IDENTIFICATION OF POLYMORPHISMS AT THE BOVINE HORMONE-SENSITIVE LIPASE LOCUS

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfilment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Animal Science

2001

ABSTRACT

IDENTIFICATION OF POLYMORPHISMS AT THE BOVINE HORMONE-SENSITIVE LIPASE LOCUS

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Genomic DNA was isolated from four beef breeds (Angus, Brahman, Simmental, and Tarantaise), one dairy breed (Holstein), and one dual-purpose breed (Shorthorn). One forward primer and one reverse primer was designed to amplify part of exon 8 (based on the human sequence) of the hormone-sensitive lipase (HSL) gene, downstream of the phosphorylation activation site. The amplified fragments were sequenced and polymorphisms were found at base 51 (A/G) and base 93 (A/G) from the start of the sequenced fragment. Changes in the two bases result in a change in amino acids from isoleucine to valine (base 51) and from glutamic acid to lysine (base 93). These changes may change the 3D-structure of the protein and, therefore, affect enzymatic activity. The polymorphisms were found in all breeds. This study suggests that there may be an association between polymorphisms at those sites and phenotypic traits, such as rate of lipid hydrolysis.

To my wife
for her love and support

ACKNOWLEDGMENTS

I am grateful to the individuals who contributed to the completion of this research. First of all I would like to thank my major professor, Dr. Robert M. Cook, for his patience, support and understanding.

To the members of my guidance committee: Drs. Loran Bieber who shared with us his lab and knowledge, Mel Yokoyama,, John Kaneene, and Karen chou, I am grateful for their contributions to my research project as well as my graduate education as a whole.

I would especially like to thank Dr. Cathy Ernest for her technical guidance, and for providing me with space and equipment in her laboratory.

Also I would like to thank Dr. James Jay for financial support through most of my graduate program. To my colleagues Melvin Pagan and Nancy Raney, and to Dr. Jeane Burton, I appreciate their help and support.

My deepest appreciation is expressed to Dr. Ahmed M. Rafaat who taught me basics in molecular biology. Dr. Bill Thomas for giving me the opportunity to learn molecular biology and interact with many scientists by providing a fellowship to attend the Molecular Biology and PCR summer

workshop in Northampton (Thomas Enrichment Fellowship). My thanks to Dr. Peter Saama for his assistance with the cluster analysis and Jim Liesman for his useful comments on this dissertation.

I express sincere gratitude to my family and friends in Lansing and at home in Sudan.

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LIST OF ABBREVIATION

DNA:	Deoxyribonucleic acid
EDTA:	Ethylenediaminetetraacetic acid
ETBr:	Ethedium bromide
PCR:	Polymerase chain reaction
RFLP:	Restriction fragment length polymorphism
SDS:	Sodium dodecyl sulfate
SSC:	Sodium chloride/Sodium citrate
SSCP:	Single-stranded conformation polymorphism
SSPE:	Sodium chloride/sodiumhydroxyphosphate/EDTA
TBE:	Tris-borate/EDTA
TE:	Tris-EDTA
TEMED:	Tetramethylethyl-enediamine

INTRODUCTION

Lavoisier was the first to suggest that "life is a chemical process" (Maynard et al., 1979). We now know that sustenance involves a series of chemical reactions and physiological processes in which food is transformed into body tissues and activities. In a broad sense the chemistry of life can be considered a cycle. Food is consumed, and following digestion in the gut, nutrients are absorbed. These nutrients are utilized by body tissues and in turn through several mechanisms, play a role in regulating food intake (Baile, 1971), thus completing the cycle. In mammals, nutrients are utilized by tissues for maintenance and growth and for establishing body reserves including energy stores (lipids), glucose reserves (glycogen), and amino acid reserves (labile protein).

Short-term fluctuations in energy balance are to some extent buffered by glycogen stores. However, the capacity to store glycogen is limited. Longer-term imbalances between energy intake and expenditure are translated into changes in the body's store of lipids, mainly in the form of intracellular triacylglycerol (TG) in adipose tissue. Adipose tissue lipolysis is the major regulator of the supply of lipid energy because it controls the release of fatty acids into the plasma.

The rate limiting step in adipose tissue lipolysis is the hydrolysis of TG by hormone-sensitive lipase (HSL). This enzyme is so-named because it is susceptible to regulation by phosphorylation and dephosphorylation in response to hormonally controlled cAMP levels (Vaughan et al., 1964). Phosphorylation activates HSL, thereby stimulating lipolysis in adipose tissue, raising blood fatty acid levels, and ultimately activating the 2-oxidation pathway in other tissues such as muscle and may also lead to the accumulation of fatty acids in liver that cause fatty liver. Two additional tissues utilizing a substantial portion of maternal nutrients are the developing fetus and the lactating mammary gland. One should not underestimate the importance of partitioning nutrients to support pregnancy and lactation, because these physiological states are the essence of survival of the species and, of course, the foundation of the dairy industry. However, these tissues differ from other body tissues in that they confer no special advantage to the animal. Instead they make tremendous demands such that the total metabolism of the pregnant or lactating animal must be altered to accommodate these needs.

To appreciate the remarkable synthesizing capacity of dairy cows, it is appropriate to examine the adaptive changes that occur during the periparturient (30 days before and after parturition) period. Fetal growth curves (Bauman and Curie, 1980) show that over 50% of fetal development occurs during the last month. The additional nutrients are directed toward

fetal membranes, the gravid uterus, and the mammary gland (Bauman and Curie, 1980). Lactation in many, if not all species brings rapid changes in adipose metabolism resulting in decreased lipogenesis and increased lipolysis, which increases the nutrient supply for milk production. Fatty acid oxidation is regulated in part by the concentration of fatty acids in the blood, which is, in turn, controlled by the rate of hydrolysis rate of triacylglycerols in adipose tissue by hormone sensitive lipase (HSL).

During the first one-third of lactation, cows are in negative energy balance and use body reserves to meet their energy needs. In fact a zero net energy balance (i.e., intake sufficient to meet requirements) is not achieved until a point in lactation where milk production has decreased to less than 80% of peak production (Bauman and Curie, 1980). During the first 10 weeks of lactation the net energy deficit of cows is energetically equivalent to approximately 50 kg pure lipids or an average daily production of 9 kg milk (Bauman and Curie, 1980). During the first month of lactation the body reserves being utilized (i.e., net energy deficit) are energetically equivalent to about 33% of the milk produced (Bauman and Curie, 1980).

Chilliard and coworkers (Chilliard et al., 1977; Chilliard et al. 1978), working with goats, demonstrated that both lipoprotein lipase and acetyl CoA carboxylase (a regulatory enzyme in de-novo synthesis of fatty acids)

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decreased in activity in adipose tissue during late pregnancy and remain low during lactation. Investigations with cows in late pregnancy and early lactation have demonstrated the same increased rates of lipid mobilization (Metz and Van den Bergh, 1977; Sidhu and Emery, 1972) and decreased rates of pathways of lipid synthesis in adipose tissue (Metz and Biolabs® Inc., 1977; Shirley et al., 1973; Sidhu and Emery, 1972). Rabbits (Mellenberger, 1972), sheep (Noble, 1971), and humans (Kaplan and Grumbach, 1974) are other species in which these coordinated changes in adipose tissue metabolism and in concentration of lipid in serum have been observed during this physiological transition.

Energy requirements for maintenance and pregnancy increase 23% during the last month prepartum (Moe and Tyrrell, 1972). During this time, feed intake typically decreases ~30% (Grummer, 1993). An inadequate energy intake causes mobilization of body lipids.

Explicit recognition of constraints on the evolution of molecules, and the growing realization that there are limits to how much we can learn about selection from studying it's footprints on DNA sequences, should force biologists to rediscovered the phenotype. Identification of polymorphisms at the HSL locus may lead to factors regulating the rate of adipose tissue metabolism which would be useful in a number of ways.

LITERATURE REVIEW

Adipose Tissue:

Adipose tissue is a dispersed organ consisting of adipose cells (adipocytes) in a matrix of connective tissue. Triacylglycerol stored as fat droplets in adipose tissue, is the most important energy depot in mammals. During fasting, starvation, or negative energy balance, mobilization of lipids provides most of the energy needs of the body. HSL catalyzes the first, rate-limiting, and the second step in this process (Fig. 1). The enzyme is under tight hormonal and neural control. Its activity is regulated through phosphorylation/dephosphorylation of a single activity controlling phosphorylation site (Belfrage et al., 1986). The third step in adipose tissue lipolysis is catalyzed by a separate enzyme, monoacylglycerol lipase (Tornquist et al., 1978), the activity of which is not regulated by hormones or nutritional state. The term hormone-sensitive lipase was first coined by Vaughan et al. (1964). Early purification efforts were unsuccessful (Tsia et al., 1970). HSL turned out to be difficult to purify mainly because of its low tissue concentration, general lability, and properties resembling

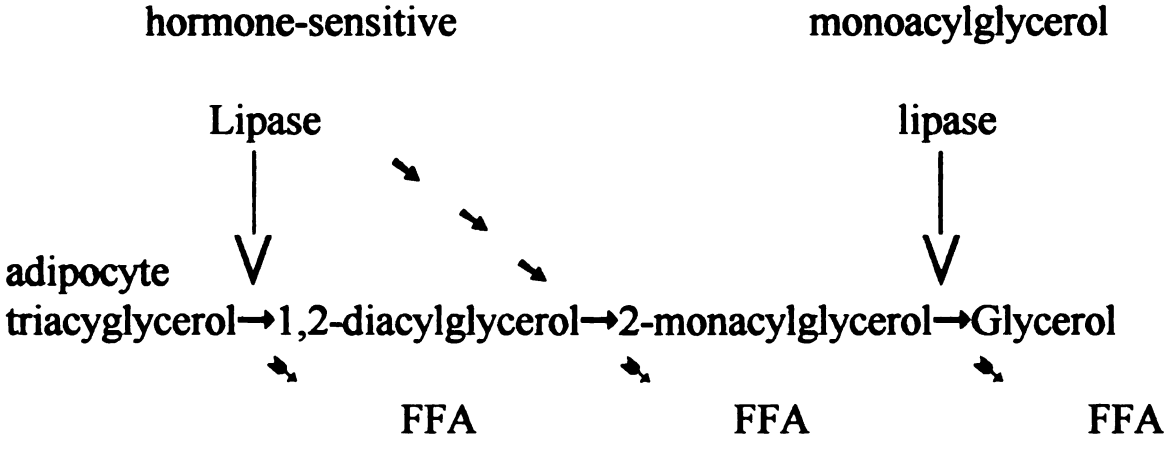

Figure 1. Adipose tissue lipolysis

those of intrinsic membrane proteins (Holm et al., 1986). Although some of the properties of the enzyme were described earlier (Vaughan et al., 1964) it was not until 1977 (Belfrage et al., 1977) that HSL was first identified after its detergent solubilization and partial purification. Furthermore, as revealed by the work of Cordle et al. (1986), bovine adipose tissue HSL was found to be similar to the rat, mouse, and swine enzyme (Kawamura, et al. 1981; Lee et al. 1985). Moreover HSL has been detected in a variety of other tissues, e.g., heart and skeletal muscle (Holm et al., 1987).

Since fatty acids can be transferred among different tissues or mobilized for use by other organs, synthesis is not a prerequisite to deposition in a given tissue. Fatty acid synthesis in adipose tissue of cows may increase during lactation in the absence of fat deposition presumably to supply fatty acids for production of milk fat (Baldwin, et al., 1976).

The three basic functions of ruminant adipose tissue are synthesis, storage, and mobilization of lipids. Adipose tissue is a dynamic energy store through which 10 to 80% of the daily energy flux passes depending on productive function, type of feed and animal requirements in relation to intake (Emery, 1979; Emery, 1969; Belferage, et al., 1980; Allen, et al., 1976). Adipose and lactating mammary tissues are the major sites of fatty acid synthesis in cattle, sheep, and pigs in contrast to significant hepatic synthesis of fatty acids in

rats, mice, and human (Allen et al., 1976; Bauman, 1976). Mobilized fat not only serves as a source of energy and as a precursor for other components, e.g., membranes, prostaglandin, but in the lactating cow it is a major contributor to milk fat. These roles partly explain the importance of adipose tissue to the lactating cow. Bauman (1976) illustrated the immense capacity cows have for fat mobilization in his summary of an energy balance study on one cow for an entire lactation. During the first 62 days of lactation, the average net energy loss was 20.6 Mcal/day. If 80% of this loss was met by body fat, mobilization of approximately 2 Kg/day of body TG would be required. During late lactation, as the need for energy subsides, energy balance becomes positive with cows depositing 10 to 15 Mcal/day (1-1.5 Kg/day)in adipose tissue while still producing milk (Bauman, 1976). Bauman and Davis (1974) indicated that adipose tissue is the major site of fatty acid synthesis in non-lactating ruminants, with a variable but minor contribution from the liver.

It is not clear whether disappearance of mammary adipose tissue with initiation of lactation in cows and reappearance of mammary fat after prolonged cessation of lactation is due to loss and formation of adipocytes or due to depletion and refilling of the central vacuole of the same adipocytes with triacylglycerol(Bauman and Davis, 1974).

Fatty acid composition of adipose tissue triacylglycerol

(TG) is rather complex, it composed of fifty to sixty individual fatty acids (FA) varying in chain length, degree of saturation and position of double bonds (Lafontan and Langin, 1995). In non-ruminants adipose-tissue FA profiles roughly correlate with dietary lipid composition. But in ruminants, adipose tissue contains a higher proportion of saturated FAs compared with polyunsaturated FAs (PUFA) relative to other tissues and organs because of biohydrogenation in the rumen by microorganisms.

Baird *et al.* (1977) found increased glycerol production in lactating cows fasted six days. Surprisingly, stimulated lipolysis was positively correlated with lipogenesis in steers where both basal and stimulated lipolysis were reduced by feed restriction (Pothoven, et al., 1975). Glycerol synthesis seems to be an important long-term control for increasing deposition of fat in growing ruminants. Reduced glyceride synthesis also permits fat mobilization with initiation of lactation (Metz, et al., 1977). Adipose tissue from lambs with a greater propensity to fatten due to breed (Southdown) or sex (ewes and wethers) had more glyceride synthesizing activity than tissue from leaner lambs (Sidhu, et al. 1973).

In ruminant as well as nonruminant adipose tissue, the major pathway of TG synthesis is acylation of glycerol-3-phosphate to form phosphatidic acid from which phosphate is hydrolyzed and the resulting diglyceride is acylated by a third acyl transferase to form triglyceride (Benson and Emery,

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1971; Bickerstaffe, et al., 1974). Glyceride synthesis in bovine mammary gland has been extensively studied (Patton and Jensen, 1975). Mammary glyceride synthesis increased six-fold with initiation of lactation (Shirely, et al., 1973). A study of adipose tissue is complicated by the fact that it is composed of nonadipocytes and adipocytes that vary widely in size and lipid content (Hood, 1982; Hirsch and Gillian, 1968). There are only limited reports on adipocyte size and density in bovine adipose tissue during lactation (Bauman and Curie, 1980; Chilliard, et al., 1984).

Lipid Pathways:

The lipid digestion products absorbed by the intestinal mucosa are converted by these tissues to triacylglycerols and then packaged into lipoprotein particles called chylomicrons. These are released into the bloodstream via the lymph system for delivery to different tissues. Similarly triacylglycerols synthesized by the liver are packaged into very low density lipoproteins (VLDL) and released into the blood. The triacylglycerol components of the chylomicrons and VLDL are hydrolyzed to free fatty acids and glycerol in the capillaries of adipose tissues and skeletal muscles by lipoprotein lipase. The resulting free fatty acids are taken up by the different tissues while glycerol is transported to liver and kidneys.

Lipoprotein lipase and HSL are two major enzymes,

negatively correlated, in lipid metabolism. Lipoprotein lipase (LPL) is an adipocyte enzyme that cleaves fatty acids from circulating lipoproteins (intercellular). Fatty acids enter the cell to be oxidized or esterified. Hormone-sensitive lipase (HSL) is an adipocyte enzyme that cleaves fatty acids from intracellular triacylglycerol. Lipoprotein lipase is not a large contributor to peripartum bovine adipose adaptation (McNamara and Bauman, 1987), but is important in retainment of body composition in mid and late lactation. Hormone-sensitive lipase activity is increased during peak and mid-lactation (McNamara and Bauman, 1987).

In ruminants, the liver is the major source of endogenous plasma triglyceride, with intestinal denovo synthesis being of little consequence (Pullen, et al., 1988). In contrast to many nonruminant species, however, ruminant liver is quantitatively unimportant in fatty acid synthesis (Ballard, et al., 1969). Most synthesis of fatty acids in ruminants occurs in adipose tissues. The liver is involved in numerous metabolic processes central to the maintenance of homeostasis and productive function. Because of this central role in metabolism, energy expenditure per kilogram of liver tissue is much greater than that of other major tissues in the animal (Baldwin et al. 1976). Most of the incoming dietary nutrients pass via the portal blood system to the liver where they are packaged according to the specific requirements of the animal at that time and then either stored or distributed to

peripheral tissues. It should be emphasized that ruminant liver is not an important site of fatty acid synthesis but it is central to formation of lipoproteins and transport of lipid among organs.

Lipolysis refers to the hydrolysis of TG, via di- and monoacylglycerol intermediates, to fatty acids and glycerol. The result of changes in adipose tissue metabolism during early lactation is a tremendous increase in the ability of adipose tissue to mobilize non-esterified fatty acids (NEFA). During early lactation, dairy cows mobilize subcutaneous fat which increases the supply of blood NEFA to the liver. Fatty acids are taken up by liver in proportion to their blood flow and concentration reaching the liver (Emery, et al., 1992). The liver then esterifies fatty acids and exports triglycerides. Metabolically, VLDL and chylomicron fractions are the main fractions active in providing triglyceride-fatty acids to peripheral tissues, most of which will be taken up by the mammary gland in lactating cows (Palmquist and Mattos, 1978). With the onset of lactation, metabolism of adipose tissue is redirected toward providing fatty acids to other organs of the body (Vernon, 1980). During the last month before parturition, lipolysis increases and lipogenesis ceases (McNamara and Hillers, 1986;,Pike and roberts, 1980).

