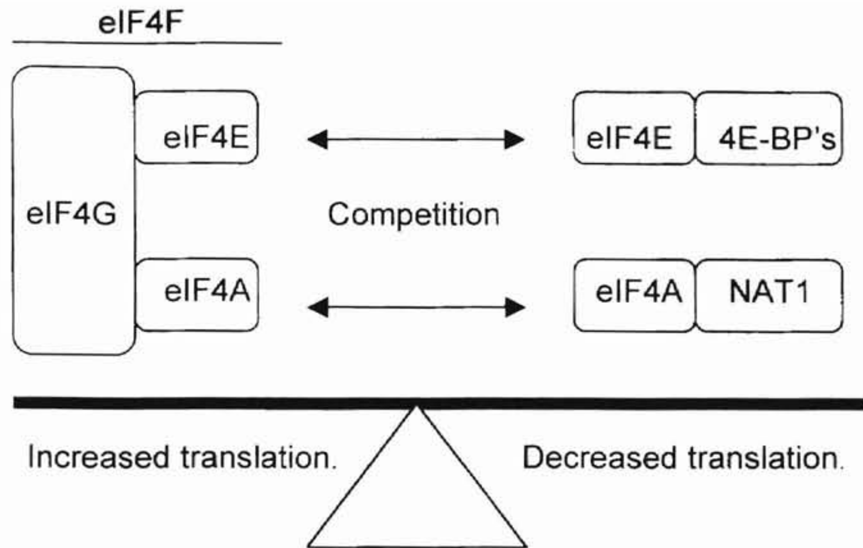


Figure 10. Illustration of normal translation and the repressors that can inhibit translation. Eukaryote Initiation Factor4F (eIF4F) is a complex consists of eIF4G, eIF4E and eIF4A for normal translation of proteins to occur. Cap-dependant translation can be repressed by 4E-BP's by binding eIF4E. NAT1 can repress both cap-dependant and cap-independent translation by competing for eIF4A. Adapted from Yamanaka et al., (1997).

expression in leaner animals suggests that NAT1 may function to repress specific genes involved with differentiation during the early part of the finishing period. Northern hybridization confirmed that during the latter stages of the finishing period, NAT1 gene expression is depressed which may release adipocytes into active lipogenesis. Further research is needed to provide a clear understanding of the role that NAT 1 might play in developing adipocytes and the genes it may be repressing.

Even though no known markers of adipocyte differentiation were identified with the first 31 genes that sequence was obtained in the present study, several amplicons did follow patterns that would be expected of a developing adipocyte. A number of enzymes involved with lipogenesis were identified through DD-PCR. ATP citrate lyase was observed with DD-PCR to be greater in expression in more finished cattle versus the younger, leaner animals (Martin et al., 1999, Bernlohr et al., 1985). Another amplicon that follows a previous report is the enzyme 6-phosphofructo-2-kinase, an enzyme involved in glycolysis. Glycolysis metabolism can shift in response to an increase or decrease in substrates such as glucose or ketone bodies (Vidal et al., 1993). Cattle that are coming from a forage-based diet usually weigh 157-276 kg when they are placed on a high concentrate grain based diet. Cattle that are 157-276 kg would be expected to have smaller organs (i.e. rumen and liver) than cattle that weigh over 394 kg and have been on feed for an extended period of time (Church, 1988). Therefore, during cattle's adaptive phase from a forage diet to a grain diet the limited number of adipocytes must store the incoming increase of energy in the small

number of adipocytes. As days on feed increase as well as the number of adipocytes in the animal the burden of storing energy is spread across a greater number of adipocytes. Gluconeogenesis in ruminants is greatest shortly after a meal when gluconeogenic substrates are most available (Church 1988). So enzymes that are involved in glycolysis such as 6-phosphofructo-2-kinase should increase in expression as seen in group 1 which was only fed for 20 d, due to the limited amount and size of the adipocytes reflected from a forage based diet and due to their young age (Martin et al., 1999). However, after the animals have adapted to a high-energy grain diet glycolysis enzyme expression should plateau due to an increased number and size of the adipocytes available to execute glycolysis.

The amplicon identified as myopodin is an interesting discovery since myopodin is associated with the podocyte family previously described only for the kidney (Martini, 1995; Barsoni et al., 1999). However, myopodin has been characterized as a structural protein in the Z-disc of skeletal and heart sarcomeres in developing embryos of mice (Barsoni et al., 1999). Myopodin, as observed in DD-PCR, had greater expression in the animals with advanced degrees of marbling compared to earlier stages of marbling development. Altiok et al. (1992) stated that adipocytes undergo structural changes during transformation from a preadipocyte to an adipocyte. However, Northern blotting analysis of myopodin showed that there was greater gene expression in the animals that were less mature in their marbling development, which is contrary to DD-PCR results. Individual animal differences in stages of adipose maturity may

have affected DD-PCR results that used pools of adipocytes, whereas during Northern blotting individual animals were analyzed.

The results of our study suggest that gene expression can be traced as animals finish. More markers of adipocyte differentiation could play a significant role in increasing the understanding of the biology of adipocyte development. Understanding the biology of marbling could lead to a better understanding of tenderness in beef and better management practices of cattle during the feedlot stage.

APPENDIX

Appendix. (A) Gene sequence of extracted, reamplified band from DD-PCR and (B) DD-PCR amplification of band across groups. Each group contains two replicates (or lanes) of 5 steers each except for group 1 which only contains one animal in each lane. Table 7 contains information for each band.

