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EVALUATION OF SERUM INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS AND THE INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-2 LOCUS FOR POTENTIAL ASSOCIATIONS WITH GROWTH, CARCASS MERIT AND MEAT QUALITY IN BEEF CATTLE

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EVALUATION OF SERUM INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS AND THE INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-2 LOCUS FOR POTENTIAL ASSOCIATIONS WITH GROWTH, CARCASS MERIT AND MEAT QUALITY IN BEEF CATTLE

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ABSTRACT

EVALUATION OF SERUM INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS AND THE INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-2 LOCUS FOR POTENTIAL ASSOCIATIONS WITH GROWTH, CARCASS MERIT AND MEAT QUALITY IN BEEF CATTLE

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The presence of growth-promoting factors in serum and tissues has been recognized since the beginning of the last century. Recent advances in protein chemistry and the development of molecular biology techniques have resulted in the identification, at both the gene and protein level, of several biological activities suspected to belong to the insulin-like growth factor (IGF) family. The IGFs (IGF-I and –II) are unique among peptide growth factors in that they circulate in blood tightly bound to a group of proteins known as the insulin-like growth factor binding proteins (IGFBP), which modulate IGF activity. The objectives of this study were to evaluate relative abundance of serum IGFBP in Angus cattle divergently selected (high vs. low) for serum IGF-I concentration, to identify DNA sequence variation (polymorphisms) at the bovine IGFBP-2 locus, and to investigate potential associations between IGFBP and traits of economic importance. No significant effect of line was observed in the expression of specific IGFBP species in periods before and after the selection lines were divergent for serum IGF-I concentration. However, heifer calves consistently expressed higher levels of the 34kDa IGFBP species than bulls. This protein

was determined to be bovine IGFBP-2 by immunoblot analysis. When the lines were divergent for serum IGF-I, IGFBP-2 was negatively correlated with serum IGF-I concentration and body weights measured throughout the postweaning performance test. Also, two restriction fragment length polymorphisms (RFLP) were identified at the IGFBP-2 locus and both were found to be segregating in different breeds of cattle. IGFBP-2 RFLP alleles identified with the restriction endonuclease *Hind* III were found to be associated with growth and carcass traits. Results of this study support IGFBP-2 as an important regulator of somatic growth in cattle and further investigation of this gene is warranted.

This dissertation is dedicated to my lovely wife Marisol Figueroa-Rodríguez for all her unconditional love and support since the beginning of my college education and also for giving me the most precious gift in life my son Melvin Omar Pagan-Figueroa. Also, I want to dedicate this work to my parents who endowed me the benefit of a college education and who have been always there for me with love, support, and guidance.

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LITERATURE REVIEW

Introduction

Production of meat is almost entirely dependent on the animal growth process, even in an era that has produced great advances in meat production and processing technology. However, many aspects of growth are not completely understood, especially the mechanisms involved in initiation of growth, regulation of growth rate, and termination of growth at maturity (Hedrick et al., 1994). A basic understanding of animal growth has potential for solving problems of efficiency in meat production.

The hormonal regulation of growth is multifactorial and a group of proteins known to significantly affect animal growth and metabolism is the insulin-like growth factor (IGF) system. The IGF system (IGF-I, IGF-II, IGF cell surface receptors and insulin-like growth factor binding proteins) has been implicated as being involved in many biological processes. These include pre- and postnatal growth, lactation, reproduction and immune function. The IGF system plays an important physiological role in the growth and development of mammals by acting locally in specific organs or systemically through circulating IGF-I (Werner et al., 1994). Insulin-like growth factors are present in relatively high concentrations in the blood of most animals and have potent anabolic and differentiation promoting effects. Similarly, it is known that genes encoding IGF-I receptors are expressed in all organs and tissues and that IGF-I acts via endocrine, paracrine, and autocrine mechanisms (Pankov, 1999). Consequently,

IGF-I is believed to play a significant role in regulating tissue and cellular growth (White et al., 1998). In most cells and tissues, the growth promoting action of IGF-I is mediated via the type I IGF receptor (IGF-I-R). This plasma membrane receptor has tyrosine kinase activity and transduces mitogenic and metabolic signals via multiple intracellular pathways (Bach, 1999).

The actions of IGF-I and -II are mediated by specific insulin-like growth factor binding proteins (IGFBP), which compete with IGF receptors for IGF binding, and as a consequence, can affect cell growth. IGFBP directly modulate the IGF-I and -II actions at the target cell either by enhancing or blocking their activity (McGuire et al., 1992; Florini et al., 1996). Therefore, the IGF system plays significant roles in areas directly applicable to animal production systems.