The flux of fatty acids from blood to tissues depends upon local concentration of fatty acids and the enzymic capacity of the tissue. Lipoprotein lipase is sequestered on

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the capillary endothelium where it acts upon triacylglycerol contained in the circulating lipoproteins to release fatty acids. The extra supply of fatty acids provided by lipoprotein lipase comes predominantly from triacylglycerol but some may also come from phospholipids. Glycerol is poorly utilized in adipose tissue, therefore its release reflects lipolysis (Allen, et al., 1976). Since fatty acids can be reacylated within adipocytes, their release may be less than total lipolytic activity.

Details of biochemical pathways of adipose tissue have been reviewed (Bauman, 1976; Bauman and Davis, 1975). The temporal relationship between metabolism of adipose tissue and onset of lactation has been studied most extensively in rats (Graham, 1964; Knopp, et al., 1975). At about day 19 of pregnancy, lipoprotein lipase (necessary for the uptake of preformed fatty acids) activity in adipose tissue decreases, and both flux rates and activities of key enzymes in the regulation of denovo fatty acid synthesis decrease rapidly (Knopp, et al., 1973;McNamara and Bauman, 1978; Fain and Scow, 1966).

Hormone-Sensitive Lipase:

Lipolysis in ruminants occurs by a mechanism similar to that of non-ruminants. The initial step involves the hydrolysis of TG to diacylglycerol and monoacylglycerol by HSL

that is activated by cAMP (Vernon, 1980). The monoacylglycerol is hydrolyzed by non regulated lipases to free fatty acids and glycerol (Vernon, 1980).

The hallmark of HSL, which distinguishes this enzyme from all other known lipases, is the control of its activity through phosphorylation/dephosphorylation. A single serine residue (regulatory site) is phosphorylated by cAMP-PK (Stralfors and Belfrage, 1983).

Adipose tissue metabolism is coordinated during pregnancy and lactation to ensure that the energy needs of the mother and young are met. In the bovine, the adaptation to lactogenesis by adipose tissue is characterized by an increase in catabolic reactions and a decrease in anabolic reactions (Metz and Van den Bergh, 1977). Numerous studies have shown that HSL is involved in the adaptations of adipose tissue during lactation, by increasing blood FFA levels (McNamara, et al., 1987; Smith and MaNamara, 1990). The increase of basal lipolysis during very-low calorie intake was caused by an increase in HSL expression (Sztalryd and Kraemer, 1994). Hormone-sensitive lipase mRNA levels increased twofold in rats after 3-5 days of fasting (Sztalryd and Kraemer, 1994).

Adipose tissue HSL is thus one of the enzymes determining whole body lipid fuel availability. In the post-absorptive state, HSL activity accounts for most of the detectable lipolysis (Frayed, et al., 1995). Exercise and short term fasting increase hormone-sensitive lipase activity in non-

ruminants (Masoro, 1977).

Adaptations in tissue metabolism taking place just before parturition, include a decrease in rate of lipid synthesis, and an increase in rate of lipolysis. The occurrence of this coordination of metabolism in the rat (Flint, et al., 1983), sheep (Chilliard, et al., 1979), human (Kalkhoff, et al., 1978), pig (McNamara, et al., 1985), and cow (Metz and Van den Bergh, 1977), along with the consistency of biochemical and endocrinological adaptations among species, has led to the concept of homeorhesis (Bauman and Curie, 1980). Homeorhesis is the orchestration of a coordinated set of adaptations in support of a dominant physiological process.

Subcutaneous adipose tissue cell size and density and HSL activity were determined during late pregnancy and first lactation in Holstein heifers (Smith and McNamara, 1990). HSL activity was found to be similar in high and low genetic lines for milk production prepartum and increased in both at 60 day postpartum (Smith and MaNamara, 1990).

The adipose tissue activities of HSL seemed to differ between Brahman and Angus heifers (Sprinkle, et al., 1998). Brahman heifers seem to have greater basal activities of subcutaneous adipose tissue HSL than Angus. Therefore, non-lactating Brahman heifers may have the ability to more readily adapt to changes in nutritional status by increasing the activities of those enzymes.

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binding of HSL to a component of the lipid droplet is not known, the primary structure of HSL does not show regions with high hydrophobicity which would clearly explain the propensity of phosphorylated HSL to bind directly to the lipid droplet. Reconsidering previous reports attributing a putative role to the lipid droplet substrate (Wise and Jungas, 1978), it may be that a protein component of the lipid droplet surface may undergo some kind of substrate activation, occurring concomitantly with HSL activation, and governing droplet-driven translocation of HSL. Or, it may be that perilipins, specific adipocyte lipid-droplet-associated proteins, which could have a role in the packaging and/or the trafficking of lipids, are possible candidates in docking proteins for HSL (Greenberg, et al., 1991). These proteins probably contribute to the organization of lipid droplets and lipid vacuoles found in mature adipocytes (Hare, et al., 1994). Perilipins are phosphorylated by cAMP-PK.

Phosphorylation/Dephosphorylation of HSL:

Definitive evidence correlating hormone-stimulated lipolysis with accumulation of cyclic AMP came from the work of Butcher and Sutherland (Butcher, et al., 1966). Stimulation of lipolysis by cAMP in cell-free systems was noted (Rizak, 1964). The activity controlling phosphorylation site was localized to Ser^{551} in a markedly hydrophilic domain, and a

lipid binding consensus site was tentatively identified (Langin and Holm, 1993) .

The understanding of molecular mechanisms underlying cellular variations of lipolysis is important with respect to milk and energy production in dairy cows. It is generally accepted that lipolysis is controlled mainly by sympathetic nervous system activity and plasma insulin levels. Basically, the lipolytic response of the fat cell depends on the balanced action of stimulatory and inhibitory pathways on HSL activity. The first step leading to activation of the lipolytic cascade involves this multi-regulated enzyme, adenylate cyclase, which produces cAMP (Lafonton, et al., 1993; Sidhu and Emery, 1993). An important point in the metabolic actions initiated by endocrine and paracrine regulators concerns the functional significance of intracellular cAMP elevations promoted by receptor- mediated adenylate cyclase control. In fat cells, the lipolytic hormones (catecholamines) increases concentration of cAMP which activates cAMP-PK (Fain Garcia-Sainz, 1983). Phosphorylation of HSL by the activated cAMP-PK causes HSL activation and lipolysis (Fig. 2). Monoacylglycerol lipase hydrolyze the breakdown of monoacylglycerol into fatty acid and glycerol. The abundance of this enzyme, which is not under hormonal control, is sufficient to avoid accumulation of intermediary products of lipolysis (Fredrickson, et al., 1986). Because HSL has 10 fold higher activity toward diacylglycerol than

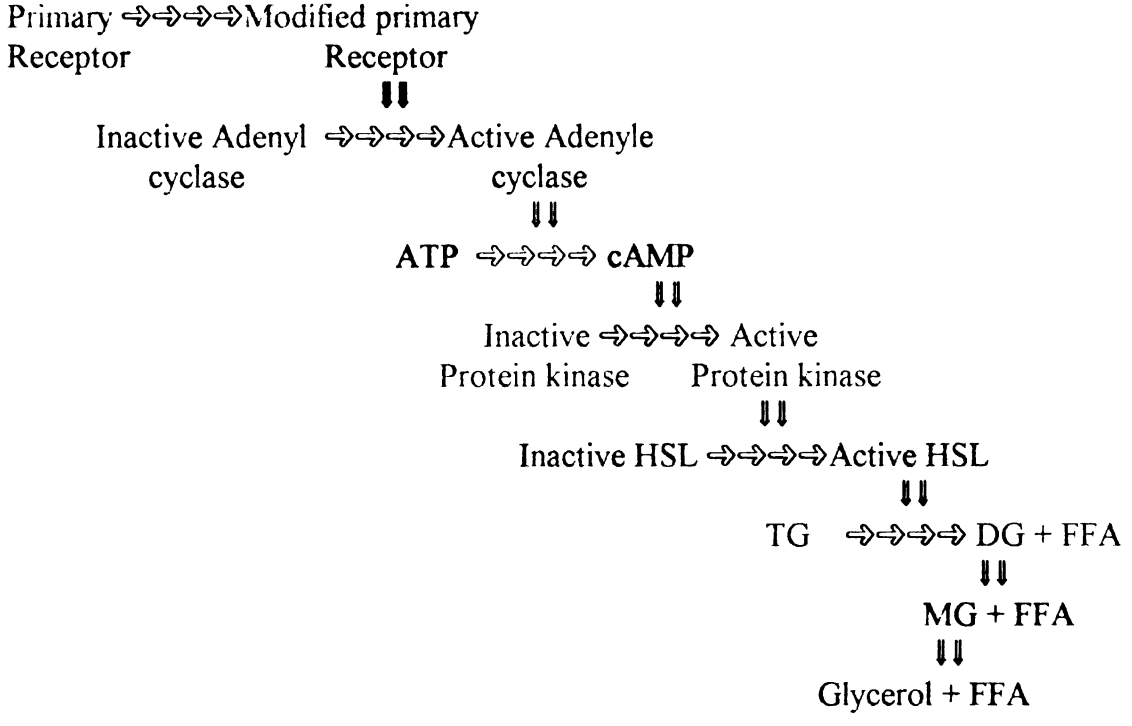

Figure 2. Cascade of reactions involved in activation (phosphorylation) of HSL and hydrolysis of triglycerides

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triacylglycerol, when not activated, the diacylglycerol lipase activity is not dependent upon the phosphorylation state of the enzyme (Stich, et al., 1997).

Peptide mapping studies have suggested that HSL is phosphorylated at only one site in vitro by cAMP-PK (Stralfors and Belfrage, 1983). This site is also that which is phosphorylated in isolated adipocytes in response to noradrenaline, consistent with the conclusion that this hormone acts via activation of cAMP-PK (Stralfors, et al., 1984). A second site was found on HSL that is phosphorylated in the absence of lipolytic hormones. However, the extent of phosphorylation of this site does not change in the presence of lipolytic hormones (Stralfors, et al., 1984) and its phosphorylation does not apparently have a direct effect on the activity of HSL (Olsson, et al., 1986).

Hormone-sensitive lipase was purified from bovine adipose tissue (Cordle, et al., 1986). The 27-residue region of the HSL polypeptide containing the serine residue phosphorylated by cAMP-PK was sequenced. The sequence, K T E P M R R S (P) V S E A A L T Q P E G P L G T D S L K, is consistent with the known specificity of this protein kinase in that two adjacent basic residues are present on the amino-terminal side of the phosphorylated serine (Cohen, 1985).

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Hormonal Regulation of HSL:

Adipose tissue lipolysis is under acute hormonal and neural control. Lipolytic agents include noradrenaline (released from sympathetic nerve endings), and adrenalin. Their action is opposed by insulin, the major anti-lipolytic hormone, which reduces phosphorylation of HSL (Nilsson, 1980), and acts, at least in part, by lowering the level of cyclic AMP (Londos, 1985). The activity of the enzyme, and thus, the over all lipolysis rate in the adipocyte, changes in response to a variety of hormones (Vaughan and Steinberg, 1965).

The different steps of the lipolytic process leading to the activation of HSL are well defined (Lafontan and Berlan, 1993). The first cellular action of catecholamines, and a number of endocrine-paracrine regulators of lipolysis, is their binding to plasma membrane receptors. The stimulatory effect on lipolysis is strictly connected to the receptors-controlled increment of intracellular cAMP concentrations which in turn promotes activation of cAMP-PK (Honnor, et al., 1985).

Glucose and insulin are homeostatic regulators of lipid metabolism in adipose tissue. Insulin, the anti-lipolytic hormone, causes dephosphorylation of HSL and thereby it's deactivation. Insulin is the most physiologically important and potent inhibitor of basal and hormone-stimulated lipolysis. Consistent with the continuous need for lipid

mobilization during early lactation in a dairy cow, adaptation has occurred in adipose tissue such that lipolytic rates are unaffected by glucose during this period (Metz and Van den Bergh, 1977).

Several investigators have proposed that prolactin coordinates lipid metabolism in adipose and liver in a manner to partition nutrients to the mammary gland (McNamara and Bauman, 1978; Zinder, et al., 1974). Blocking release of prolactin during lactogenesis or in early lactation produces a negative effect on mammary synthesis of milk as well as a reduction in the rates of lipid mobilization from adipose tissue (McNamara and Bauman, 1978).

Noradrenaline, released from sympathetic nerve endings, and circulating adrenalin, and glucagon all stimulate lipolysis by raising the intracellular concentration of cyclic AMP. This leads to phosphorylation of HSL, causing activation of the enzyme and subsequent lipolysis (Nilsson, et al. 1980).

Molecular Biology of HSL:

The expression of HSL gene has been detected in a variety of tissues including adrenal glands, adipose tissue, heart, skeletal muscle, and steroidogenic tissues (Sztalryd and kraemer, 1994; Kramer, et al., 1991; Holm, et al., 1988B). In rat adipose tissue, HSL mRNA abundance was reported in steady state through development and aging (Kramer, et al., 1991),

and the levels increase in rats during starvation (Sztalryd and Kraemer, 1994) and in patients with hyperlipidaemia (Holm, et al., 1988 A). The HSL mRNA in testes and corpus luteum varies by 200-600 nucleotides in length from that in adipose tissue (Holm, et al., 1988 B). There has been little investigation into the regulation of the HSL gene in organs other than adipose tissue.

Nucleotide sequence of the region surrounding the serine residue in bovine hormone-sensitive lipase that is phosphorylated in vitro by cyclic AMP-dependent protein kinase was reported (Garton, et al., 1988).

The human HSL gene encodes a 786 amino acid polypeptide (85.5 Kda) (Langin and Holm, 1993). It is composed of nine exons spanning ~ 11 Kb, with exons 2-5 clustered in a 1.1 Kb region. The putative catalytic site (Ser^{423}) and a possible lipid-binding region (a short hydrophobic stretch) in the C-terminal part are encoded by exons 6 and 9, respectively. The phosphorylation site sequence (Met-Arg-Arg-Ser^{551}-Val-Ser^{553}-Glu-Ala-Ala) is completely preserved in bovine, rat and mouse HSL. Exon 8 encodes the phosphorylation site (Ser^{551}) that controls cAMP-mediated activity and a second site (Ser^{553}) that is phosphorylated by 5′-AMP activated protein kinase. The human HSL gene sequence showed 83% homology with the rat enzyme and contained a 12-aa deletion immediately upstream of the phosphorylation sites with an unknown effect on the enzyme activity. Site-directed mutagenesis of Ser^{423} leads to a

complete abolition of both lipase and esterase activity (Holm, et al., 1994). Besides the catalytic site motif (Gly-X-Ser-X-Gly) found in most lipases, where 'X' is any amino acid, HSL exhibits no homology to other known lipases or proteins, except for a recently reported unexpected homology between the region surrounding its catalytic site and that of the lipase 2 of *Moraxella* TA144, an antarctic psychrotrophic bacterium (Langin, and Holm, 1993). The catalytic role of Ser^{423} of the consensus pentapeptide (Gly-X-Ser-X-Gly), was analyzed by site-directed mutagenesis and substitution of ser^{423} by several different amino acids. This resulted in complete abolition of both lipase and esterase activity, whereas mutation of other conserved serine residues had no effect on catalytic activity (Holm, et al., 1994). This shows that the pentapeptide serine is analogous to the active site serine in several lipases, including hepatic lipase, lipoprotein lipase, and pancreatic lipase.

HSL exhibits cholesterol hydrolase activity in steroidogenic tissue and macrophage. Thus, common genetic variations in HSL gene could affect both energy metabolism and atherogenesis.

Using overlapping single-stranded conformation polymorphism analysis (SSCP), a common single base change (T/C) in intron 4 of the human gene, and variable CA repeat in intron 6 with 9 alleles were identified (Talmud, et al., 1998). The 5′ sequence shows 57-59% homology with the mouse

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promoter with higher homology at potential regulatory motifs. It was concluded that 1.7 kb of 5′ sequences is well conserved and may play a role in the regulation of HSL gene expression. Grober *et al.* (1997) re-characterized the promoter of the human adipose HSL gene and defined a short and a long 5′ UTR; one of 54 bp about 1.5 kb upstream of the first coding exon and the other of 150 bp about 13 kb upstream of the translation start site.

Laurel *et al.* (1996, 1997) found that in human adipocytes, instead of a full-length 88 kDa enzyme, an 80 kDa truncated form of HSL is synthesized as a result of skipping exon 6 which encodes the active serine. The extent of exon skipping varied from 5 to 40% in different human fat biopsies (Grober, et al., 1997). The skipping of exon 6 is human specific and could represent an important additional control mechanisms in human adipose tissue or macrophage since the truncated form still encodes the lipid binding domain but not the active site (Laurel, et al., 1996).

To investigate whether mutations in the HSL gene are associated with non-insulin dependent diabetes mellitus (NIDDM), Shimada *et al.* (1996) reported polymorphisms for HSL in 35 Japanese human subjects with NIDDM. Single stranded conformation polymorphism analysis identified a variant pattern in exon 4, and the sequence at this variant pattern resulted from amino acid polymorphisms (Arg309Cys). They identified a variation in HSL gene at two sites. The first is

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an amino acid change R309C identified at a carrier frequency of 0.17 in the Japanese population. In their small study in type two diabetics and healthy controls, the C309 allele was shown to be associated with higher plasma total cholesterol but not plasma TG or obesity (Shimada, et al., 1996). The second variant is a hyper-variable CA repeat in intron 7 with 7 alleles (Levitt, et al., 1992) which has been used in linkage analysis in families with familial combined hyperlipidaemia (Pajukanta, et al., 1997).