A. Band 2

```
CTTTANCGCTAGCATGGCATCATGCGTGGACCTCCTCAGTGGGACAGGAAA
GTGGGAGAAAGCATGGCTATAGTANCACTAGGANAANCAGCTTCACACATT
CTTCAAGGANTAATGGTGGATCAAGAGGATGTTCTGGGNGGNATAATGGTG
GTGCNGGAGGTCCCTCACCTTGGTTTCACAACCATAGTAATTCTGGNGGTG
GTTGGCNTTCAAACAGNGGGACNGNTGATTGGAATCATAATGGTNCAGGAA
GGANTTCCAGTTGGCATTCTNNAGGAGCNGGTGGNTTTTNCAGNTGGCNTA
TGAACAGCTGTANCGGAAATTGGANATCCAGTGCACGTGGTACANATAGNT
GGAATTACAGTGGCCCCNGAGACAAATTTCANCCGGGGCNGAAACAGANAT
TCTATCTGNNAAATGGAAGACATGANTATGCTNTTGAATACNAANTCTANGT
TAAACATATCCANTCAAGACAGGTTTAAGNGGCTGCGNGCACCCNAAANTG
ACAAANTTGGNGCCTGTTGCCNCATACATAGGTCCTTCTGAAGGANTNGCA
ANTGGNTAGNTACATTCNTGACGCTTNGTTGACNTCANCN
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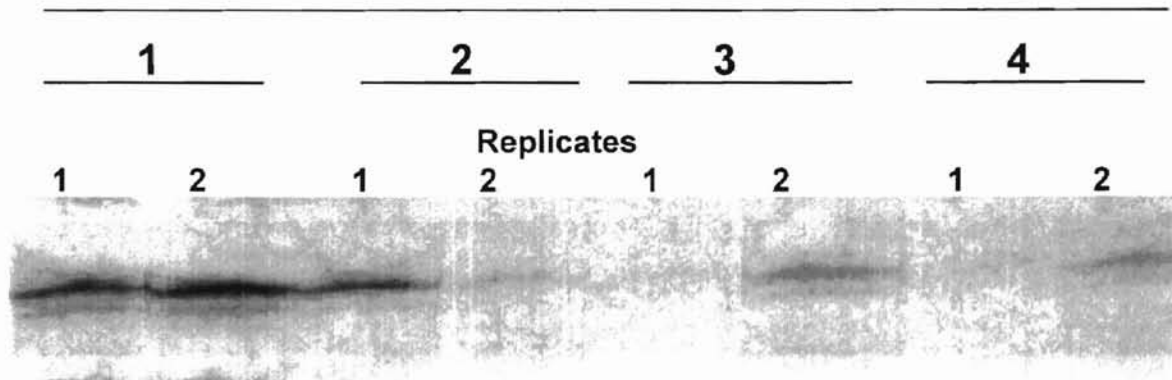
B.



A. Band3

```
TTNNNTCTNAACCTTNNAACGACTCCAAGGAGGTGCACTACAAGNAACCGC
TACTGGCACGACACCTGCTTCCGCTGCTCTAAGTGCCTCCAGCCCTTGGCC
AGTGAGACCTTCGTGGCCAAGGACAACAAGATCCTGTGCAACAAGTGCACC
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AGGAGATCAGAACGTGGAATACAAGGGCACTGTCTGGCACAAAGACTGCTT
CACCTGCAGCAACTGCAAGCAAGTCATCGGGACAGGAAGCTTCTTCCCTAA
AGGGGAGGACTTCTACTGCGTGACTTGCCATGAGGCCAAGTTTGCCAAGCA
CTGCGTGAAATGCAACAAGGCCATCACATCTNGGAGGAATCACTTACCAGG
GATCAGCCCTGGCATGCCGAGTGCTTTGTGGNGTGTTACCTGCTCTAAGAA
GCTGGCTGGGCAGCGTTTACCCNCTGNGGAGGACCAGNATTACTGCGNGG
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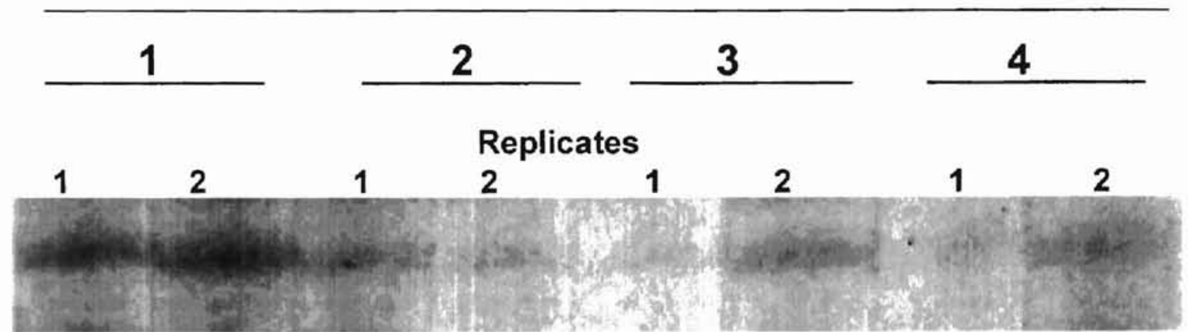
B.



A. Band 7

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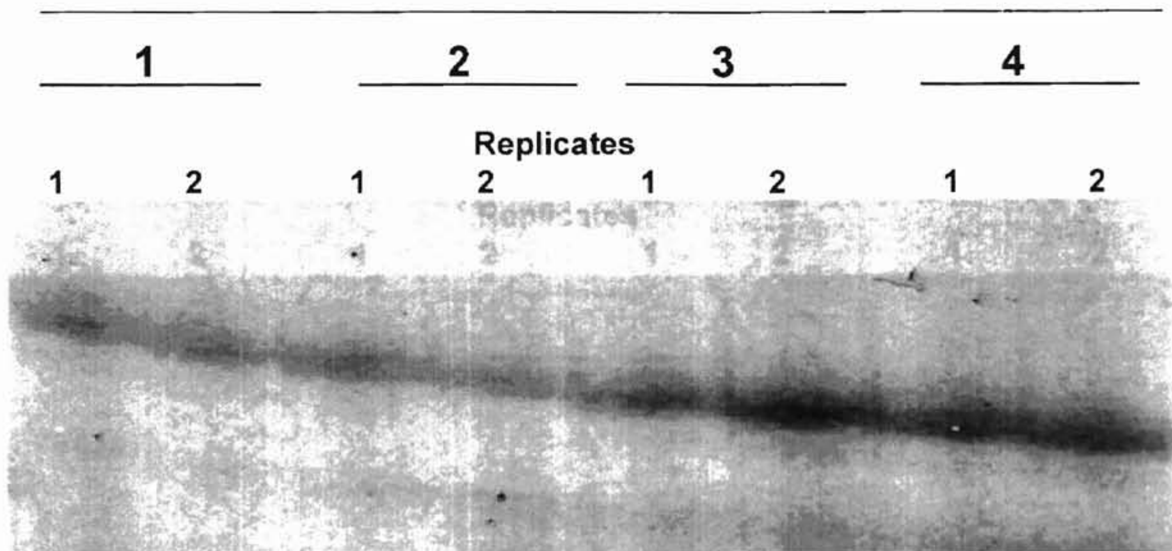

A. Band 9

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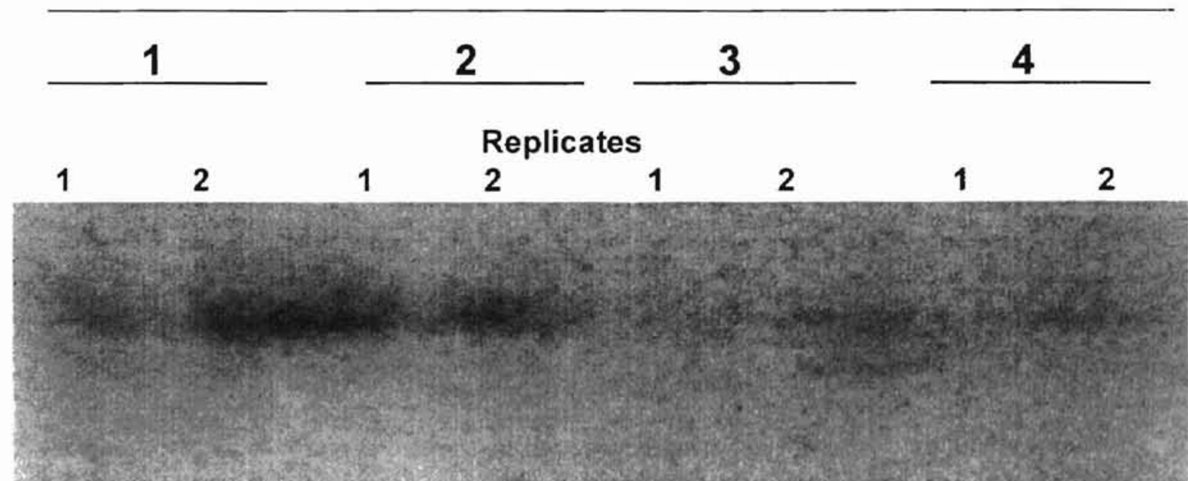
B. **Groups**



A. Band 10

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AAGTAAAAGCCAGAACCAGAGAAGTAATTACGACAAAACAAGAGCAGGTGC
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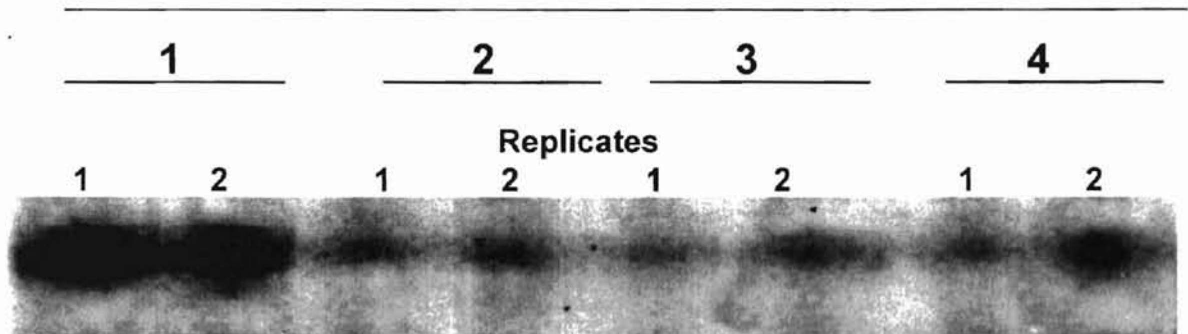
B.



A. Band 11

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GCGCATCGACACCAGGCTCAACAAAGCCGTCTGGGCCAAAGGGATCAGGA
NTGTCCCATACCGGATNCGTGTGNGGTTGTCCAGAAANCGTANTGAAGACG
AAGACTTGCCAAACAAGCTCTATACTTTGGCTACCTACGNGCCCGTCACCA
CCTTCAAAAATCTACAGACNGTCANTGCGGATGAGAACTAATTGCTGNTGGT
CCAATNAAGCTATTGAACNGCCAAAAAAAAAACG
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B.

A. Band 14

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TTATTGTTNTNGNACTTNCTAGGACANCCNCGATTGCCTACCNTAGTTAATA
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AGCCTGTTNGCNGGATACATCATTTTCAACANTATTCCTCCAACAACANTTC
CGCAAANAACTATGCCCTACTACCTAAAAACAACAGGCCTAATTGTNACAAT
CCTAGGCTTNATNTTAGCCCTAGAAATCAGTAATATAACTAAAAATNTAAAAT
ATACTACCCCTCANACGCCTTCAAGTTCTCAACCTTGCTAGGGNATTTCCCC
ACAATTATACATCGCCTAGCTNCATCATAAATTTATCAATAAGCCAAANATCA
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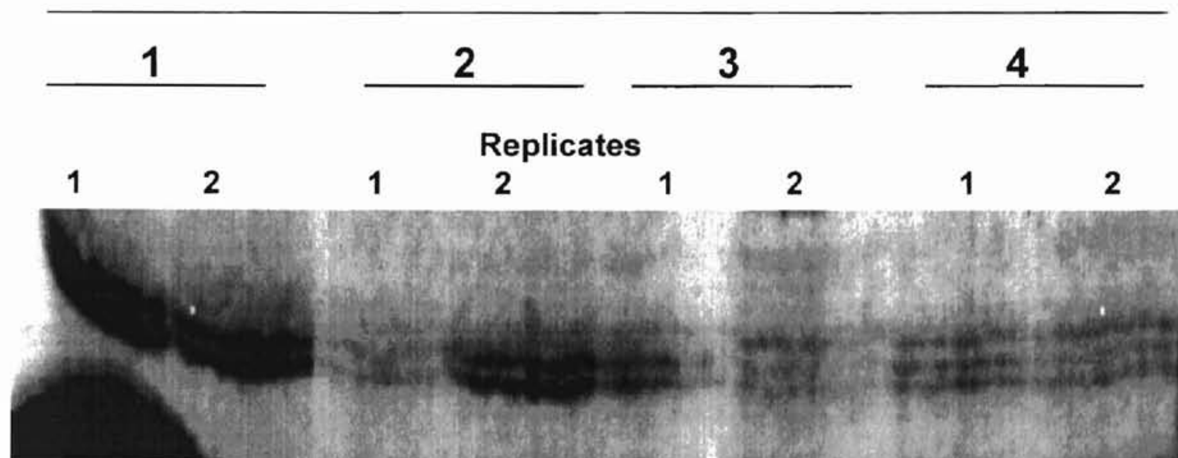
B. **Groups**