Another study (Li, et al., 1994), examined the 5′ flanking region for nucleotide sequences that may modulate HSL gene transcription in the mouse. Using primer extension, a major transcription initiation site 593 nucleotides upstream of the protein coding sequence was identified. Homology with several known gene regulatory elements are present in the HSL 5′ flanking region, including a sequence motif that appears to direct expression in adipose, testis, adrenal, and myeloid tissues (Li, et al., 1994).

Part of bovine HSL has been sequenced. Lee, et al. (1997) from the university of Minnesota (unpublished data) submitted the partial cDNA (429 bp) of bovine HSL sequence to the GenBank (Accession # U78042), which is part of exon 8 in the human HSL gene. Also, Einspanier, R., and Kettler, A. (1999; unpublished data) have recently submitted 311 bp of the bovine sequence to the GenBank (accession # AJ237675) which represents the end of exon 1 and beginning of exon 2 in the

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human HSL gene.

The predicted primary translation product of rat HSL is 757 amino acid in length and is 82,820 Dalton in size (Holm, et al., 1986). Rat HSL shares no homology with either the members of the lipase gene family (lipoprotein lipase, hepatic lipase, and pancreatic lipase) or any other sequence lipase (Kirchgessner, et al., 1987).

The human (Langin and Holm, 1993) and rat (Holm, et al., 1988A) HSL genes have been cloned, and the phosphorylation site in the human gene has been determined (Langin, et al., 1996). A 3.3 kb HSL mRNA was found in swine adipose tissue, liver, heart, testis, skeletal muscle, and spleen (Liu, et al., 1995), and the cDNA was 86% similar to the rat HSL gene. The mouse HSL gene has also been cloned and it shows 94% sequence identity with the rat gene and 85% with the human sequence (Li, et al., 1994).

The nucleotide sequence of rat adipose HSL cDNA (accession no. J03087; Holm, et al., 1988A) was used to measure mRNA levels in rat adipose tissue, testes, adrenals and heart (Kramer, et al., 1991). The 757-amino acid sequence of hormone sensitive lipase predicted from a cloned rat adipocyte cDNA showed no homology with any other known lipase or protein (Holm, et al., 1988A). One of several mRNA species was expressed in adipose tissue, steroidogenic tissues, heart and skeletal muscle.

HSL shares no homology with known eukaryotic proteins. In

particular, HSL does not belong to the so-called lipase gene family that includes lipoprotein lipase, hepatic lipase and pancreatic lipase. However it does show sequence similarity to five prokaryotic enzymes from distantly related eubacteria (Langin, et al., 1993). The region of similarity is bordered by the catalytic site motif which is identical in the six proteins and a His-Gly dipeptide which may constitute one of the hydrophobic 'wings' flanking the catalytic site. The strongest sequence similarity is found with lipase 2 of *Moraxella TA144*, an antarctic psychrotrophic bacterium. Since lipase 2 is active below 4 °C, the cold adaptability of HSL, an unexpected property for a mammalian lipase, was investigated. HSL retained distinctly more catalytic activity at low temperatures than either lipoprotein lipase or carboxyl ester lipase. The so-called 'cold adaptability' of HSL has been discussed (Langin, et al., 1993). This unexpected property of HSL could be of critical survival value when lipids mobilized at low temperatures are the primary energy source. e.g. in hibernators or poikilotherms.

Recent studies have shown that HSL gene is differentially expressed in tissue during growth (Huttenen, et al., 1970) and fasting (Sztalryd and Kraemer, 1994). HSL levels increased in subcutaneous adipose tissue of cancer patients (Thompson, et al., 1993). This increase could be one of the mechanisms involved in the depletion of lipid from adipose tissues in these patients.

Determining the 3-D structure of HSL and mapping of functional domains by site-directed mutagenesis will elucidate structure-function relationships of HSL. A recent expression system utilizing recombinant baculovirus arraying a full rat HSL cDNA downstream from the polyhedrin promoter in insect cell has allowed production of large amounts of functional recombinant HSL protein. It is expected that determination of the 3-D structure of HSL by crystallization studies will be considerably facilitated (Holm, et al., 1994).

The chromosomal mapping of HSL gene was performed in human and mice. The human HSL gene is located in chromosome 19q13.1 (Holm, et al., 1988B; Schonk, et al., 1990), while the mouse gene is positioned approximately 8 cM from the centromere of chromosome 7 (Warden, et al., 1993). However, a recent analysis showed that at least one portion of the HSL gene or a pseudogene could also be mapped to 19p13.3 (Holm, et al., 1988B). Precise knowledge of the HSL gene chromosomal location and the possibility of gene duplication are critical for genetic linkage analysis and studies of regulatory elements involved in HSL gene expression.

It has been speculated that HSL is an intrinsic membrane protein (Holm, et al., 1986). However, its predicted amino acid sequence does not appear to contain a membrane-spanning region. The combined knowledge of the gene structure and its localization to chromosome 19cent-q13.3 (Holm, et al., 1988B) would clearly be important for future genetic studies.

Existing genetic variation provides the material for genetic change, be it between or within breeds.

Rationale:

The rate-limiting step in lipolysis, namely the hydrolysis of triacylglycerol to diacylglycerol and free fatty acid is catalyzed by the enzyme HSL. The activity of this enzyme is controlled by reversible phosphorylation. Activation of the enzyme results from phosphorylation catalyzed by cAMP-dependant protein kinase. Inactivation by dephosphorylation. The bovine HSL enzyme has been purified near homogeneity (Cordle, et al., 1986). It was identified as a polypeptide of M_r 84,000 by affinity labeling with [^{3}H] diiso-propyl fluorophosphate.

Cattle breeds that originated in different climates of the world may have unique cellular control of energy retrieval and storage through their enzymes, enabling them to adapt to their environments (Sprinkle, et al., 1998).

Evaluation of allelic differences of specific genes among animals of different genetic merit offers potential for identification of markers associated with genetic merit (Lee, et al., 1996). Development of these molecular markers could result in marker-assisted selection programs and methods of determining, at an early age, the potential genetic merit of individual animals. Comprehensive investigation of the

relationships among genetic polymorphisms and phenotypic traits requires evaluation of distribution frequencies of alleles of various proteins known to have critical roles in the phenotypic traits of interest. Polymorphism refers to the simultaneous occurrence in the population of genomes showing allelic variations (as seen either in alleles producing different phenotypes or, for example, in changes in DNA affecting the restriction pattern).

The identification of polymorphisms at the bovine HSL locus between and within different cattle breeds is useful for increasing our understanding of the mechanism responsible for differences in body fat mobilization between and within different breeds. This will permit identification of potential associations between sequence polymorphisms in the bovine HSL gene and economically important traits and diseases such as milk production and fatty liver. The specific aim of this dissertation is to identify polymorphisms at exon 8 (phosphorylation site) of the bovine HSL locus.

MATERIALS AND METHOD

The steps taken to isolate the bovine HSL gene are illustrated in Figure 33.

Isolation of Bovine Genomic DNA:

Blood samples were collected from one Holstein lactating cow into two different vacutainers (one heparin and the other was EDTA), and held at 4 °C. Bovine genomic DNA was isolated and purified using Promega kit (Wizard Genomic DNA Purification System cat. # A1120). The Promega kit provides a rapid way to isolate double-stranded genomic DNA from fresh whole blood. It is based on a four-step process (Miller, et al., 1988). The first step in the purification process lyses red blood cells in a Cell Lysis Solution. This leaves the white blood cells intact. In the second step, the white blood cells and their nuclei are lysed and solubilized in the Nuclei Lysis Solution. RNase digestion followed by protein precipitation, leaves the high molecular weight genomic DNA in solution. Finally the genomic DNA was concentrated and desalted by isopropanol precipitation. The genomic DNA was loaded in 1% agarose gel [50 ml TBE buffer (1x) + 0.5 g agarose] along with 1.0 ul of 9 cut with Hind III (heated at 65 °C for 10 minutes before loaded). 6x loading dye [0.25%

bromophenol blue, 0.25% xylene cyanol, and 15% Ficoll (Type 400; Pharmacia)]was used (Figure 3; Images in this dissertation are presented in color).

DNA Electrophoresis:

1.0% agarose solution was prepared in TBE buffer (0.089 M Tris-base, 0.089 M boric acid, 0.002 M EDTA, pH 8.0) by heating in a microwave oven. The solution was cooled to 50 °C and ethidium bromide added to a final concentration of 0.5 ug/ml. The solution was poured into the gel plate. After the solution gelled, the comb was removed and TBE electrophoresis buffer was added to the apparatus to a volume of about 0.25 ml above the gel. DNA samples were placed in the sample wells and a potential difference of 100 V applied until the tracking dye moved about two thirds of the length of the gel (Sambrook, et al., 1989). The gel was examined under UV light. Gels were photographed using a Polaroid camera.

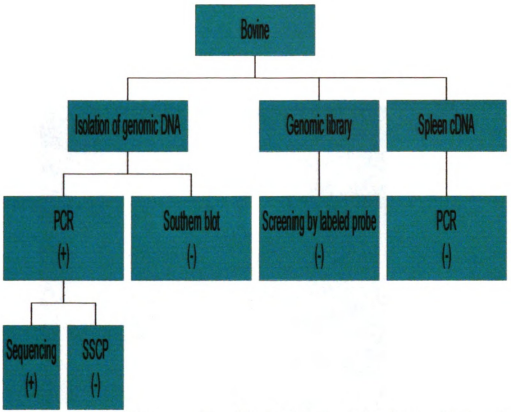

Figure 33. A flow-chart showing the steps followed to isolate the bovine HSL gene.
(+) means the experiment produced positive products.
(-) means the experiment produced negative products.

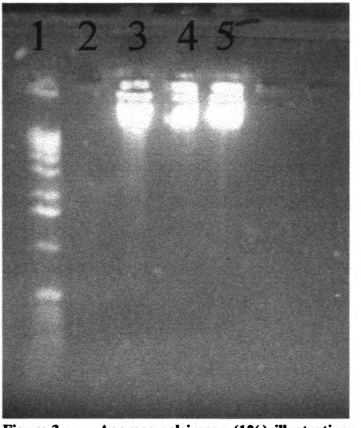

Figure 3. **Agarose gel image (1%) illustrating bovine genomic DNA obtained from whole blood which was collected in heparin and EDTA tubes**

Lane 1	⇨	1 Kb DNA Ladder
Lane 3	⇨	7.0 ul genomic DNA (heparin tube)
Lane 4	⇨	3.5 ul genomic DNA (EDTA tube)
Lane 5	⇨	7.0 ul genomic DNA (EDTA tube)

Strategy to Amplify HSL Gene:

Human HSL gene sequence was divided into 5 different fragments (Figure 34), assuming that there is a high homology between the human and bovine HSL gene, so that each fragment could be amplified separately, by the polymerase chain reaction (PCR), and then the whole gene could be identified by overlapping the different fragment sequences. The fragments were determined as follows:

1) Fragment 1 (≈516 bp) that includes exon 1.
2) Fragment 2 (≈2707 bp) that includes exon 1 (≈516 bp) and exon 2 (≈91 bp) and the intron between them (≈2.1 kb).
3) Fragment 3 (≈ 2991) that includes exon 2 (≈ 91 bp), exon 3 (≈ 146 bp), exon 4 (≈186 bp), exon 5 (≈295 bp), exon 6 (≈228 bp), exon 7 (≈177 bp), intron 2 (≈110 bp), intron 3 (≈183 bp), intron 4 (≈137 bp), intron 5 (≈800 bp), and intron 6 (≈620 bp).
4) Fragment 4 (≈3002 bp) that includes exon 7 (177 bp), exon 8 (≈425 bp), and intron 7 (≈2.4 kb).
5) Fragment 5 (≈1503 bp) that includes exon 8 (≈425 bp), exon 9 (≈558 bp), and intron 8 (≈520 bp)

Note: whenever exon 8 is mentioned in this dissertation, it refers to the human exon 8.

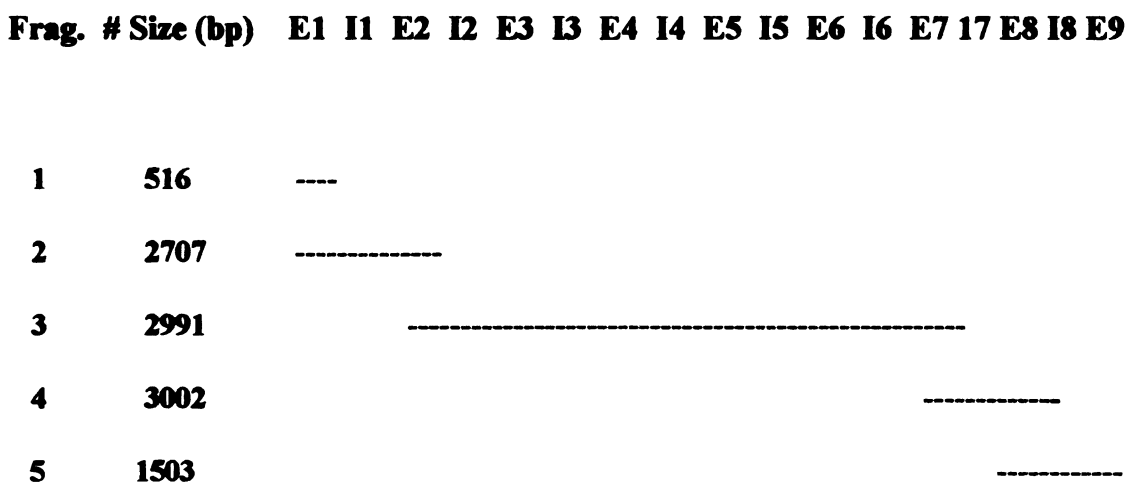

Figure 34. Schematic diagram illustrating the arrangements of the 5 fragments to be amplified

E	=	**Exon**
I	=	**Intron**
-----	=	**DNA fragment**

Primer Design:

To design a primer for each of the 5 fragments described above, the HSL sequences for human, rat, swine and the published part of the bovine were lined up in the GCG (Genetic Computer Group; **http://www.gcg.com/**) program, using the bestfit command, in order to find conserved sequences among the different species to be used as primers. The Oligo software program (National Biosciences Incorporation NBI, version 5) was used to design and fine tune the primers, e.g. free from primer dimers and hairpins, and to make the GC% as close as possible to 50%. Also the expected product lengths were noted.

The designed primers (forward and reverse) were then entered into the database of the NCBI (National Center for Biotechnology Information; http://searchlauncher.bcm.tmc.edu) home page (which includes Genbank, PubMed, Genomes, Locus Link, OMIM, Proteins, and Structures). The blast command was then used, and the primers were confirmed after they hit, with high-scoring, the hormone-sensitive lipase sequence for any of the species. After all designed primers hit HSL sequences as described above, they were used in PCR as described below.

Polymerase Chain Reaction (PCR):

About 80 different polymerase chain reactions were performed for the 5 fragments above using different

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combinations of primers and adjusting reaction conditions. Another six polymerase chain reactions were performed using a new set of primers designed for exon 8 (Figure 5, panel 1) in a 50 ul total reaction volume [Reaction 1 to 6: 5.0 ul Taq DNA Polymerase buffer (10x), 1 ul dNTPs (10 mM), 2.5 ul upper primer (7.6 pM/ul), 2.0 ul lower primer (10.4 pM/ul), 34.25 ul milliQ water, 4.0 ul MgCl (25 mM), 1.0 ul template DNA (25 ng/ul), 0.25 ul Taq DNA Polymerase (5 u/ul)]. Also a new set of primers, designed for the fragment includes exons 2, 3, 4, 5, and the introns between them, were used in four polymerase chain reactions (Figure 5, panel 2). The reactions condition for Figure 5, panel 2 is as follows: 5.0 ul Taq DNA Polymerase buffer (10x), 1.0 ul dNTPs (10 mM), 3.0 ul upper primer (6.6 pM/ul), 1.5 ul lower primer (13.4 pM/ul), 35.25 ul milliQ water, 3.0 ul MgCl (25 mM), 1.0 ul template DNA (25 ng/ul), 0.25 ul Taq DNA Polymerase (5 u/ul)].

Robocycler:(Stratagene Gradient 96) **Thermocycler:(MJ Research model PTC-200)**

Thermal Cycler Conditions:

Reaction 1	**Reaction 2**
94 °C for 5 minutes	**94 °C for 5 minutes**
94 °C for 50 sec ⇨ ⇨ ⇨ 30 Times	**94 °C for 50 sec. ⇨ ⇨ ⇨ 30 Times**
55 °C for 50 sec. ⇨ ⇨ ⇨ 30 Times	**55 °C for 50 sec. ⇨ ⇨ ⇨ 30 Times**
72 °C for 50 sec. ⇨ ⇨ ⇨ 30 Times	**72 °C for 1:05 minutes⇨ ⇨ ⇨ 30 Times**
72 °C for 10 minutes	**72 °C for 10 minutes**
6 °C for ∞	**4 °C for ∞**

The PCR products were then purified with the Micron kit (Amicon cat # 42413), and run in a 2% agarose gel.

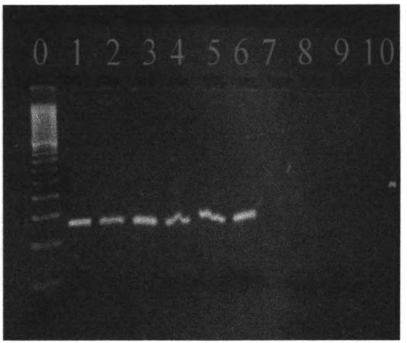


Figure 5. **Agarose gel image (2%) illustrating HSL PCR products' results. Panel 1: part of exon 8 (263 bp); Panel 2: exon 2, 3, 4, 5, and the introns between them (nothing being amplified).**

Lane 0 ⇨ 100 bp PCR ruler
Lane 1 ⇨ Sample 1 (part of exon 8; panel 1)
Lane 2 ⇨ Sample 2 (part of exon 8; panel 1)
Lane 3 ⇨ Sample 3 (part of exon 8; panel 1)
Lane 4 ⇨ Sample 4 (part of exon 8; panel 1)
Lane 5 ⇨ Sample 5 (part of exon 8; panel 1)
Lane 6 ⇨ Sample 6 (part of exon 8; panel 1)
Lane 7 ⇨ Sample 7 (fragment 2; panel 2)
Lane 8 ⇨ Sample 8 (fragment 2; panel 2)
Lane 9 ⇨ Sample 9 (fragment 2; panel 2)
Lane 10 ⇨ Sample 10 (fragment 2; panel 2)

Cloning of the Bovine PCR Product:

The pGEM-T Easy Vector Systems (Promega pGEM-T Vector cat. # A3600), which has a 3′ thymidine added to both ends, was used as a plasmid to clone the PCR product. These single 3′-T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerase (Robles and Doers, 1994).