A. Band 20

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CCCCCNCCCGCTCCCNCACNNCNCCCCTCCCNCCCNNCTNCNTTCCCTCTT
CCCNTNNTTNNCCNTCNCCCTCCCCCCTCCNTCNNCCCNCTTCCTNTACCT
CCCCCCTCCCCCCCCCNCCNTCCACCTCTCTCNCCCTCTCTCCCNNNCTTN
CTTCCCNTCCCACCCTCCCNCTCNNTTCTCTTTNTTTACTCCCTCTCCCTCTT
CCCACCNTCTACATNCCACTNCTCCTTTCNTCCCCCCNCNNTCCCTTTCCTA
NCCTCCCNCCTCCTTCCCTTCCNCTCATTCCCNCGNTTCTCCCTCCNCCNC
CTTTNCTCTTCNCACCCTCNNTCNCCCNCCCTCCCCNNCNTNTCNCCNCCC
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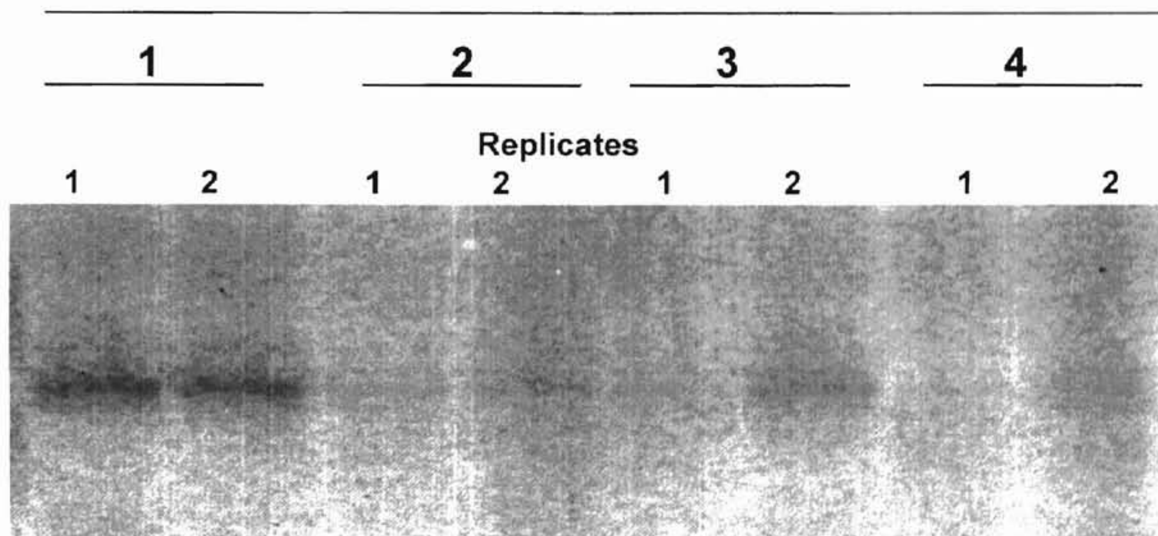

A. Band 23

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CTAAAATGAAGACAGAAAAAAAAAAAACANAAA
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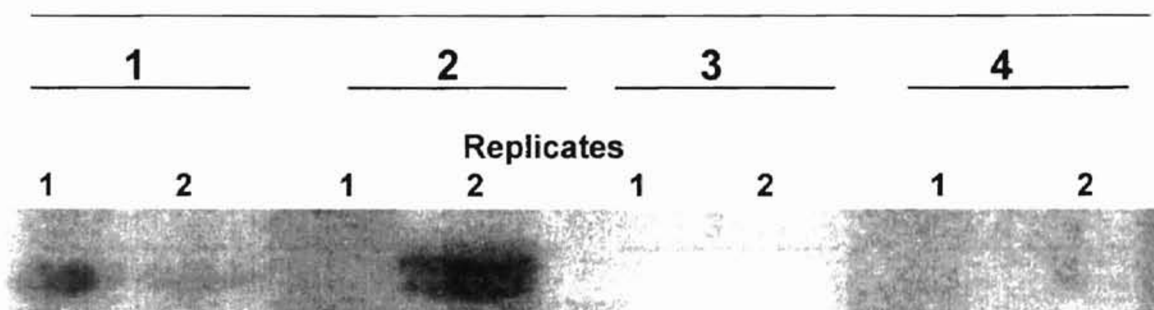
B.

Groups



A. Band 26

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GGCCTCTAGCAATAATATGATTTATCTCAACACTAGCAGAAACAAACCGAGC
TCCATTTGATTTAACTGAAGGAGAATCAGAGCTAGTCTCGGGCTTCAACGTA
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CACACATCCAGAACTCTACACAATCAATTTTACCATTAAATCCCTACTGTCAC
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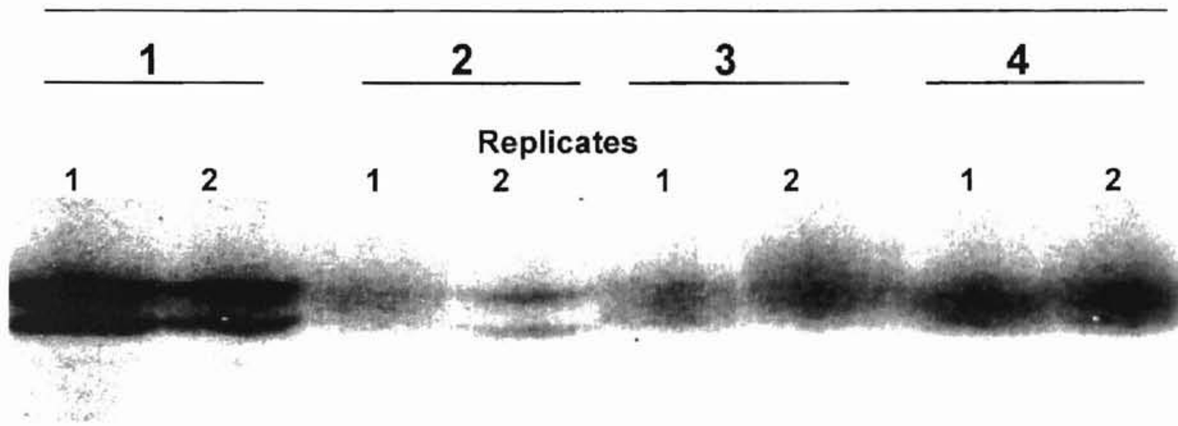

A. Band 39

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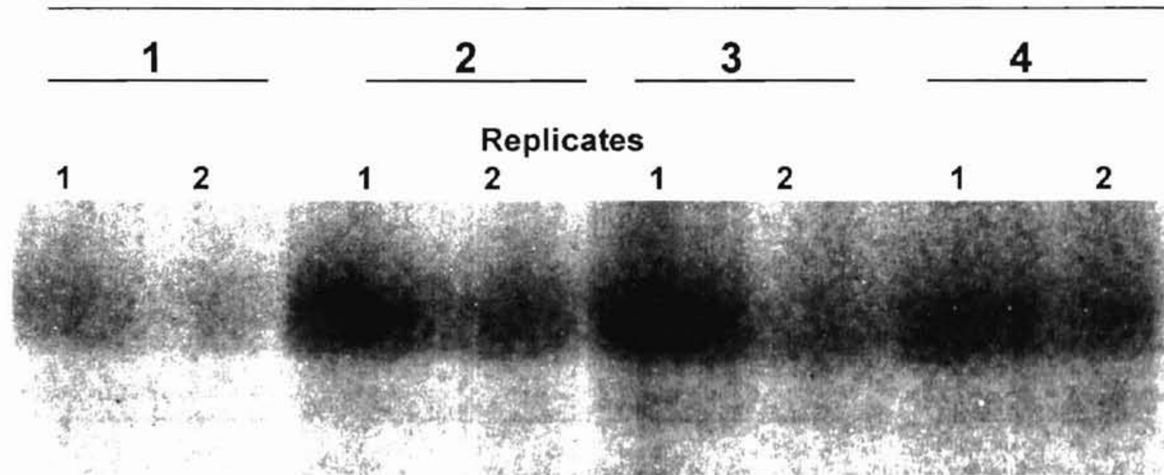
TTAATTTATCAAGTCATGTTCATGCCCTGATTTTATATACTTGTATCTATCAAT
AAACATTGTGATACTTGAAAAAAAAAAANGCCNTNTNGGGGGTNGTTTTNCA
CC

B.

Groups



A. Band 40

CANNCTTTTTNGNNTTGGTTNAGCCTAGCCGTATACTCCATTCTATGACAGG
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CCTAATAAGTGGGTCCTTTACCCTCTCCACATTAATTACTACACAAGAACAAA
TATGGTTAATCCTNCCAGCATGGCCTCTAGCAATAATATGATTTATCTNAACA
CTAGCAGNAACAAACCGAGCTCCATTNGATTTAACTGAAGGAGAATTAGNG
CTAGTGTTGGGCTTCATCGTAGAATCTGCANCAGGACCATTCGCCCTCTTCT
TCATAGCNGCGTACGCNNCTATTNTNCANCATNNANATCNTTNCAGCAACNT
ATTTCNTNGGGNCATCCCCCATTNNCCACACATACCNNNNANCTCTCCCCC
ATCCAACTTCTNNCCATGTAAANTCNCTNCNGNGTANACACACATNCCTCCC
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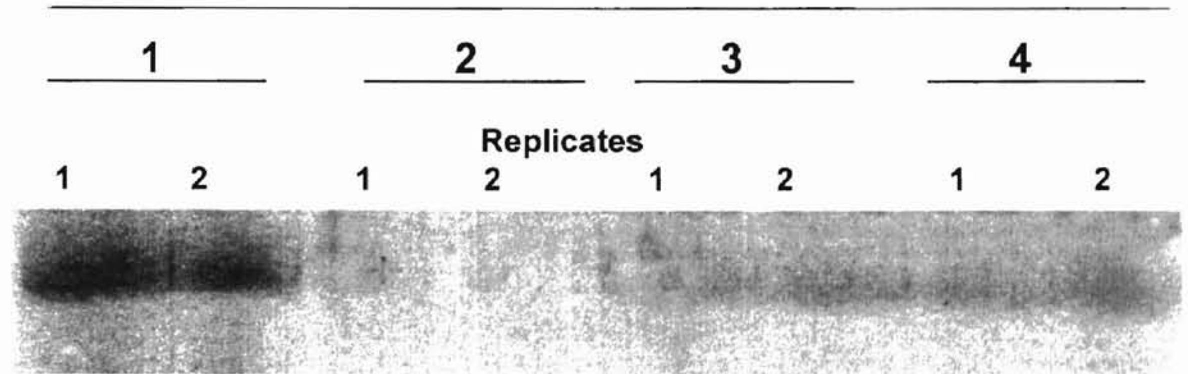
B.