Three reactions were performed as follows:

Reaction (A): I ul of pGEM-T Easy Vector (50 ng) was added to 1 ul T_4 DNA Ligase 10x buffer and 2 ul of the PCR product, 1 ul T_4 DNA ligase (3 Weiss unit; Wiess, et al., 1968), and 5 ul of MilliQ water to a final volume of 10 ul.

Reaction (B): same as reaction (A) except that 1 ul of the PCR product and 6 ul of MilliQ water were added.

Reaction (C): This was the positive control which was the same as reaction (A) except that instead of the PCR product, 2 ul of control insert DNA were added.

The tubes were mixed by pipetting and were incubated overnight at 4 ^{0}C. 40 ul of MilliQ water were added to each reaction after the ligation was completed.

Preparation of Agar Plates:

950 ml of milliQ water, 10 g Bacto-trypton, 5.0 g Bacto-yeast extract and 10.0 g Nacl were mixed. The pH was adjusted to 7.0 with 5 N NaOH, and 15.0 g bacto-agar were added, the volume made to 1.0 liter with milliQ water and autoclaved for 20 minutes at 15 lb / sq. inches on liquid cycle. The medium was cooled to about 50 °C before adding ampicillin (50 ug / ml) . Plates were then poured directly from the flask, 30-35 ml of medium per 90-mm plate. After the medium had harden completely, the plates were inverted and stored at 4 °C. The plates were incubated at 37 °C 2-3 hours before they were used.

Transformation:

Most methods for bacterial transformation are based on the observations of Mandel and Higa (1970), who showed that bacteria treated with ice-cold solutions of $CaCl_2$ and then briefly heated could be transfected with bacteriophage lamda DNA. The same method was subsequently used to transform bacteria with plasmid DNA (Cohen, et al., 1972) and E. coli chromosomal DNA (Olishi and Cosloy, 1972).

Subcloning of JM109 Competent cells (Promega cat. # A3610 JM109) was used for routine subcloning into plasmid vectors according to the manufacturer's procedure. JM109 competent cell stored at -70 °C were thawed on ice. 5.0 ul of

the ligation product above were added to 50 ul of the competent cells, mixed gently with pipet tip, and incubated 30 minutes on ice. The cells were heat shocked by incubation for 20 seconds at 37 °C followed by 2 minutes on ice. 950 ul of LB medium were added and the tube incubated for 1 hour at 37 °C. LB medium was prepared by mixing 950 ml of milliQ water, 10 g Bacto-trypton, 5.0 g Bacto-yeast extract, and 10.0 g Nacl, the pH was adjusted to 7.0 with 5 N NaOH. The volume was adjusted to 1.0 liter with milliQ water and autoclaved for 20 minutes at 15 lb/square inch on liquid cycle. The cells were isolated by centrifuging 20 seconds at 3,000 x g. Eight hundred ul of supernatant were removed and the cells were suspended in the remaining 200 ul of LB medium. To isolate cells containing recombinant pGEM-T Easy Vector, 40, 60, and 100 ul of the cell suspension were spread on agar plates containing 40 ul of 100 mM isopropyl 2-D thiogalactopyranoside (IPTG) in water, 40 ul of 2% 5-bromo-4-chloro-3-indolyl-2-D-galactopyraniside (X-gal) in dimethyl-formamide, using sterile rod and incubated overnight at 37 °C. Positive colonies were selected and cultured in 10 ml medium with ampicillin (50 ug / ml) and then incubated aerobically Overnight at 37 °C in a 225 rpm shaker. The DNA miniprep kit (Promega Wizard *plus* Minipreps DNA Purification System, cat. # A7500) was used to purify the product. The plasmid with the insert was then quantified and submitted for sequencing.

Restriction Enzyme Digest:

Restriction enzyme digest was performed to check for the presence of the amplified fragment in the cloning vector. Restriction enzymes bind specifically to and cleave double-stranded DNA at specific sites within or adjacent to particular sequences known as recognition sites (Roberts, 1988).

An EcoRI digest (New England Biolabs® Inc. cat. # 101S) was performed using 0.25 ul of each of the cloned samples above (250 ng/ul) placed in a 1.5 ml eppendorf tube containing 7.75 ul MilliQ water and 1 ul EcoRI enzyme buffer (10x buffer) and 1 ul of EcoRI enzyme. The tube was incubated at 37 °C for 4 hours. The digestion product was then run in 1% agarose gel for 1½ hours.

Sequencing:

ABI automated sequencer (Perkin-Elmer Applied Biosystems, model 373; protocol P/N 402114) was used to sequence the DNA in both direction, which implies the chain termination method (Sagner and Coulson, 1977).

Microcon-100's chromatography columns (Amicon cat # 42413) were used to purify and concentrate 100 ul of PCR product. The DNA was then quantified by comparing it with known bands of 9 cut with Hind III.

1) sequencing reaction:

To 8.0 ul ABI (sequencer) Ready Reaction Mix in a 200 ul PCR tube, 10 ul milliQ water, 1.0 ul PCR product (10 ng / 100 bp), and 1.0 ul primer (3.2 pmole) were added. Because samples were light sensitive, all subsequent steps were done in reduced light. All samples were put in a thermal cycler and a sequencing program was run.

2) Post sequencing reaction preparation:

The above PCR was stopped, and then 74 ul of 70% ETOH with 0.5 mM $MgCl_2$ was added to the 20 ul sequencing reaction tube. The product was then transferred to 1.5 ml eppendorf tube. Samples were mixed and remained at room temperature for 15 minutes, then centrifuged for 20 minutes at 4 °C at 10,000 x g with the back edge of the tubes pointed to the outside of the centrifuge. The supernatant was removed carefully with a vacuum and the samples were dried for 3 minutes using a speed vac. Samples were stored at room temperature until they were loaded in the sequencing gel.

3) Preparation of samples for loading:

To each DNA sample tube, 4.0 ul loading buffer, 5.0 ml formamide, and 0.5 g AmberLite Resin were added and vortexed for 1 minute. The tubes were heated at 90 °C for 3 minutes to denature the DNA. The samples were centrifuged briefly to bring the solution to the bottom of the tube. The tubes were

placed immediately on ice before being loaded into the ABI sequencing gel. The loading buffer was made by mixing 0.5 ml EDTA (0.5 M), 9.5 ml Sterile water, and 0.5 g Blue Dextran.

PCR of HSL for 6 Bovine Breeds:

Six different breeds of cattle, obtained from Michigan Cattlemen's Association / Michigan State University Farm-to-Fab Program, were used to amplify a partial sequence of exon 8 for HSL gene from their genomic DNA (10 ng /ml). In a 200 ul PCR tube, 0.25 ul Taq DNA polymerase(5 u/ul) was added to 47.70 ul PCR premix (5.0 ul Taq DNA polymerase buffer (10x), 0.5 ul dNTPs (10 mM), 2.5 ul Upper primer (7.6 pM/ul), 2.0 ul Lower primer (10.4 pM/ul), 4.0 ul $Mgcl_2$ (25 mM), and 34.75 ul MilliQ water were vortexed briefly and then 2 ul genomic DNA (10 ng / ul) were added. The thermal cycler machine was set as follows:

Robocycler:

94 °C for 10 minutes

94 °C for 50 sec. ⇨ ⇨ ⇨ 30 Times

55 °C for 50 sec. ⇨ ⇨ ⇨ 30 Times

72 °C for 1 minutes ⇨ ⇨ ⇨ 30 Times

72 °C for 10 minutes

6 °C for ∞

1% agarose gel, 2 ul 100 bp ladder, 6 ul PCR product, running

time was 1 hour.

Since this was the only part of the bovine HSL gene being amplified by the different PCR trials, other approaches were then tried to see if the HSL gene could be isolated from a bovine genomic DNA library.

Titering Bovine Genomic Library:

Since it was first used as a cloning vehicle (Murray and Murray, 1974), bacteriophage 9 has occupied a central role in molecular cloning. It adsorbs to receptors in the outer membrane of the bacteria that are normally used to transport maltose into the cell.

A bovine genomic library (adult male liver digested with MboI) was obtained from Clontech (cat. # BL1015j). The fragments (average size was 15 Kb) were cloned at the BamH1 site in phage EMBL3 SP6/T7. The titer of the phage was determined by standard procedures using the bacterial host K802. The Clontech library protocol was followed for titering the phage (protocol # PT1010-1; version # PR47377). 2, 5, 10, and 0 ul of the diluted phage (1:250,000 dilution) were incubated with 100 μl (2 X 10^8 cells) of the bacteria for 20 minutes at 37 °C. The phage-infected bacteria were added to 4 ml of sterile top agarose (10 g bacto trypton, 5 g bacto yeast extract, 10 g NaCl, and 8 g of agarose in 1 liter of milliQ

water) in 15 ml tubes at 50 °C. Top agarose was layered onto 90 mm agar Petri dishes[top agarose: 10 g bacto trypton, 5 g bacto yeast extract, 15 g bacto agar, and 5 g NaCl in one liter of milliQ water which was autoclaved and cooled to Room temperature]. The plates were incubated overnight at 37 °C. The individual lysed bacterial colonies were not in contact with each other.

Plate Lysate:

Adsorption of bacteriophage particles to the receptors is facilitated by magnesium ions and occurs efficiently and rapidly (within a few minutes) both at room temperature and at 37 °C. However, penetration of the cell by bacteriophage DNA and the subsequent events in the lytic cycle do not occur efficiently at room temperature.

450 ul of the diluted titered samples were plated after infecting 600 ul of bacteria (K802) at 37 °C for 25 minutes. The plates were incubated at 37 °C for 11 hours, until fully lysed. Phage were eluted by adding 12 ml 1x dilution buffer [100 ml of 10x dilution buffer: 5.83 g of 1.0 M NaCl, 2.47 g of 0.1 M $MgSO_4$, 3.5 ml of 1.0 M Tris-HCl (pH 7.5) and MilliQ water to a final volume of 100 ml, autoclave and store at 4 °C] and 2 ml of 2% gelatin to each 150 mm plate and left overnight at 4 °C. The plates were then placed on a rocker and slowly shaken at room temperature for 1 hour. The 1x dilution buffer

was collected from each pair of plates and put in 50 ml tubes. The plates were washed with 2 ml of 1x dilution buffer and then added to the 50 ml tubes. These 50 ml tubes were centrifuged at 2,000Xg for 10 minutes at 4 °C. DNase 1 (1 ug/ml) and RNase A (5 ug / ml) were added to the supernatant and incubated at 37 °C for 1 hour. Then 5 g of PEG_{800} and 2.92 g of Nacl were added to each tube and the volume brought to 50 ml with 1x dilution buffer. The tubes were left on ice Overnight to precipitate. The next day PEG_{800} crystals were dissolved and small amounts of the white cloud of the phage were seen. The supernatant was decanted after centrifuging (2000 xg) for 30 minutes at 4 °C.

The precipitate was resuspended in 1x dilution buffer and transferred to 15 ml tubes. The solution was extracted three times with CH_3Cl by centrifuging at 2500-3000 xg for 15 minutes at 4 °C until the white interface disappeared. 20 ul of EDTA (0.5 M), 20 ul 10% SDS, and 10 ul proteinases K (10 mg/ml) were added and the tubes were incubated for 1 hour at 50 °C in a shaking water bath and cooled at room temperature The tubes were then phenol / chloroform extracted twice by centrifuging each time at 2,500 xg for 15 minutes at room temperature. The tubes were extracted again with CH_3Cl. The solution was aliquoted to 1.5 ml eppendorf tubes. 1/10 volume NaOAc pH 7.0 and two volume absolute ETOH (freezer cold) were added to each tube, then centrifuged at 12,000 xg for 15 minutes at 4 °C. The supernatant was decanted and the pellet

was rinsed with 70% ETOH and centrifugated (microcentrifuge to max. speed) for 5 minutes. The pellet was dried at room temperature, resuspended in 80 ul TE buffer pH 8.0 and kept overnight at 4 °C. 5 ul of the sample was run in a 1% agarose gel to check for the presence of the library DNA.

1 ul RNase (10 mg/ml) was added to 1 ul of the phage lysate above and then incubated at 37 °C for 1 hour, the 2 ul were used in a 50 ul PCR reaction (5 ul Taq Polymerase buffer, 1 ul dNTPs (10 mM), 2.5 ul upper primer (7.6 pM/ul), 2 ul lower primer (10.4 pM/ul), 33.25 ul milliQ water, 4 ul MgCl (25 mM), 2 ul of bovine library above, and 0.25 ul Taq Polymerase (5 u/ul))

The thermal cycler was adjusted as follows:

94 °C for 5 minutes
94 °C for 50 sec ⇨ ⇨ ⇨ 30 Times
55 °C for 50 sec. ⇨ ⇨ ⇨ 30 Times
72 °C for 1 minutes ⇨ ⇨ ⇨ 30 Times
72 °C for 10 minutes
6 °C for ∞

The PCR product was then run in a 1% agarose gel

Screening The Bovine Genomic Library:

1) Plaque Lifting:

Oligonucleotide screening was performed as described by Grunstein and Hogness (1975).

Nylon membranes (Hybond-N from Amersham part # 71720-31) were used for plaque lifting from 25 plates of bovine genomic library (Clonetech cat # BL1015j). Two membranes were used for plaque lifting from each plate which were then treated with a UV cross-linker and left overnight to air dry. They were packed in Zipper plastic bags and shelved for further processing.

Agar plates containing the bacteria infected with the phage were incubated overnight at 37 °C. Lysed colonies grew large enough till they come in contact with each other. The petri-dishes were then cooled for at least 30 minutes at 4 °C before the dry nylon membranes were placed on the agar . Each membrane was marked 'DNA' and the plate numbered with a blunt ended pencil. The membranes were folded in half with the DNA side outward. The resulting troughs were placed across the center of the petri-dishes. The troughs were released and the membranes were allowed to sit on the surface for 30 seconds, the disk position was marked at three positions by using a sterilized sharp pin. The membrane was removed from the petri-dishes after 30 seconds in one continuous movement using sterilized forceps. The same process was repeated with another

membrane. Membranes were put in a denaturation solution (1.5 M NaCl and 0.5 N NaOH in water) for 3 minutes, then in a neutralization solution (1.5 M Nacl, 0.5 M Tris-HCl pH 8.0) for 3 minutes. The membranes were then washed for 5 minutes in 2x SSC (1 x SSC is 150 mM NaCl, 15 mM Na citrate pH 7.0) and transferred, DNA side up, to a pad of 3MM paper to air dry. DNA was fixed to the membrane by using UV cross-linker C_3 (HyBaid model # H9320; Benton and Davis, 1977) and left to air dry overnight. They were packaged in zipper plastic bags and kept for further processing.

2) Probe Labeling:

DNA fragments were routinely labeled to a high specific activity by the random hexamer priming method (Fienberg and Vogelstein, 1983). The concentration (70 ng/ul) of the bovine HSL PCR product (264 bp) to be labeled with ^{32}P was measured at 260 nm. The concentration was confirmed with 9 cut with Hind III and run in 1.5 % agarose gel for 40 minutes.

Amersham standard protocol (cat. # 1601Z) was used for probe labeling. 1 ul Probe DNA (70 ng) to be labeled was dissolved in 4 ul MilliQ water in a 1.5 eppendorf tube, heated at 95 °C in a boiling water bath for 2 minutes, and then chilled on ice for 10 minutes. The tube was centrifuged briefly to concentrate the contents at the bottom of the tube, then the tube was placed on ice. 10 ul of random primer buffer were added to the 5 ul of denatured probe above. 5 ul of

random hexanucleotide primers, 23 ul milliQ water, 5 ul of a mixture of ATP, dGTP, dTTP, and [^{32}P]dCTP containing 50 uCi (≈3000 Ci/mmol), and 2 ul Klenow enzyme (exonuclease free) were added consecutively.

The reaction was mixed gently by pipetting up and down and spun for a few seconds. The reaction was carried out overnight at room temperature under the hood. A second reaction was run for 30 minutes at 37 °C . Micro Bio-Spin chromatography columns (Bio-Rad cat. # 732-6202) were used to isolate the product from the reaction mixture. The exclusion limit for these columns was 5 base pairs. Samples were diluted 1:10 before placing on the column.

Micro Bio-Spin Chromatography Columns:

The columns were inverted sharply several times to resuspend the settled gel and remove any bubbles. The tips were snapped off and the columns were placed in a 2.0 ml microcentrifuge tube, then the top caps were removed. The excess packing buffer was allowed to drain by gravity to the top of gel bed (about two minutes). The drained buffer was discarded and then the columns were placed back into the 2.0 ml tube. The columns were then centrifuged for 2 minutes in a microcentrifuge at 1,000xg to remove and discard the remaining packing buffer. The columns were placed in 1.5 ml microcentrifuge tubes. The samples (20-75 ul) were carefully applied directly to the center of the column. The columns were

centrifuged for 4 minutes at 1,000xg. The purified samples are now in Tris buffer. Molecules smaller than the column's exclusion limit are retained by the column.

3) Hybridization:

When using oligonucleotides as probes to hybridize with complimentary DNA sequences , the aim is to find conditions that are stringent enough to guarantee specificity and sufficiently flexible to allow formation of stable hybrids at an acceptable rate.