Groups



A. Band 41

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AGAGGGCGGAAGCTCCAGTGTCTTCTCCATGTTTGATCAGACCCAAATCCA
GGAGTTCAAGGAGGCCTTCACAGTAATTGACCAGAACCGGGATGGCATTAT
TGACAAGGAAGACCTGCGGGACACTTTTGCAGCCATGGGGCGTCTCAATGT
GAAGAATGAGGAGCTAGACGCCATGATGAAGGAAGCCAGTGGGCCCATCA
ACTTCACTGTCTTCCTGAACATGTTTGGGGAGAAGCTCAAAGGTGCCCGAC
CCTGAGGATGTGATCACTGGAGCCTTCAAGGTCTTAGACCCTTGAGGGAAA
AGGCACCATCAAGAAAAAANTTCTGGNGGANCTGCTNACCACTCACTGNGA
CCGTTTNTCCACGCAAGNAGATCNACNANCATGTNGGCCTGCCTTCCCCCC
CCCCCCCNGTCCGGCAACNCANNCTNCNACAACATTTNCTTCCNNATTNCC
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CNNTNCC
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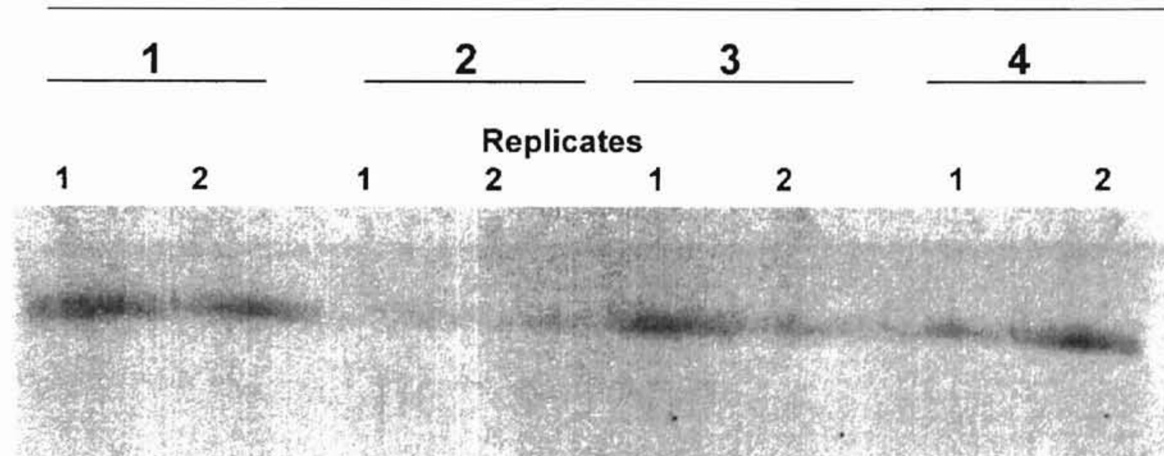
B.

Groups



A. Band 48

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ACACAGAGAATGTGNTGAAATTAAACTCTGNAAGCTTGAAAAAAAAAAAAAGC
CNTNTTNNGNGTNNTTTTCCCCCCNCCCCCCCCCCCCTCNNNTCNNCNCNCC
CNCNCNCNCNCNACCNCCNNCCCNCTCCCCCNCCNTCNCNCCCTNCCTCC
CCANNTNNTTNNNNNCCTNCNCNCCNCCCNNCNCCCNCCTTCNCTCCTCNT
NNCCCTNNCNTCNCCCNNNCNTNCNNCNCCTCNTNCCNATNCCNCTNNCTN
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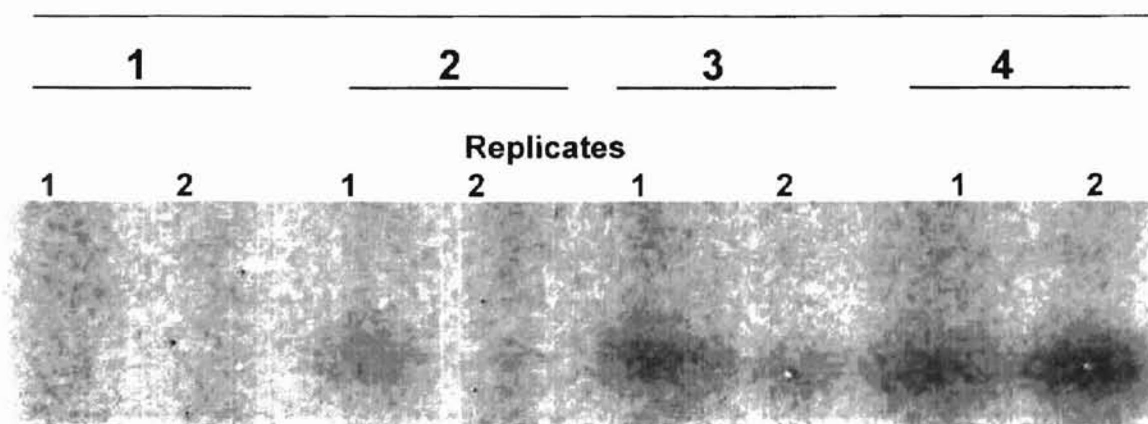
B.



A. Band 49

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TTATGCACCTGACTTTTGTCTGNTATTGACTCTTCTCCAACCAGTTTGCATCT
GTAATCCATAANGATTCCAGGCAGCTGCCACCTCNGTCTTCCCTCTGTATTA
TCATAGCNTGNTNTAAATNAACTATNTAGCCNCNCCNCAAACNCANCANCCC
CNTCTCNCCNCTTCCCNTCTCACCCCNCCCCTCCCCTCNCACACTCTCCCC
ACCCCNCCCCTCTCNCCCCCCCCCNCTNCTCCCCCCCTCCCCTCACTCNNN
CTTCCCCCTTTCCCCCCCTCCCCCACCTCCCCCCCCCNCCNCCCTCNCNTA
NCCTCCACCTCTCCCCTCACCTCCTCCTTCCTCNCTNNTCCTCTCATCCCCN
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CCTCCTCTCCCCNCNNCCCNCCCCG
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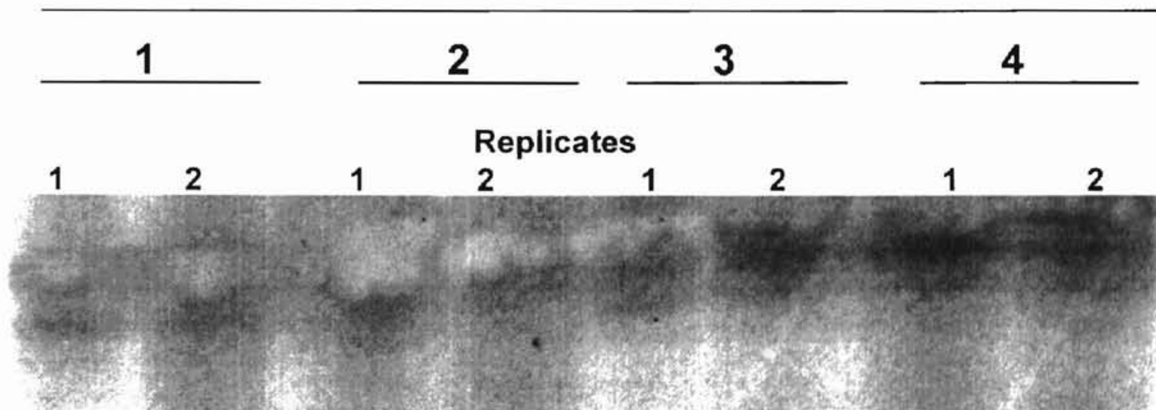
B.