Each 6-8 nylon membrane, prepared as described above, were placed in a UV cross linker's cylinder. The labeled probe was denatured in a 100 °C water bath for 10 minutes and then put on ice, 30 ml prehybridization solution [50 ml Formamide, 25 ml SSPE 20x (Clonetech manual), 5 ml Denharts 100x, 0.3 g SDS, and 20 ml water] were poured into the cylinders, containing the membranes, and the temperature was adjusted to 50 °C . After rocking the cylinder for two hours the prehybridization solution was dicarded and 5 ml of hybridization solution [5 ml Formamide, 2.5 ml SSPE 20x, 0.5 ml Denharts, 0.01 g SDS, 0.1 ml Denatured Salmon Sperm DNA (denatured at 95-100 °C for 2 minutes), 1.9 ml water] were poured in the cylinders. The denatured labeled probe was pipetted gently to the center of each cylinder and left on a rocker overnight.

The next day the hybridization solution was removed, the

membranes were washed with wash buffer 1 (50 ml 20x SSC, 2.5 g SDS, and 450 ml sterile water) for 20 minutes at 50 °C in the cylinders. The membranes, were transferred to tupperware where they were washed with wash buffer 2 at 65 °C (50 ml 20x SSC, 1.0 g SDS, and 950 ml sterile water), in a shaking water bath, for 1 hour. Then the membranes were put in wash buffer 3 (5 ml 20x SSC, 495 ml sterile water) at 65 °C in a water bath for 1 hour. Membranes were taken individually and placed on 3MM Whatman papers cut to the size of the membrane and wrapped with Saran Wrap.

Autoradiography:

Autoradiography produces permanent images on photographic film of the distribution of radioactive atoms on a two-dimensional surface. In molecular cloning, autoradiography is used for a variety of purposes, including the visualization of bands of radioactive nucleic acids in Southern and Northern hybridization and localization of bands of DNA in gels.

Wrapped membranes were placed in film cassettes and taken to the dark room to add the film. Between 4-8 membranes were put on one film for autoradiography. The membranes were exposed to a Kodak X-ray film in cassettes at -80 °C for 72 hours(Sambrook, et al., 1989).

Positive plaques were picked using blue pipet tips cut from the edge and then sucked with a pipetman and put in a 1.5

ul eppendorf tube containing 1 ml sterile 1x lamda dilution buffer. A drop of chloroform was added and the tubes were vortexed briefly, and left overnight at room temperature. The next day the tubes were centrifuged at 8,000g for 2 minutes and the solution transferred to new tubes. These tubes were titered and plated again, as above, for a second screen. Positive colonies were picked again and a third screen was done.

Probe Labeling:

The PCR product, obtained by using the primers that were used successfully to amplify part of exon 8 of HSL gene from genomic DNA as described before, was used as a probe for plaque lifting and Southern blot. The PCR product to be labeled was first quantified by comparison with known bands of 9 cut with Hind III (Figure 16). The probe was then labeled with ^{32}P and the percentage of the ^{32}P incorporated into the probe was calculated as follows:

(CPM after column / CPM before column) X 100% .

When 70 ng DNA were used, the incorporation was 16.9% when the reaction was incubated overnight at room temperature compared to 5.67% when it was incubated for 30 minutes at 37 °C.

Total amount of ^{32}P was 1.36 X 10^7 cpm for the overnight sample, and 5.83 X 10^6 for the 37 °C sample.

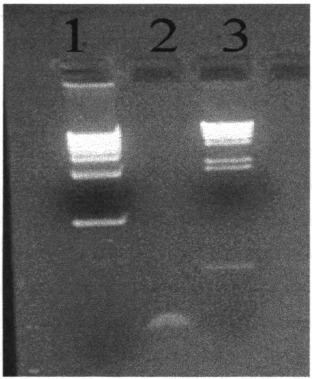


Figure 16. **Agarose gel image illustrating the quantification of PCR product (263 bp) of part of exon 8 of the bovine HSL to be used as a probe. 9 cut with Hind III was used as standard for the quantification**

Lane 1 ⇨ I Kb Ladder
Lane 2 ⇨ 5 ul PCR product
Lane 3 ⇨ 5 ul 9 cut with Hind III

Southern Blot:

Localization of particular sequences within genomic DNA is sometimes accomplished by transfer techniques described by Southern (1975).

3 ng of genomic DNA extracted from each of Simmental, Tarentaise, Shorthorn, and Holstein breeds were digested, in four different tubes, with 15 U of EcoRI at 37 °C . The digests were left overnight in a 20 ul reaction volume. The next day the samples were run in 1% agarose gel until the dye reached the end of the gel (150V; 1 ½ hour.). The gel was stained with ETBr [300 ul (10 mg/ul) in 500 ul water] for 15 minutes, destained for 1 hour in water, and photographed. The agarose gel was placed for 15 minutes in tupperware containing 250 ml depurination solution (0.25 N HCl). A nitrocellulose membrane, that was cut to the size of the gel and marked DNA on one side, was bended in half without creasing it, then the membrane at the bend was immersed into a tray of milliQ water for 5 minutes. The membrane was transferred to a tray containing 10x SSC for a minimum of 15 minutes. The depurination solution was carefully poured off and the gel rinsed once in 250 ml of milliQ water. The gel was then denatured by incubating in 250 ml of denaturing solution (0.5 N NaoH, 1.5 M Nacl) for 20 minutes. The denaturing solution then was poured off and the gel was rinsed once in 250 ml of milliQ water. The gel was neutralized by incubating in 250 ml

of neutralization solution (1.0 M Tris-HCL pH 7.5, 1.5 M Nacl) for 20 minutes with occasional rocking. The agarose gel tray was then placed upside-down in a tupperware container. 10x SSC was added so that the level was half-way up the side of the gel tray. The wick of filter paper (3MM Whatman paper cut 3 inches more than the size of the gel) was then saturated in 10x SSC and aligned on the back of the gel tray. Air bubbles were removed using a disposable glass Pasteur pipet. The gel was placed face up on the saturated filter papers and checked that no air bubbles were trapped between the gel and the wick. The wetted nitrocellulose membrane was then placed DNA side down on the gel. The membrane was checked so as not to hang over the gel and come in contact with the wick. A pasteur pipet was then used to remove air bubbles. One pre-cut filter paper saturated with 10x SSC was placed on the top of the membrane. The other two dry filter papers were then added to the stack. They were checked to make sure that they were not in direct contact with gel or the wick. The filter paper was then covered so as to not directly contact the gel or wick, with the 3-inch high stack of pre-cut paper towels. The paper towels were then covered with a rigid support. The whole apparatus was then covered with a plastic wrap to prevent evaporation. A blot weight of about 200-500 g was placed on the rigid support. Transfer of DNA was allowed to precede overnight(Southern, 1975). The membrane was removed from the gel and placed DNA side up on a clean filter paper. The gel

was then checked under the UV light to make sure that the DNA was transferred properly. The membrane was rinsed for about 5 minutes in 6x SSC. The membrane was laid DNA side up on a clean sheet of filter paper to air dry and then X-Linked. Hybridization and autoradiography with a labeled probe was done as above.

Bovine PCR for Pools and Individual:

PCR was carried out on 100 ng of DNA from each of the Holstein pools (6 animals), Angus pools (2 animals), Hereford pools (5 animals), Shorthorn cow, Tarentaise cow, and Simmental cow [10 ul genomic DNA(10 ng/ul) 1.0 ul dNTPs (10 mM), 2.5 ul upper primer(7.6 pM/ul), 2.0 ul lower primer(10.4 pM/ul), 25.25 ul milliQ water, 4.0 ul $Mgcl_2$ (25 mM), 5.0 ul Taq DNA Polymerase Buffer (10x), 0.25 ul Taq DNA polymerase (5 U/ul)].

Robocycler:

94 °C for 5 minutes
94 °C for 50 sec/ ⇨ ⇨ ⇨ 30 Times
55 °C for 50 sec. ⇨ ⇨ ⇨ 30 Times
72 °C for 1 minutes ⇨ ⇨ ⇨ 30 Times
72 °C for 10 minutes
6 °C for ∞

The PCR product was run in 1% agarose gel

The PCR products above were cloned in pGEM-T Easy vector as described above. Then the PCR products were purified using Microcon columns from Amersham (cat. # 42413).

Single-Strand Conformation Polymorphism (SSCP):

The SSCP technique is based on the fact that single-stranded DNA has a sequence-specific secondary structure. Sequence differences as small as a single base change can affect this secondary structure and can be detected by electrophoresis in a non-denaturing polyacrylamide gel. Double-stranded mutant and wild-type samples are first denatured into single strands and then loaded onto the gel. Differences in mobility of the single strands between the control wild-type DNA and the other samples indicate a mutation. SSCP is a widely used mutation screening method because of its simplicity. However, since experimental conditions cannot be predicted for a particular DNA, it is important to optimize gel electrophoresis conditions. The cold SSCP method (Hongyo, et al., 1993) and an SSCP machine (BioRad model DCode System) were used. When connected to an appropriate external chiller, the Dcode system can control the buffer temperature between 5-25 °C. The electrophoresis

cooling tank is outfitted with two cooling fingers. Tygon tubing connects the cooling fingers in the electrophoresis tank to an external chiller. The chiller recirculates a coolant through the cooling fingers which, in turn, cool the buffer. The external chiller is set to cool the coolant to approximately -20 °C, and the Dcode heater regulates the buffer temperature. 8% gel (No glycerol) reaction was prepared [8 ml 40% acrylamide / Bis solution, 4 ml 10 x TBE, and 23.5 ml MilliQ water were mixed and degassed for 15 minutes before adding 40 ml TEMED and 400 ul 10% Ammonium Persulfate(0.1 g Ammonium Persulfate in 1.0 ml milliQ water)]. The gel was cast immediately after the above step and was placed at 4 °C for 7 hours.

8 ul of SSCP 2x loading dye (0.05% Bromophenol Blue, 0.05% Xylene cyanol, 95% Formamide, 20 mM 0.5 M EDTA, pH 8) were added to 8 ul of each PCR product and the samples were placed in heating blocks at 95 °C for 10 minutes and then on ice before loading. The best running condition was 10 °C for 6 hours.

Bovine Spleen cDNA Library:

PCR was carried out, as above, on bovine spleen cDNA and bovine genomic DNA libraries.

Robocycler:

94 °C for 5 minutes

94 °C for 50 sec ➪ ➪ ➪ 35 Times

55 °C for 50 sec. ➪ ➪ ➪ 35 Times

72 °C for 1 minutes. ➪ ➪ ➪ 35 Times

72 °C for 10 minutes

6 °C for ∞

Materials

Bacto-trypton, bacto-yeast extract, and bacto-agar were from DIFCO laboratories. Agarose, tris-base, lamda cut with Hind III, and EDTA were from GIBCOBRL. Sodium chloride, sodium hydroxide, sodium acetate, chloroform, and boric acid were from J.T. Baker. T_4 DNA ligase was from New England Biolabs® Inc.. Formamide, and PEG_{8000} were from Fisher Biotechnology. SDS, IPTG, X-gal, ampicillin, RNase A, and DNase 1 were from Boehringer Mannheim. Denharts, and sheared Salmon sperms were from Eppendorf. TEMED, acrylamide/bis acrylamide solution, 1 Kb molecular ruler, 100 bp rular, and ammonium perusulfate were from Biorad. 3MM whatman papers were from Whatman. The restriction enzymes, and Taq DNA polymerase, were from Promega, BMB and New England Biolabs® Inc.. Proteinase K was from Life technology. Ethidium bromide, bromophenol blue, and xylene cyanol were from Sigma

RESULTS

Quantification of Genomic DNA:

Promega strongly suggests not to use A_{260} measurement to quantify DNA. Therefore, to estimate DNA concentration, genomic DNA was run in 1% agarose gel along with 1.0 ul 9 cut with Hind III as a marker (Figure 4). The estimated genomic DNA concentration isolated from both the heparin and the EDTA tubes was found to be the same (25 ng/ul).

Primer Design:

More than fifteen different sets of primers were not able to amplify any of the HSL 5 fragments, described above, after several experiments using different combinations of primers and adjusting reaction conditions. The only successful ones were for part of exon 8. The sequence of those primers were:

Upper primer = 21 bp; Tm= 65.7 ^{0}C; GAC ACA ACA GAC ACG CCA GAG

Lower primer = 17 bp; Tm = 64.9 ^{0}C; CAG CAG CGG TGA CAT GAA G

Expected product length was 263.

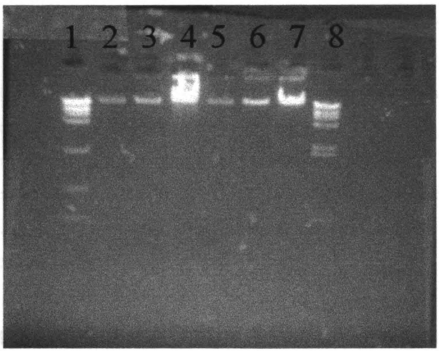


Figure 4. **Agarose gel image illustrating the same concentration of bovine genomic DNA obtained when the whole blood was collected in heparin and EDTA tubes. The lamda cut with Hind III was used as standard to measure the concentration.**

Lane 1	⇨	1 ul 9 cut with Hind III
Lane 2	⇨	1 ul genomic DNA (EDTA tube)
Lane 3	⇨	2 ul genomic DNA (EDTA tube)
Lane 4	⇨	4 ul genomic DNA (EDTA tube)
Lane 5	⇨	1 ul genomic DNA (heparin tube)
Lane 6	⇨	2 ul genomic DNA (heparin tube)
Lane 7	⇨	4 ul genomic DNA (heparin tube)
Lane 8	⇨	0.5 ul 9 cut with Hind III

Polymerase Chain Reaction (PCR):

When six PCR (Figure 5, panel 1) for part of exon 8 and 4 PCR (Figure 5, panel 2) for a fragment consisting of exons 2, 3, 4, 5, and the intron between them (according to the human sequence) were performed, the expected product lengths were 263 bp and 1062 bp, for exon 8 and exon 2-5 respectively (Figure 5). Only the fragment for exon 8 (Figure 5, panel 1) was amplified but not the other fragment (Figure 5, panel 2) that consists of exon 2, 3, 4, 5, and the introns between them although different reaction conditions and sets of primers were tried.

Miniprep and Restriction Enzyme Digest:

The PCR product (263 bp) from the above reaction (Figure 5, panel 1) was cloned successfully into pGEM-T plasmid vector and transformed into bacteria (strain JM109). The transformed bacteria were then plated onto agar plates and incubated overnight at 37 °C. Three white colonies and one blue colony (as a negative control) were picked randomly and grown overnight in a LB liquid media in a 37 °C shaker. A miniprep was done and the products were cut with EcoRI and run in 1% agarose gel for 1½ hours (Figure 6) to check for the presence of the insert. A 263 bp insert and 3.3 kb plasmid were clear on the gel when the clone was cut with EcoRI in lanes 1, 2, and 3 (Figure 6). Only the circular uncut plasmids appeared

on the other lanes.

Sequencing:

The cloned bovine PCR product was sent to the sequencing facility at Michigan State University along with both the forward (upper) and reverse (lower) primer. The sequence (Figure 7 and 8) was edited whenever the color-coded signal was clear. A bestfit command was done, to the edit sequence using the GCG program (http://www.gcg.com/), with HSL for other species and the bovine published sequence (GenBank accession # U78042). A high homology was found between the amplified part of exon 8, the published HSL gene for bovine sequence (GenBank accession # U78042; Figure 9), swine (Figures 10) rat (Figure 11), and human (Figure 12). Also the sequence was blasted into the NCBI home page (http://searchlauncher.bcm.tmc.edu) and it hit the HSL gene for different species.

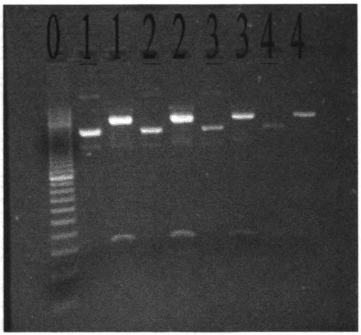


Figure 6. **Agarose gel image (1%) illustrating part of exon 8 of HSL PCR products (263 bp) cloned in pGEM-T vector and cut with EcoRI**

Lane 0 ⇨ PCR ruler
Lane 1 ⇨ Uncut cloned sample (the vector and the insert), white colony # 1
Lane 1 ⇨ The insert cut from the plasmid , white colony # 1
Lane 2 ⇨ Uncut cloned sample (the vector and the insert), white colony # 2
Lane 2 ⇨ The insert cut from the plasmid , white colony # 2
Lane 3 ⇨ Uncut cloned sample (the vector and the insert), white colony # 3
Lane 3 ⇨ The insert cut from the plasmid , white colony # 3
Lane 4 ⇨ Uncut sample from the miniprep (the blue colony)
Lane 4 ⇨ The digestion product for the blue colony.

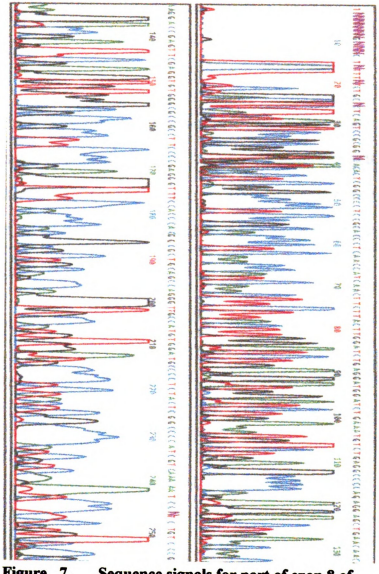


Figure 7. Sequence signals for part of exon 8 of bovine HSL gene
The red peak is Thymine
The green peak is Adenine
The black peak is Guanine
The blue peak is Cytocine

CTNTTTNTCTGCNCTCAGTCCGCGGNCACACTCGGCCCCTCCGCACCCTCAACCATCAAC

TTCTTACTTGGATCTGAGGATGGATCTGAAATGTCTGAGGCCCCAGAGGAGCTGAACAAC

AAGGACCGAGTTCGAGGTGTGGGCGCCGCCTTCCCCGAGGGTTTCCACCCACGGCGCTGC

AGCCAGGGTTGCCATGTGGATGCCCCTCTACTCGGCCCCATCGTCAAAAATCCNTCTGTC

CCGNCGGGGAA

Figure 8. The HSL PCR product sequence (251 bp) for part of bovine exon 8 before editing showing 4 more bases at the beginning and 14 more base at the end (underlined) of the sequence compared to the 233 bp sequenced on this study.

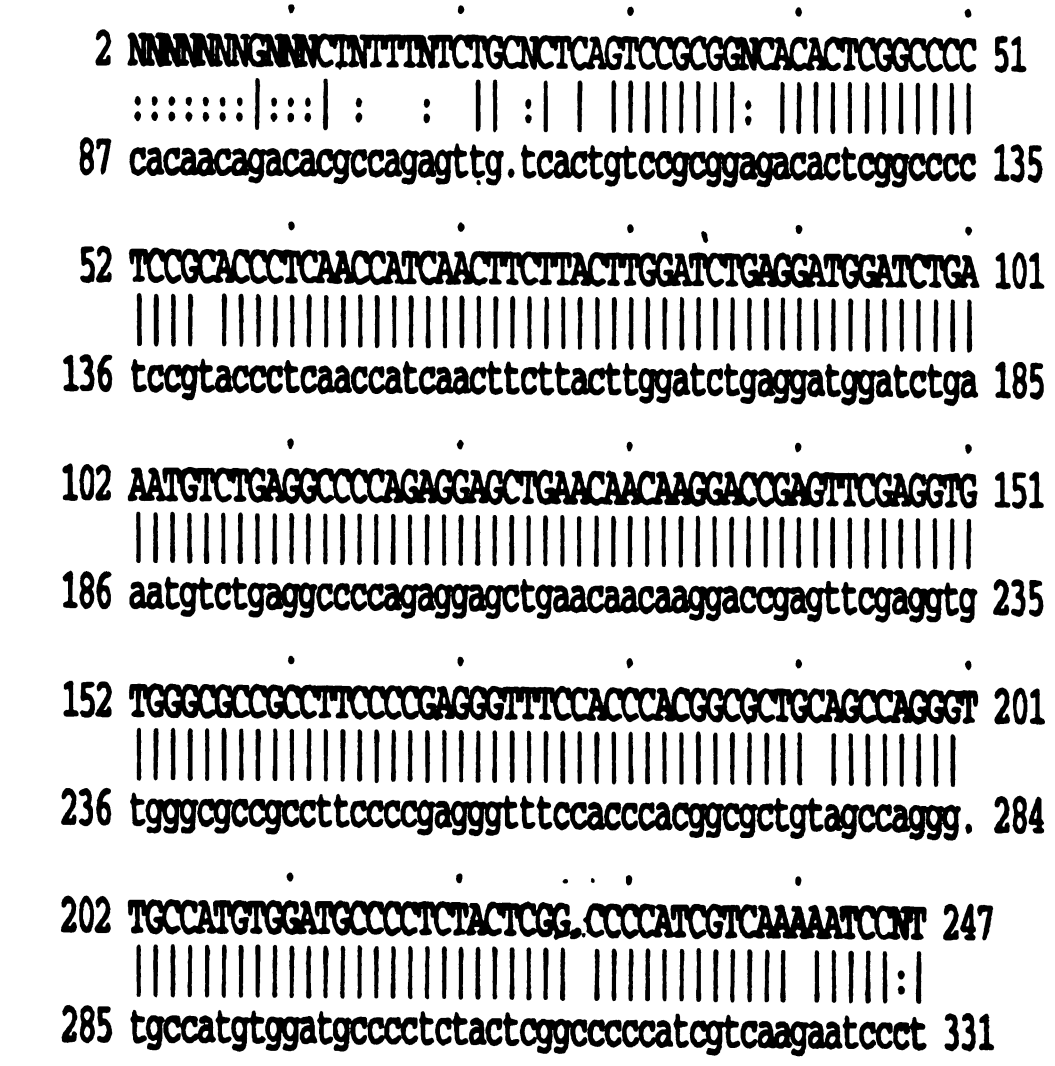


Figure 9. **Alignment of part of the bovine exon 8 HSL PCR product sequence (upper) before editing and the published bovine HSL partial sequence (lower) accession # U78042. Note the high homology between the two sequences which indicates that the sequenced fragment (upper) is part of HSL gene. The alignment was prepared using the GCG program**

Animal1 x SwineHSL

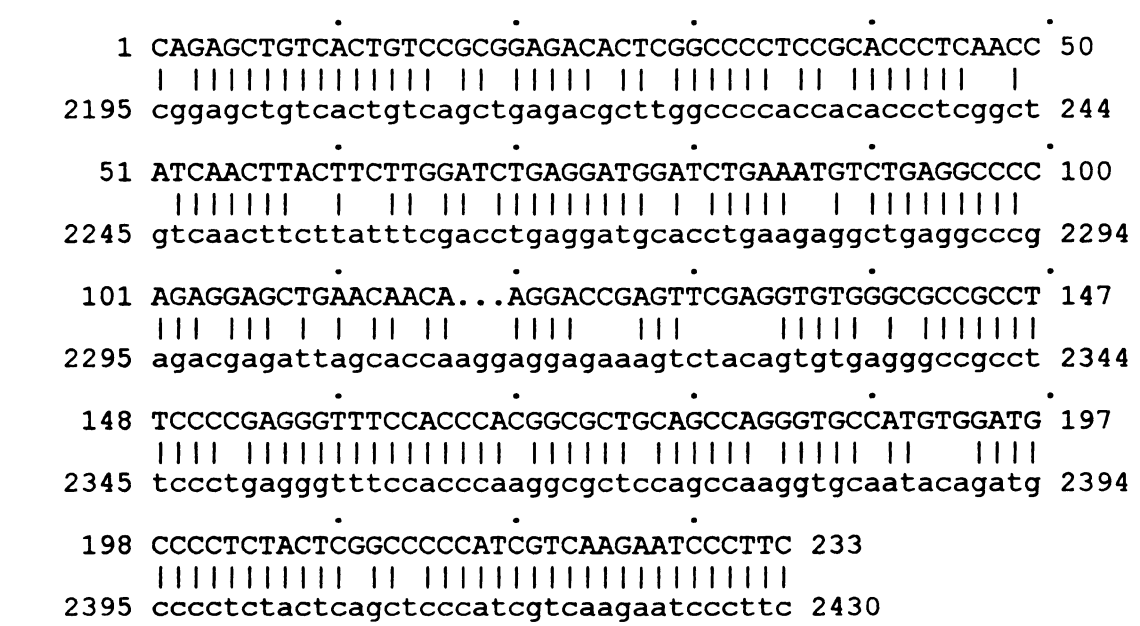


Figure 10. **Sequence comparison between part of the bovine exon 8 HSL PCR product (upper) and part of the swine HSL gene (lower) showing 78% homology.**

Animal1 x RatHSL

Figure 11. **Sequence comparison between part of the bovine exon 8 HSL PCR product (upper)and part of the rat HSL gene (lower) showing 73% homology. The alignment was prepared using the GCG program**

Animal1 x HumanHSL

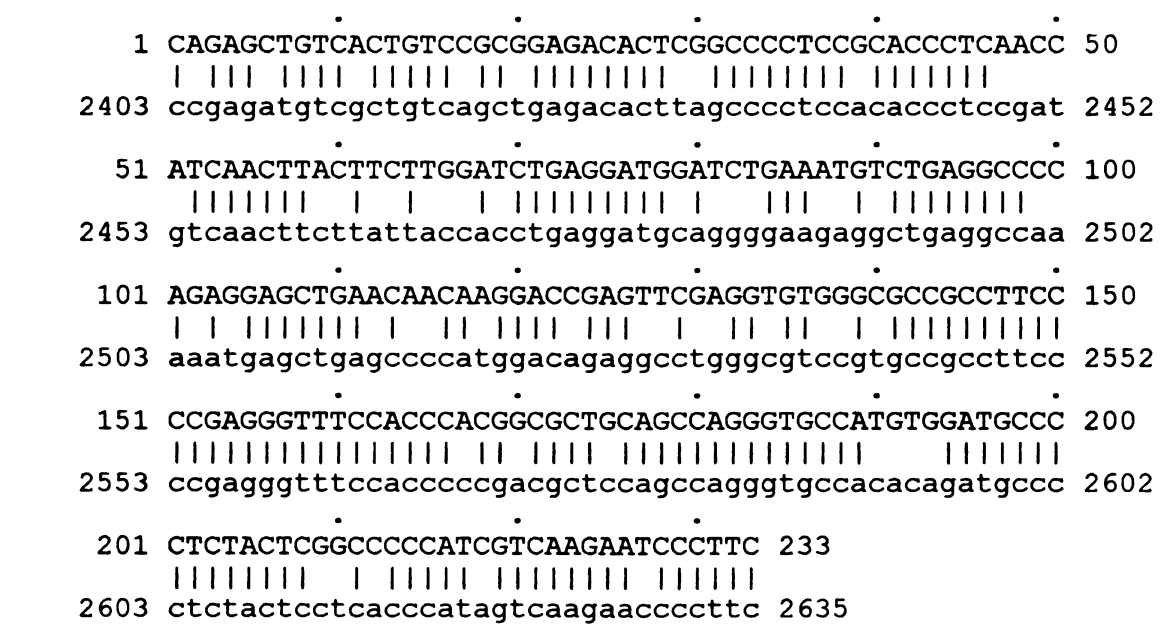


Figure 12. **Sequence comparison between part of exon 8 (233 bp) of the bovine HSL PCR product (upper)and part of the Human HSL gene (lower) showing 76% homology. The alignment was prepared using the GCG program**

PCR of HSL for the 6 Bovine Breeds :

When 32 animals from six breeds of cattle (6 Brahman, 5 Simmental, 3 Tarentaise, 5 Shorthorn, 5 Angus, and 8 Holstein cows) were used to amplify the partial sequence of HSL gene from their genomic DNA (10 ng/ml), only PCR products from 26 animals were obtained (5 Brahman, 5 Simmental, 3 tarentaise, 5 Shorthorn, 2 Angus, 6 Holstein; Figure 13).

The Holstein breed was originally from Netherlands, Tarentaise was from France, Angus was from Scotland, Brahman was from India, Shorthorn was from British Isles, and Simmental was from Switzerland.

263 bp were obtained from the PCR product of 5 Brahman, 5 Simmental, 3 Tarentaise, 5 Shorthorn, 2 Angus, and 6 Holstein as shown in Figure 13. This amplified fragment is located in exon 8, downstream of the phosphorylation site. All attempts to amplify the other parts of the gene were not successful, although different primers and reaction conditions were used.

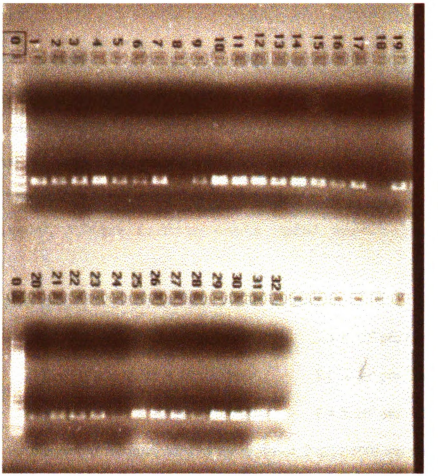


Figure 13. **Agarose gel image (1%) illustrating PCR products (263 bp) of part of exon 8 of HSL gene from 6 different bovine breeds**

Lane 0 ⇨ 100 bp Ladder
Lane 1 to 32 ⇨ The 32 animals samples (Brahman, Simental, Tarentaise, Shorthorn, Angus, Holstein)

Titer and Phage Lysate of the Bovine Genomic DNA Library:

When using 2, 5, 10, and 0 ul of the diluted bacteriophage 9 (1:250,000 dilution) from a male bovine liver genomic DNA library (Clontech corporation) to infect 100 μl (2 X 10^8 cells) of the bacteria and plated onto top agarose plates, the number of lysed colonies were 59, 171, 297, and 0 respectively. Plaque forming units (pfu) were calculated as follows:

pfu = (# of plaques / ul used) X dilution factor X 10^3 ul / ml

Plate # 1 **(59 / 2) X 25 X 10^4 X 10^3 ul / ml = 7.4 X 10^9 pfu / ml**

Plate # 2 **(171 / 5) X 25 X 10^4 X 10^3 ul / ml = 8.6 X 10^9 pfu / ml**

Plate # 3 **(297 / 10) X 25 X 10^4 X 10^3 ul / ml = 7.4 X 10^9 pfu / ml**

To use 3 X 10^4 colonies as recommended by the protocol, 2 ul (1:500) were needed.

2 ul (1:500) = (7.4 X 10^9 X 2) / (5 X 10^2 X 10^3) = 3 X 10^4

It was found that it was better to use 1 ul (1:500) in-order to obtain half that amount of colonies. The DNA from the eluted phage was run in 1% agarose gel to check for the presence of genomic DNA (Figure 14). Large DNA fragments (more than 10 Kb)were observed in lanes 1 to 7 (Figure 14), as suggested by the company (Clonetech), and the same fragments were observed, after being purified with columns, in lanes 1′ to 7′. The purified samples were used later on in PCR, library screening, and hybridization.

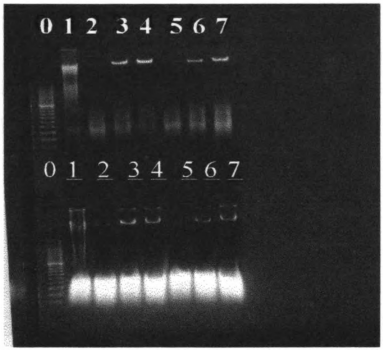


Figure 14. **Agarose gel image illustrating DNA samples obtained from bovine genomic library**

Lane 0 ⇨ 100 bp Ruler
Lane 1 ⇨ Sample after column purification
Lane 1 ⇨ Sample before column purification
Lane 2 ⇨ Sample after column purification
Lane 2 ⇨ Sample before column purification
Lane 3 ⇨ Sample after column purification
Lane 3 ⇨ Sample before column purification
Lane 4 ⇨ Sample after column purification
Lane 4 ⇨ Sample before column purification
Lane 5 ⇨ Sample after column purification
Lane 5 ⇨ Sample before column purification
Lane 6 ⇨ Sample after column purification
Lane 6 ⇨ Sample before column purification
Lane 7 ⇨ Sample after column purification
Lane 7 ⇨ Sample before column purification

PCR for the Genomic DNA Library:

No PCR product was obtained when the genomic DNA library was used as a template in a PCR as shown in lanes 1 to 6 (Figure 15). The bovine genomic DNA was used as a positive control in lane 7. The primers were the same primers that were used successfully to isolate the HSL gene fragment from the genomic DNA as described above (Figure 13).

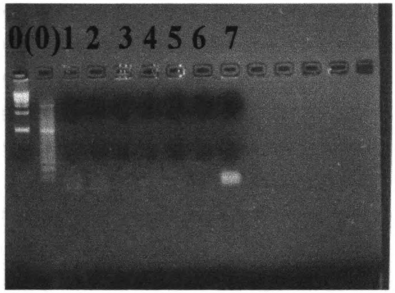


Figure 15. **Agarose gel image (1%) illustrating part of exon 8 PCR products of bovine genomic DNA library (nothing being amplified) and bovine genomic DNA (263 bp) using the same primers and reaction conditions**

Lane 0 ⇨ 1 kb Ladder
Lane (0) ⇨ 100 bp Ladder
Lane 1 ⇨ Phage Lysate Sample (genomic library)
Lane 2 ⇨ Phage Lysate Sample (genomic library)
Lane 3 ⇨ Phage Lysate Sample (genomic library)
Lane 4 ⇨ Phage Lysate Sample (genomic library)
Lane 5 ⇨ Phage Lysate Sample (genomic library)
Lane 6 ⇨ Phage Lysate Sample (genomic library)
Lane 7 ⇨ Bovine Genomic DNA

Hybridization and Autoradiography:

The PCR product of part of exon 8 (Figure 16) was used as a probe, after being labeled , to screen the genomic DNA library by hybridizing to the nylon membranes that were used in plaque lifting. The membranes were then exposed to the X-ray film to later identify positive signals. About four false positive plaques were found (Figure 17). The positive signals were then picked for a second and a third screening to check for false positives. No positive signals were found after the third screening. Therefore, this procedure was rejected as a way to isolate the HSL gene.

Southern Blot:

Genomic DNA from each of Simmental, Tarantaise, Shorthorn, and Holstein breeds were digested with EcoRI and examined using Southern blot. The resulting fragments were separated, according to their sizes (<100 bp - >10,000 kb), by electrophoresis (1% agarose gel; Figure 18 and 19). After the Southern blot was completed, the gel was observed under a UV light to make sure that the DNA was transferred successfully. No DNA was left behind as shown in Figure 20. After hybridization with the probe, the autoradiography showed positive signals (Figure 21). The probe was not able to pick up the gene after the background was eliminated.