Groups



A. Band 50

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CCCCCNNNNNNGGGTTNTCTACATAGCGTCTNAAATACTACATCATCCTCTT
CATTTGCGGTAACTATATTCTACTGAATGTCTTCTTGGCCATCGCTGTAGAC
AATTTAGCTGATGCTGAAAGTTTGAATACTGCTCAGAAGGAGGAAGCTGAA
GAAAAGGAAAAAAAAAAAANGCCNTATNGNGNGTNGTNTTACANNNNCNCCN
NNCNCNNNCNNNNNNCNCNCNCCNNCCCCCCNCCCNNNNNNNCNNCCNCN
CNCCNCNCNNNNNNNCNNNCCNCCCCNNNNNNNNCCNCCNNCCNNNCCNC
NCCCNCNCNCNCCNNNNNNCNCCCNCNNNCNCANNNCNNNNCCCNCNNNC
NNNCCCCCNCNNNNCCNCCCCCNCNNCNCCCCNCCCCNNCCNNCNNCCNC
NNCTNCCCNCNNNNNCCCCCCNNCNCCNCNNCCNNNCNCNCNNNNCCNCC
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CCANCCCCCN
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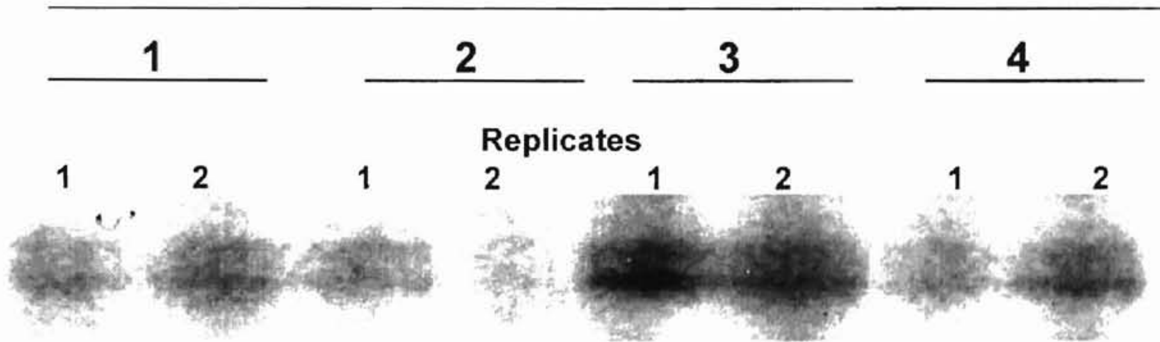
B.



A. Band 54

CNNNCNCNACCCGACTCCAAGGAAAACCATCTTCTCAATAGAAGAGATATG
TCTAGAGACTTCTCATAAAAGCTTGCCTGGTGTGAGGGTACATGGTACCATG
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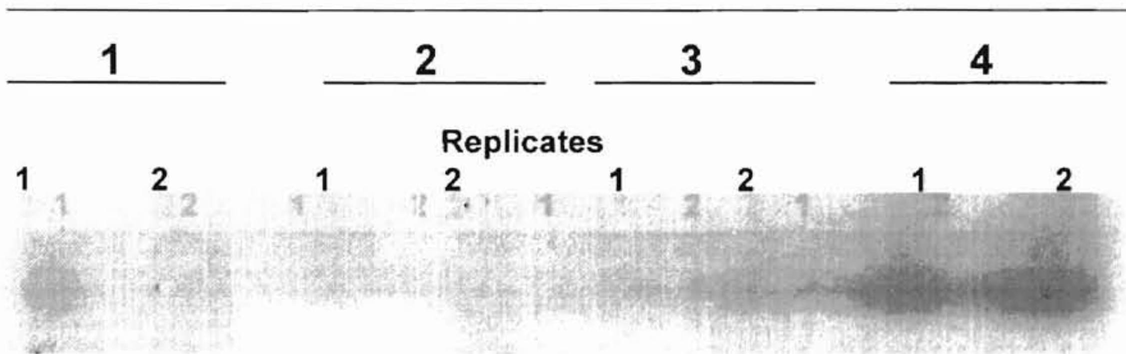
NCCCTATAGNGNGTCGTNTTACA

B.



A. Band 56

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AAAGCAAAAAGCTGTGAAATGTCTTGTGTCTTATGTTCTCTGTATTTATGCAC
CTGACTTTTGTCTGTTATTGACTCTTCTCCAACCAGTTTGCATCTGTAATCCA
TAAAGATTCCAGGCAGCTGCCACCTCANTCTTTCCTCTGTATTATCATAGTTT
GGNTTAAATAAACTATATAGTAACACCNAAAAAAAAAAAANCCCTATAGNGA
GTCGTATTACAN
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B.