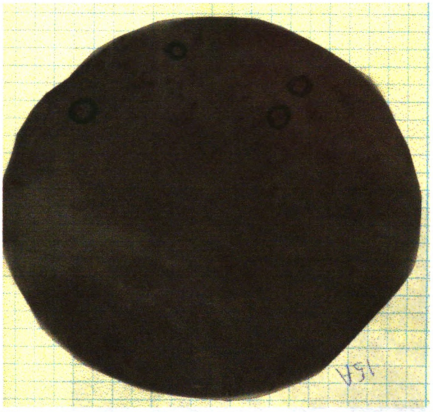


Figure 17. **X-ray film illustrating signals for HSL gene picked up by P^{32} labeled probed from the first genomic library screening**

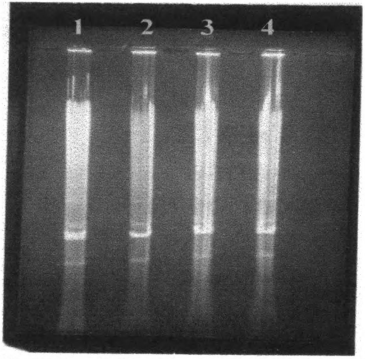


Figure 18. **Southern blot analysis (1). Agarose gel image illustrating the separation of genomic DNA bands from 4 different breeds after digestion with EcoRI for hybridization with labeled probe**

Lane 1 ⇨ Simmental genomic DNA
Lane 2 ⇨ Tarantaise genomic DNA
Lane 3 ⇨ Shorthorn genomic DNA
Lane 4 ⇨ Holstein genomic DNA

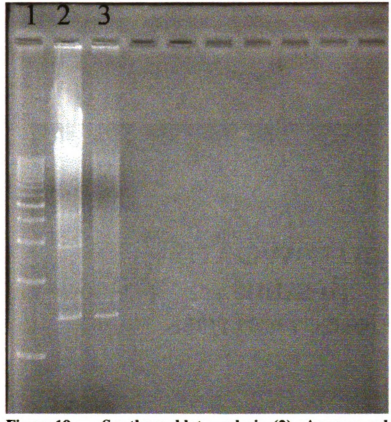


Figure 19. **Southern blot analysis (2). Agarose gel image illustrating bands separated of Holstein and Shorthorn genomic DNA after digestion with EcoRI for hybridization with labeled probe**

Lane 1 ⇨ I Kb Ladder
Lane 2 ⇨ Holstein genomic DNA
Lane 3 ⇨ Shorthorn genomic DNA

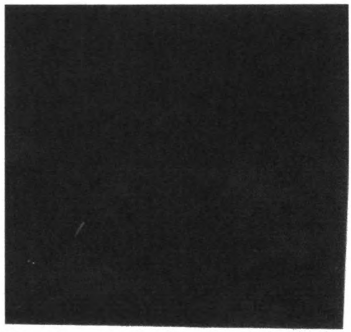
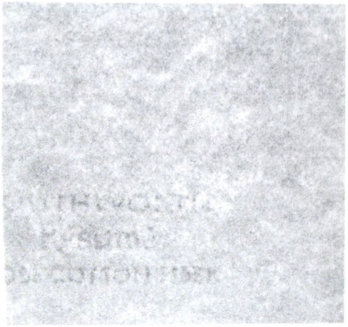


Figure 20. **Southern blot analysis (1). Agarose gel image illustrating the lack of DNA bands from digested genomic DNA of 4 different cattle breeds as a result of a complete transfer of the bands to the nylon membrane**



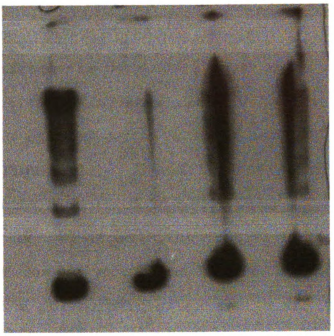


Figure 21. **Southern blot analysis (1). X-ray film illustrating DNA bands of 4 different cattle breeds after labeled probe hybridization and before eliminating the background**

Bovine PCR for Pools:

PCR, using the set of primers described in the primer design section, was also done for the genomic DNA of Holstein pool, Angus pool, Hereford, Shorthorn cow, Tarentaise cow, and Simmental cow. The PCR product was run in 1% agarose gel, about 263 bp fragments were observed as expected (Figure 22).

The amplified fragments above were then quantified by running in an agarose gel with known bands of lamda cut with Hind III (Figure 23). The fragments were purified with Microcon columns and cloned in pGEM-T Easy vector as described before and then sequenced. The results were then used as a second proof for polymorphisms due to the undetermined (N) signals at the polymorphic sites and to the presence of two expected bases (A and G) at the same polymorphic position.

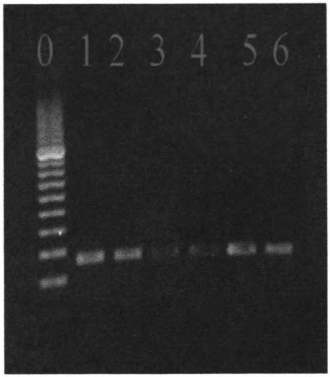


Figure 22. Agarose gel image illustrating part of exon 8 HSL PCR products (263 bp) for bovine genomic DNA pools and individuals

Lane 0 ⇨ 100 bp Ladder
Lane 1 ⇨ Holstein Pool
Lane 2 ⇨ Angus Pool
Lane 3 ⇨ Hereford Pool
Lane 4 ⇨ Shorthorn Cow
Lane 5 ⇨ Tarentaise Cow
Lane 6 ⇨ Simmental Cow

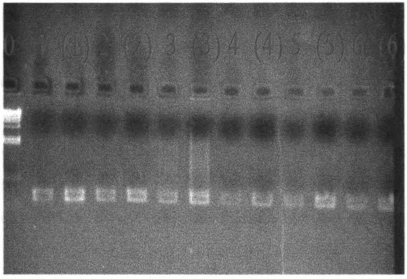


Figure 23. **Agarose gel image (1%) illustrating the quantification of the genomic DNA PCR product (263 bp) of part of exon 8 of HSL gene for individual and pooled bovine breed. lamda cut with Hind III was used as standard for the quantification**

Lane 0 ⇨ 9 cut with Hind III (0.5 ug)
Lane 1 ⇨ 0.5 ul Holstein Pool
Lane (1) ⇨ 1.0 ul Holstein Pool
Lane 2 ⇨0.5 ul Angus Pool
Lane (2) ⇨1.0 ul Angus Pool
Lane 3 ⇨0.5 ul Hereford Pool
Lane (3) ⇨ 1.0 ul Hereford Pool
Lane 4 ⇨ 0.5 ul Shorthorn Cow
Lane (4) ⇨ 1.0 ul Shorthorn Cow
Lane 5 ⇨ 0.5 ul Tarentaise Cow
Lane (5) ⇨ 1.0 ul Tarentaise Cow
Lane 6 ⇨ 0.5 ul Simmental Cow
Lane (6) ⇨ 1.0 ul Simmental Cow

Single-Strand Conformation Polymorphism (SSCP):

When SSCP is used , it is expected to see a difference in mobility in a single stranded DNA when there is a polymorphism at one or more bases of that DNA fragment. The bovine PCR fragments, obtained from individual cows, were denatured and run in 8% non-denaturing polyacrylamide gel. No difference in band migration was observed, and all the 263 bp fragments were seen at the same migration position in all lanes (Figure 24). 6 different SSCP reactions had been tried under different reaction conditions but polymorphism was not detected.

Bovine Spleen cDNA Library:

PCR products were not obtained from a bovine spleen cDNA library using the same primers that amplified the genomic DNA isolated from six different breeds of cattle (Figure 25). It may be that the HSL gene is not expressed in the spleen.

Figure 24. **Single-strand conformation polymorphism gel image (8% non-denaturing polyacrylamide gel) illustrating the same distance of band migration of part of exon 8 HSL PCR products (263 bp) of 6 different bovine breeds**

Lane 0 ⇨ 8 ul Undenatured PCR product
Lane 1 to 32 ⇨ 8 ul Denatured PCR products for six different cattle breeds (Brahman, Tarentaise, Angus, Semintal, Shorthorn, and Holstein)

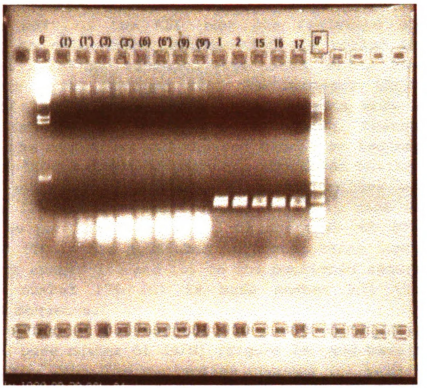


Figure 25. **Agarose gel image (1%) illustrating PCR products of part of exon 8 of HSL from bovine spleen cDNA library (nothing being amplified) and bovine genomic DNA (263 bp) from 4 different bovine breeds**

Lane 0 ⇨ ul 9 cut with Hind III (0.5 ug)
Lane (1) ⇨ 4 ul PCR product of Spleen cDNA lib.
Lane (1/) ⇨ 4 ul PCR product of Spleen cDNA lib.
Lane (3) ⇨ 4 ul PCR product of Spleen cDNA lib.
Lane (3/) ⇨ 4 ul PCR product of Spleen cDNA lib.
Lane (6) ⇨ 4 ul PCR product of Spleen cDNA lib.
Lane (6/) ⇨ 4 ul PCR product of Spleen cDNA lib.
Lane (9) ⇨ 4 ul PCR product of Spleen cDNA lib.
Lane (9/) ⇨ 4 ul PCR product of Spleen cDNA lib.
Lane 1 ⇨ 4 ul PCR product of Brahman genomic DNA
Lane 2 ⇨ 4 ul PCR product of Brahman genomic DNA
Lane 15 ⇨ 4 ul PCR product of Shorthorn genomic DNA
Lane 16 ⇨ 4 ul PCR product of Tarentaise genomic DNA
Lane 17 ⇨ 4 ul PCR product of Holstein genomic DNA
Lane 0/ ⇨ 100 bp ladder

Part of the Bovine HSL Gene Sequence and Polymorphisms:

When part of the HSL gene was sequenced and edited according to color-coded signals for six bovine breeds (26 cows total), 233 bp fragments were noted, which were part of HSL bovine gene (Figure 26). The sequence shows 233 bp instead of 263 bp because the PCR products were sequenced directly and not cloned before sequencing, therefore, few bases at the beginning and the end cannot be sequenced. When this sequence was compared with the published bovine sequence (GenBank accession # U78042), five nucleotides were found different (Figure 27). Base number 6 (C) is base number 116 (T) in the published sequence, base number 40 (C) is base number 140 (T) in the published sequence, bases number 59 and 60 (AC) are number 159 and 160 in the published sequence (CT), and base number 176 (C) is base number 276 (T) in the published sequence.

Two potential polymorphic sites were noted (Figure 28). These were base number 51 (A/G) (Figure 29) and base number 93 (G/A) (Figure 30). The nucleotide changes for base 51 change the amino acids from Isoleucine to Valine and from Glutamic acid to Lysine in base 93 (Figure 31). 19 cows out of the 26 have an A instead of a G in position 51 (frequency of 73%), and 3 cows out of 26 have an A on position 93 instead of a G (frequency of 11.5%). No heterozygous alleles (a diploid nucleus that contains two different alleles for a particular gene) were found among the animals.

All the shorthorn and Angus breeds have an 'A' at position 51, and all the Tarentaise cows have a 'G' on the similar position,. The Brahman, Simmental, and Holstein breeds may have either 'A' or 'G' at position 51, but they have more 'A' than 'G'. All the Brahman, Tarentaise, Angus, and Holstein cows have 'G' on position 93. Simmental and Shorthorn have either 'A' or 'G' on that position, Simmental have more 'G' than 'A' on position 93, 'A' and 'G' are equal for Shorthorn.

Cluster Analysis:

A cluster analysis of the 233 bp fragments sequenced from 26 samples from six different cattle breeds was done according to the Wards hierarchical clusters algorithm as implemented in Clustan Graphics (http://clustan.com/). This is a computer software package developed by D. Wishart

(University of Edinburgh, Scotland, 1996). The cluster analysis is an exploratory tool for solving classification problems (Gordon, 1981). The Jukes-Cantor genetic distance metric, which is suitable for DNA sequence data (Weir, 1996), was used to compute the similarity matrix. Cows were grouped according to the 233 bp sequences of HSL in three different groups and two subgroups. Samples from all breeds except Tarentaise were clustered in one group. Samples from Holstein, Brahman, Simmental, and Tarantaise were a second group. The third group has Shorthorn and Tarentaise. Two subgroups emerged from the three groups, one subgroup contains Holstein Brahman, Simmental and Tarentaise. The other subgroup has Simmental, Tarentaise and Holstein (Figure 32).

CAGAGCTGTCACTGTCCGCGGAGACACTCGGCCCCT
CCGCACCCTCAACC **(G/A)** TCAACTTACTTCTTGGA
TCTGAGGATGGATCTGAAATGTCT **(G/A)** AGGCCCC
AGAGGAGCTGAACAACAAGGACCGAGTTCGAGGTGT
GGGCGCCGCCTTCCCCGAGGGTTTCCACCCACGGCG
CTGCAGCCAGGGTGCCATGTGGATGCCCCTCTACTC
GGCCCCCATCGTCAAGAATCCCTTC

Figure 26. **Part of exon 8 sequence of the bovine HSL gene (233 bp) illustrating the two (G/A) polymorphic sites (bold)**

Animal1 x Pub1.hsl

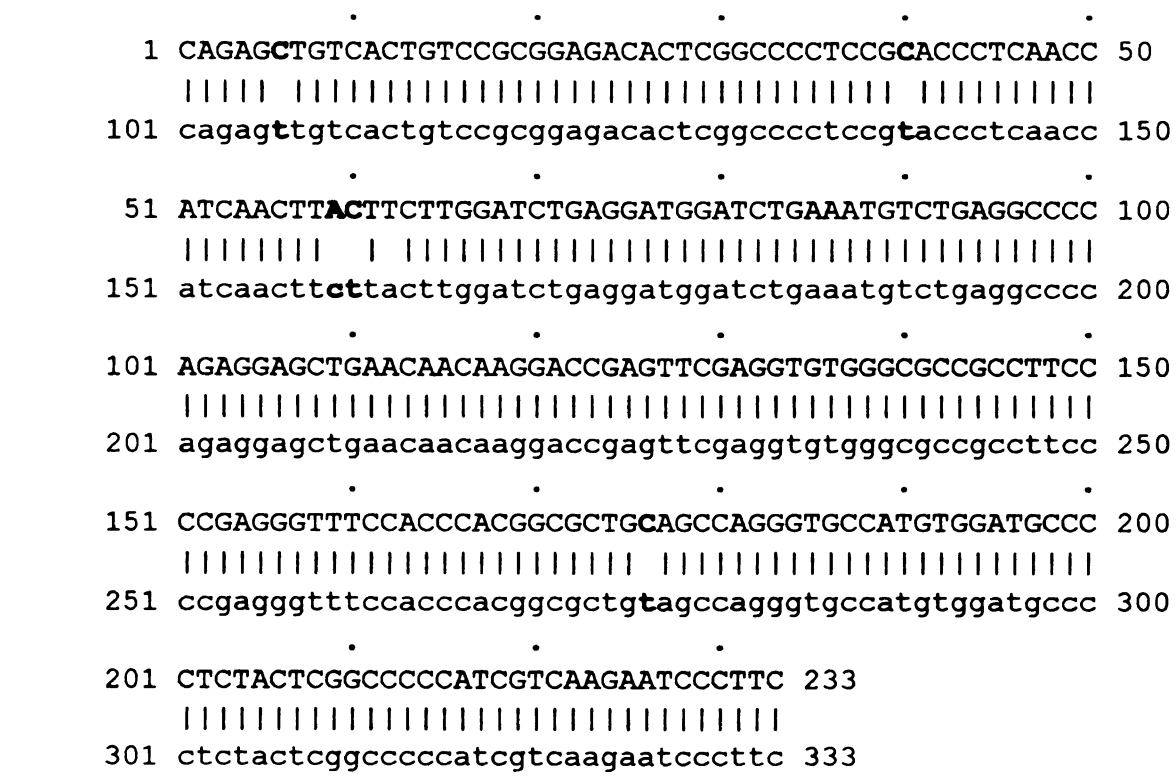


Figure 27. **Alignment of part of the bovine exon 8 HSL PCR product sequence (233) after editing (upper) and the published bovine partial HSL sequence (lower) accession # U78042. The alignment was prepared using the GCG program. Note the 5 bases difference between the two sequences (bold)**

Br ACC**A**TCAACTTCTTGGATCTGAGGATGGATCTGAAATGTCT**G**AGGCC
Br ACC**A**TCAACTTCTTGGATCTGAGGATGGATCTGAAATGTCT**G**AGGCC
Br ACC**A**TCAACTTCTTGGATCTGAGGATGGATCTGAAATGTCT**G**AGGCC
Br ACC**G**TCAACTTCTTGGATCTGAGGATGGATCTGAAATGTCT**G**AGGCC
Br ACC**A**TCAACTTCTTGGATCTGAGGATGGATCTGAAATGTCT**G**AGGCC
Si ACC**A**TCAACTTCTTGGATCTGAGGATGGATCTGAAATGTCT**G**AGGCC
Si ACC**G**TCAACTTCTTGGATCTGAGGATGGATCTGAAATGTCT**G**AGGCC
Si ACC**G**TCAACTTCTTGGATCTGAGGATGGATCTGAAATGTCT**G**AGGCC
Si ACC**A**TCAACTTCTTGGATCTGAGGATGGATCTGAAATGTCT**A**AGGCC
Si ACC**A**TCAACTTCTTGGATCTGAGGATGGATCTGAAATGTCT**A**AGGCC
Ta ACC**G**TCAACTTCTTGGATCTGAGGATGGATCTGAAATGTCT**G**AGGCC
Ta ACC**G**TCAACTTCTTGGATCTGAGGATGGATCTGAAATGTCT**G**AGGCC
Ta ACC**G**TCAACTTCTTGGATCTGAGGATGGATCTGAAATGTCT**G**AGGCC
Sh ACC**A**TCAACTTCTTGGATCTGAGGATGGATCTGAAATGTCT**G**AGGCC
Sh ACC**A**TCAACTTCTTGGATCTGAGGATGGATCTGAAATGTCT**G**AGGCC
Sh ACC**A**TCAACTTCTTGGATCTGAGGATGGATCTGAAATGTCT**G**AGGCC
Sh ACC**A**TCAACTTCTTGGATCTGAGGATGGATCTGAAATGTCT**A**AGGCC
Sh ACC**A**TCAACTTCTTGGATCTGAGGATGGATCTGAAATGTCT**G**AGGCC
An ACC**A**TCAACTTCTTGGATCTGAGGATGGATCTGAAATGTCT**G**AGGCC
An ACC**A**TCAACTTCTTGGATCTGAGGATGGATCTGAAATGTCT**G**AGGCC
Ho ACC**A**TCAACTTCTTGGATCTGAGGATGGATCTGAAATGTCT**G**AGGCC
Ho ACC**A**TCAACTTCTTGGATCTGAGGATGGATCTGAAATGTCT**G**AGGCC
Ho ACC**A**TCAACTTCTTGGATCTGAGGATGGATCTGAAATGTCT**G**AGGCC
Ho ACC**A**TCAACTTCTTGGATCTGAGGATGGATCTGAAATGTCT**G**AGGCC
Ho ACC**G**TCAACTTCTTGGATCTGAGGATGGATCTGAAATGTCT**G**AGGCC
Ho ACC**A**TCAACTTCTTGGATCTGAGGATGGATCTGAAATGTCT**G**AGGCC

Figure 28. The two potential polymorphic sites (bold) for HSL gene in 26 animals from 6 cattle breeds

Br = Brahman
Si = Simental
Sh = Shorthorn
Ta = Tarentaise
An = Angus
Ho = Holstein

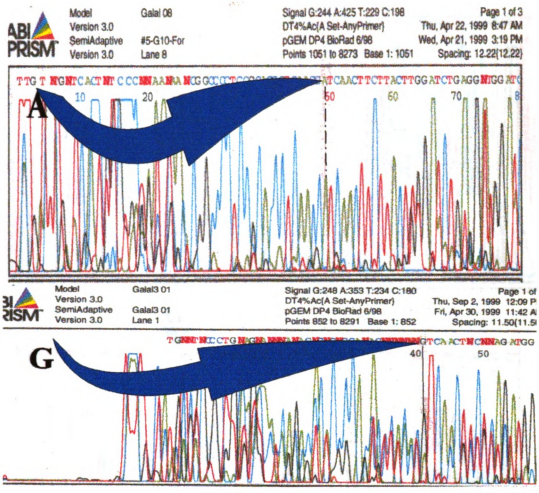


Figure 29. **Part of exon 8 of HSL gene sequence signals. The arrows illustrate an 'A' at position 51 in one animal (top) and a 'G' at a similar position in another animal (bottom)**

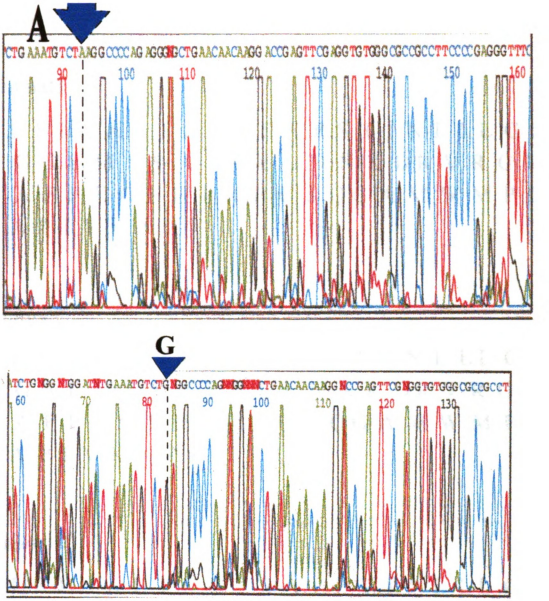


Figure 30. **Part of exon 8 of HSL gene sequence signals . The arrows illustrate an 'A' at position 93 in one animal (top) and a 'G' at a similar position in another animal (bottom)**

Animal1

E L S L S A E T L G P S A P S T **I** N L L L G S E
D G S E M S **E** A P E E L N N K D R V R G V G
A A F P E G F H P R R C S Q G A M W M P L Y
S A P I V K N P F

Animal8

E L S L S A E T L G P S A P S T **V** N L L L G S E
D G S E M S **E** A P E E L N N K D R V R G V G
A A F P E G F H P R R C S Q G A M W M P L Y
S A P I V K N P F

Animal10

E L S L S A E T L G P S A P S T **I** N L L L G S E
D G S E M S **K** A P E E L N N K D R V R G V G
A A F P E G F H P R R C S Q G A M W M P L Y
S A P I V K N P F

Figure 31. Amino acids sequences for part of exon 8 of the bovine HSL gene from three different animals illustrating amino acid differences I/V and E/K (bold and underlined) in two different positions. The amino acid sequence was obtained by using the GCG program

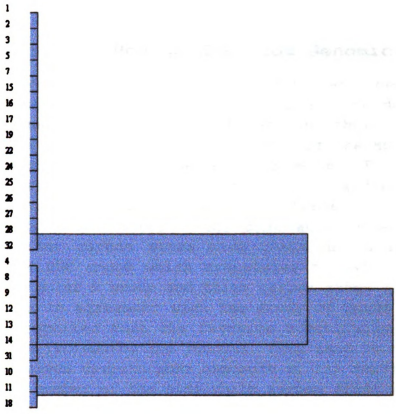


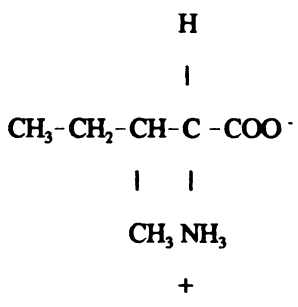
Figure 32. **Cluster analysis, using clustan graphics software, illustrating an association between 5 different groups of animals from 6 bovine breeds according to their HSL gene sequence**

DISCUSSION

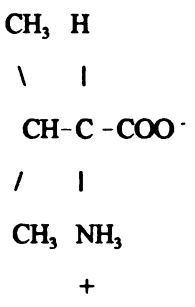
Amplification of Bovine HSL from Genomic DNA:

When blood samples from four different beef breeds (Angus, Tarentaise, Simmental, and Brahman), one dairy breed (Holstein), and one dual purpose breed (Shorthorn) were used to isolate the HSL gene, a 263 bp fragment of the HSL gene was amplified (Figure 13) and sequenced (Figure 26). Polymorphisms were found (Figure 28) in position 51 (A/G) and position 93 (A/G). The change of base 51 (A to G) changes the codon AUC (Figure 31) which translates to the amino acid isoleucine (I), that has a four carbon atoms side chain and a nonpolar R group, to the GUC codon which translates to valine (V) that also has a nonpolar R group and three carbon atoms in its side chain. This is in agreement with the study of Bordo and Argas (1991), who reported that the favorite substitution partners for valine are isoleucine and leucine. They tend to substitute amongst themselves despite some exposure at the surface. This change in the number of the side chain carbon atoms may affect the structure of the protein which could lead to a change in the function, due to the extra space occupied by the fourth carbon atom.

Isoleucine and valine have hydrophobic side chains. The hydrophobic side chains like to cluster by coming together to avoid contact with water.

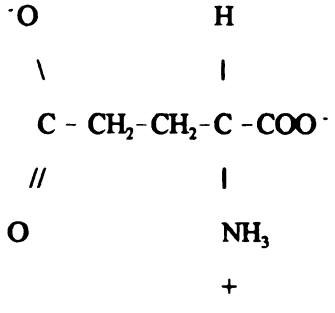


Isoleucine

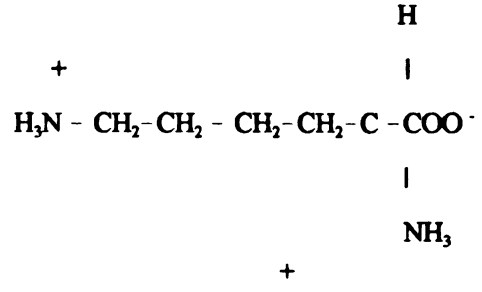


Valine

Changing base 93 (G to A), shown in Figure 28, changes the codon GAG (Figure 31) which translates to the amino acid glutamic acid (E) that has a negatively charged R group at physiological pH, to AAG which translates to lysine (K) having a positively charged R group at neutral pH. Both glutamic acid and lysine have polar side chains that make them highly hydrophilic and therefore located on the surface of the protein in contact with the aqueous solvent. In cases where these amino acids occur in the interior of a protein, they often have a specific chemical function such as promoting catalysis or participating in metal ion binding.



Glutamic Acid



Lysine

A change in an amino acid charge could change the structure of the protein which may change the function. Changing the function of a protein could lead to a desirable or undesirable change in a phenotypic trait and consequently this may be useful in selection.

The mutagenesis experiments of serine^{423}, in exon 6, is the first demonstration in HSL of a relationship between catalytic function and structure (Holm, et al., 1994). Also correlations between milk traits and polymorphism at the growth hormone gene were reported (Lucy, et al., 1993). Another example of the structure and function is in one of the different forms of acetyl-CoA carboxylase coexisting *in vivo*. An insertion of eight extra amino acids located upstream of the phosphorylation site inhibits the phosphorylation by cAMP-dependent protein kinase (Kong, et al., 1990) and consequently fatty acid synthesis. Polymorphisms found here at positions 51 and 93 could be associated with phenotypic traits, such as milk production.

The 233 bp fragment sequenced here resembles part of exon 8 in the Human gene, which is the third largest exon after exon 1 and 9 in human HSL gene. If the 3′ UTR of exon 9 is excluded, exon 8 is the second largest exon. Exon 8 encodes the serine residue (regulatory site) phosphorylated by cAMP-dependent protein kinase and a second phosphorylation site (basal site) upstream of the sequenced bovine fragments. Exon 8 is 425 bp in human and the sequenced bovine fragment is about 132 bp downstream of the start of the exon and about 117 bp downstream of the phosphorylated serine relative to the human sequence. Therefore, the sequenced bovine fragment is near the phosphorylation site in exon 8. Consequently, any changes in the sequence of that fragment could affect the function of the bovine HSL. The sequence analysis of the region downstream of the phosphorylation site has a high homology with the other species which indicates the importance of this region to the function of the protein.

The regulatory site of HSL phosphorylated by cAMP-dependent protein kinase (Garton, et al., 1988) was identified in human as Ser^{551} in exon 8. Ser^{553} has been found to be a second phosphorylation site (basal site). This basal site is phosphorylated by the 5′-AMP-activated protein kinase without concomitant increase of enzyme activity (Garton, et al.,

1989). Phosphorylation of Ser^{553} prevents the phosphorylation of the regulatory site (Garton et al., 1989). The hydroxyl group on the serine makes it much more hydrophilic (water-loving). Therefore, when found on the surface of the protein, serine is more reactive than some other amino acids.

Although Figures 10, 11, and 12 show homology between the HSL bovine sequenced fragment, the swine (78% homology), rat (72% homology), and human (76% homology), all attempts to amplify the whole bovine gene from bovine genomic DNA, using the conserved sequences of other species, was not successful. This may be because the primers were designed according to the human HSL gene sequence with the assumption that there is a high homology between the human and bovine HSL gene sequences and also the number and the positions of the exons and introns were assumed similar. Therefore, there could be an interruption of the different bovine exons by large introns which are not found in other species. This is probably why most of the primers failed to amplify the HSL gene from the bovine genomic DNA.

An example of HSL gene difference is that the phosphorylation site sequence (Met-Arg-Arg-Ser-Val-Ser-Glu-Ala-ala) is completely conserved in bovine (Garton, et al., 1998), rat (Holm, et al., 1988B), mouse, and human HSL (Langin, et al., 1993). The region surrounding these residues is predicted to be hydrophilic and K-helical, consistent with a position at the surface of the protein accessible to the protein kinases (Kyte and Doolittle, 1982). The human HSL differs from the rat sequence in the region immediately upstream of the phosphorylation site (Langin, et al., 1993), where the phosphorylation site is at the beginning of exon 8. The 12 amino acids deleted in human HSL modifies the HSL secondary structure since a connecting loop present in the rat HSL between a hydrophilic region and the K-helix containing the phosphorylation sites is not found in human HSL. The contact between the triacylglycerol substrate and the active serine probably requires a conformational change in this segment. An appropriate folding flexibility is important for catalyzing lipolysis and could be achieved by a relatively high number of small-sized amino acids that are known to be involved in peptide chain *2* -turn structures.

It is speculated that HSL may exist in two conformational

states, an active form corresponding to the "open" form exposing a large hydropbobic area observed in other lipases, and inactive("closed") form (Holm, et al., 2000). Phosphorylation of HSL would be required to trigger the transition from the closed to the open form, exposing a hydrophobic area that would interact with the lipids. Any disturbance in the structure of this region could affect the function of the protein.

PCR for the Spleen cDNA:

The gene may not be highly expressed in the bovine spleen, this may be why the primers, that were used to amplify the 263 bp fragment, were not successful in the PCR for the spleen cDNA (Figure 25).

Bovine Genomic Library Screening:

Selection of recombinant clones based on hybridization to specific nucleic acid sequence, is perhaps, the most widely used screening method since it does not require protein expression but only the presence of a correct DNA sequence. Nucleic acid hybridization is the most general approach to screening libraries since the selection of probes and stringency of hybridization can be readily altered to suit the requirements of specific sequence.

Nylon membranes were used to immobilize the DNA fragments. Their advantage over the nitrocellulose membranes is that small nucleic acid fragments (<250 bp), about the size of the probe used here, bind inefficiently to nitrocellulose membranes (Meinkoth and Wahl, 1984).

The HSL gene was not detected by the above method when genomic DNA library was screened. The reason could be that the mammalian genome contains approximately 3 X 10^6 Kb of DNA per haploid genome, so that screening for single copy gene in a mammalian genomic library would be a very difficult task. An alternative is screening a cDNA library which contains only the exons of the genes as compared to the whole gene (introns and exons) in the genomic library. Because a bovine cDNA library from organs that express HSL protein was not found in

the market, bovine genomic library was used. Therefore, there was a very low probability that the gene could be found using the genomic library. This is confirmed when samples of the genomic library were used in the PCR (Figure 15) using the same primers that amplified the 263 bp from the genomic DNA (Figure 13).

Isolation of a clone from a cDNA or genomic library often involves screening the library by several rounds of plating and filter hybridization. This is not only laborious and time-consuming, but also is prone to artifacts, such as false positives commonly encountered in filter hybridization. The false positive signals seen in the first and second screening (Figure 17) could be because background signals are generally higher for nylon membranes than with nitrocellulose as concluded by Johnson et al.(1984).

Southern Blot:

An advantage of using nylon membranes for Southern blots is that high salt is not required, so the DNA transfer can be done directly in 0.5N NaOH (Reed and Mann, 1985). This permits less handling of the gel and filter and more rapid transfer. As shown in Figures 18 and 19, the digestion of the genomic DNA produced separated fragments by electrophoresis. Although a complete transfer of the digested DNA to the Nylon membrane was accomplished (Figure 20), no hybridization to the labeled probe had occurred which remains unexplained. The failure to recover the gene in both the genomic library screening and the Southern assay could also be due to a loss of the hybrid from the Nylon membrane for an unknown reason.

Single-Strand Conformation Polymorphism (SSCP):

Polymorphism was not detected by the PCR-SSCP method, where PCR products, as shown in Figure 13, were used in the SSCP assay (Figure 24) to detect the presence of mutations. This may be because there were many parameters in the SSCP assay that needed to be optimized. Detection of polymorphisms by sequencing rather than the SSCP method is the most accurate method.

Restriction Fragment Length Polymorphism (RFLP):

Restriction fragment length polymorphism (RFLP) is another method to identify polymorphism. The ability to score genetic variation at the recognition sites of restrictions endonucleases has enabled new ways to detect polymorphisms between individuals. RFLP involves a series of steps involving restriction digestion, electrophoresis, and visualization of bands. Restriction endonucleases recognize specific DNA sequences and cut in a specific manner relative to that sequence, but not necessarily within that sequence. RFLP was not used here because when the sequence was entered in the GCG program (http://www.gcg.com/) and a 'map' command used to show all the recognition sites, no restriction enzyme recognition sites that included the polymorphic sites were found. Restriction analyses are usually unable to provide information about the patterns of substitutions that caused observed differences in RFLPs. Actual DNA sequences do provide this information. Therefore, this study relied mainly on the DNA sequence.

Bovine Pool HSL Sequence:

Further evidence for polymorphisms was from the sequences of the Holstein and Angus DNA pools. It was clear that there were signals for both A and G at the same polymorphic site for both positions but one of them (A or G) was stronger than the other (G or A). If we consider the occurrence of the nucleotide A as genotype AA and G as BB, no heterozygous individual (AB) was found. This may be due to the small number of samples or to the absence of heterozygosity among those breeds.

Cluster Analysis:

The objective of the cluster analysis is to group either the data units or the variables into clusters such that the elements within a cluster have a high degree of natural association among themselves while the clusters are relatively distinct from one another. Cluster analysis may be used to

reveal structure relations in the data. One of the principal applications of cluster analysis is to construct taxonomies. Cluster analysis is a device for suggesting hypothesis. The cluster analysis, in this experiment, did group the cows, which means there could be an association between each group of samples. Since we don't know much about the phenotypic traits of those cows relative to production, reproduction, and diseases associated with body fat mobilization, further studies are needed to relate the genotypic differences to different phenotypic traits such as milk production, disease resistant, and cold or heat tolerance for selection purposes. Effect of different polymorphisms on protein function could be determined by studying enzyme activity at different physiological states.

SUMMARY AND CONCLUSION

The objective of this study was to determine if there is polymorphism at the bovine hormone-sensitive lipase locus. It was found that there are two polymorphic sites about 168 bp and 210 bp downstream of the phosphorylation activation site. One polymorphism at base 51 (A/G) and the other at base 93 (A/G) from the start of the sequenced fragment. These changes in bases result in the amino acid substitution (Iso/Val) and (Glu/Lys) at sites 51 and 93, respectively. The amino acid changes may alter enzyme activity by changing the 3D-structure of hormone-sensitive lipase. Variation in the structure of hormone-sensitive lipase may affect rate of lipid hydrolysis. Further studies are needed to establish the functional significance of these polymorphisms. It is also expected that the exon and/or intron positions and lengths of the bovine HSL gene are different than the human HSL gene.

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