A. Band 57

CNNNNNACCNTGCANCCTTAGCCNTGTTACAGAGTANGGGAAGTGGTAATT
TAAAATGTAAACTTGTAACATACATATGTTTAAAGTGGAAGCGCAAAGCAAA
AAGCTGTGAAATGTCTTGTGTCTTATGTTCTCTGTATTTATGCACCTGACTTT
TGTCTGTTATTGACTCTTCTCCAACCAGTTTGCATCTGTAATCCATAAAGATT
CCAGGCAGCTGCCACCTCAGTCTTTCCTCTGTATTATCATAGTTTGGTTTAA
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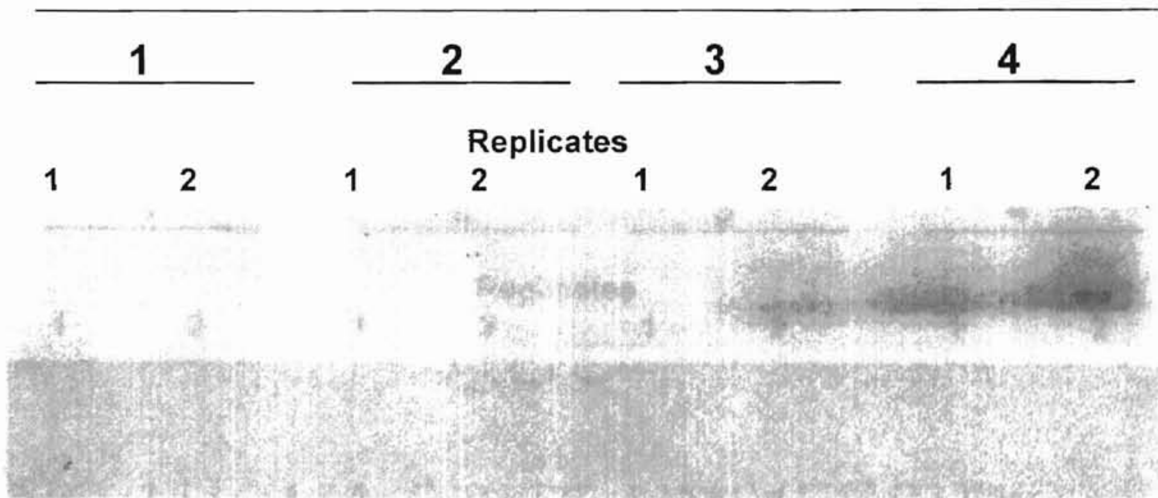
B. **Groups**



A. Band 61

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ACTGCAGGAGACACTTTCCCTGCATCCTGTGAAAGTCAACAACAACAAAAAA
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AACTCTGTAAGCTTGTATGNGGTTGCTGATCTTTTTTTCTGACAGACACCCA
TAANANAAGANCATNATAANACGNANAAAAANANAGCCCTCTCNTNNGNCC
CTTCCCCCCCTCCACCCCCCCTNTANCCCTTCACCCCCNCCCCCNCCCCTCC
CCCTTCCCCTCACCCTCTTCCTCCNCCCCCCNCCCCTCCNNNNNCNCCCNC
TCCCCTCCCNCCTCCNTNCCTNCACCCCTCTCCTNCNCCCCCCTCTNCTCC
CCTCTCCNCTCCNTCNCCCCCCCCCTTNTCCNCTCCTCCTCCCCCNACCCC
CCCNTACCCCACCCCCCCCCCCTTCCTCANCCCCTCCCCNTCTCCTTCTCC
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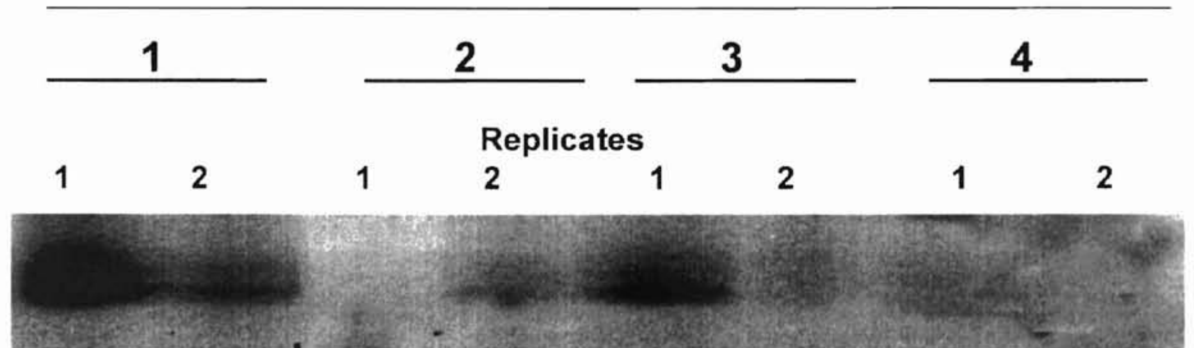
B.

Groups



A. Band 70

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GNAGAGTGNNTNCATNTGCTTGGATTNTANNTACTGCAAACTCTNAAATGGT
TANTTNGATTANAATGGTGAATGTAATTAGAGCTGTTGTCCTGCTAATGCTTA
TTAGNGCAAGTGTANCTNCTCCNNTTAGGNGAATNAANAGGNGTCCTGCAC
NGATNTTNNCTGTTAACCNNNCGGNNAGGGNTATNGNTTGANTAAAANGNC
TCTNGCNTCCAANANTTACTNGTATTGGGATTAGTGNANNGGNNNTTCCTTN
NNNTAANANANGGNCAAATNATNCTNNCTTTTNATNNCNNNNNCNTNTTNTT
ACCGCTCCTNCTAACAGGGGGATNGATANCCNTAANCTTATTGGAAGCCCT
GTGGNNNGNTTTTAATAAANGGGGCTNAAGACCTGTTGGAATGAAAACCCC
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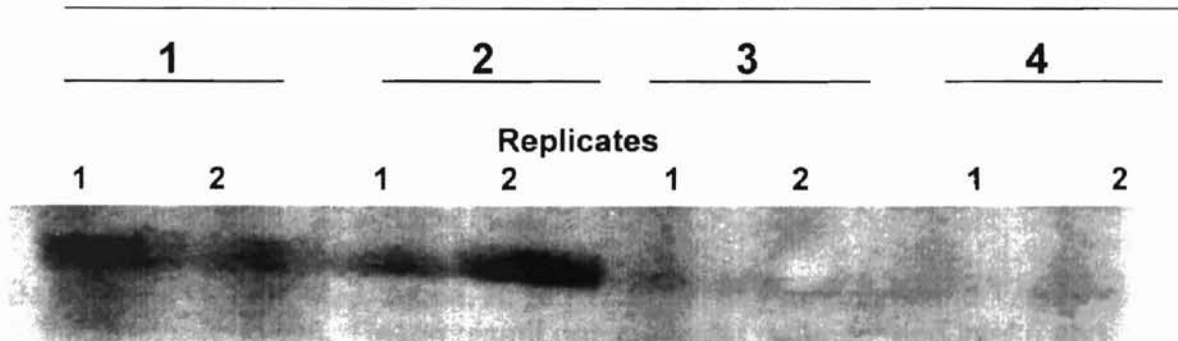
B.



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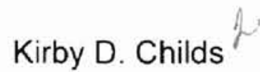

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