Despite advances in basic knowledge of the IGF system, relatively little is known about its expression, regulation and effects on livestock growth. Furthermore, this system has not been explored for molecular genetic markers associated with variation in growth traits that could be used in marker-assisted selection. Until recently, genetic variation and improvements in growth have been expressed in terms of phenotypic differences between lines of individuals. Recent developments in molecular genetics now enable one to monitor variation directly at the DNA level, thereby greatly expanding the total amount of observable variation, and also enabling genotype to be determined directly.

The application of molecular biology tools and the development of genome maps for livestock species are making it increasingly possible to identify and characterize specific genes associated with traits of economic importance such

as growth. Increased awareness of the mode of action of the IGF system as well as the factors that regulate this system should enhance our ability to optimize animal production (McGuire et al., 1992). Moreover, the identification of molecular genetic markers within the IGF system that are associated with variation in growth traits would provide additional benefits by enabling producers to identify precise genotypes in breeding stock that are associated with superior production. Such markers could be used as biological indicators of genetic merit in groups of animals and to support breeding decisions that would enhance growth traits. This would allow genetic improvements to be achieved in a cost-effective manner and in a reasonably small number of generations.

The IGF System

A. IGF Ligands

The insulin-like growth factors (IGF-I and -II) are mitogenic and differentiation-inducing hormones with structural homology to proinsulin (Stewart and Rotwein, 1996). Both IGF-I and -II consist of a small hydrophobic core of approximately 7 to 7.5 kDa formed by three helical segments corresponding to the B-helix and two A-helices of insulin, which are connected by a 12-residue linker known as the C-region and stabilized by three intramolecular disulfide bonds (Vajdos et al., 2001). Also, in humans, the amino acid sequence homology between IGF-I and IGF-II is 62% (Rinderknecht and Humbel, 1978b). These single chain proteins have amino acid sequences that are highly conserved in mammals (Stewart and Rotwein, 1996). For example, bovine IGF-I is identical to human IGF-I (Honegger and Humbel, 1986). IGF-I from the sheep

is identical to human and bovine IGF-I, except for a substitution in the sheep of alanine for proline at residue 66 in the human and bovine polypeptides (Francis et al., 1989). IGF-II from sheep also differs from bovine IGF-II by a single amino acid, with an alanine residue at position 62 in the ovine and threonine at this position in the bovine (Francis et al., 1989). Francis et al. (1988) reported that bovine IGF-II differed from human IGF-II at three amino acid residues of the C-terminal domain. Serine, isoleucine, and asparagine in human IGF-II were substituted for alanine, valine and serine, respectively at positions 32, 35, and 36 of the C-terminal domain.

The most significant functional difference between the two IGFs is that IGF-I is directly regulated by growth hormone (GH), whereas IGF-II is not (Baxter, 1986). Lemal et al. (1989) reported that bovine GH injections in growing heifers progressively increased serum levels of IGF-I over a six-week period. In beef steers, bovine GH given by a daily injection (Mosley et al., 1992) or as a slow-release implant (Dalke et al., 1992; Schwarz et al., 1993) resulted in a dose-dependent increase in IGF-I concentration. In addition to growth hormone, the expression of the IGFs is modulated by a number of physiological factors, including developmental stage, nutritional status and hormones.

The levels of IGF-I are low prenatally in humans and rise to a peak during puberty before declining slowly throughout adult life (Bach, 1999). In cattle, fetal serum levels of IGF-II are much greater than those of IGF-I and this pattern is reversed in adults such that IGF-I predominates in the circulation (Holland et al.,

1997). In calves, postnatal IGF-I concentrations progressively increase until weaning (Breier et al., 1988).

Unlike GH, which is secreted in a pulsatile manner, IGF-I levels remain fairly constant throughout the day (Bach, 1999) and can be determined from a single blood sample (Bishop et al., 1989). At the same time, IGF-I serum concentration is associated with growth traits in many livestock species (Anderson et al., 1988; Graml et al., 1994) and may be useful as a physiological indicator trait in selection programs designed to improve weight and growth rate in pigs (Buonomo et al., 1987), sheep (Roberts et al., 1990) and cattle (Lund-Larsen et al., 1977; Bishop et al., 1989; Davis and Bishop, 1991).

B. IGF Receptors

Two distinct subtypes of plasma membrane receptors for the IGFs have been identified (Rechler and Nissley, 1990; De Meyts et al., 1994). These receptors are known as the type I and type II IGF receptors. The type I IGF receptor (IGF-I-R) binds IGF-I with equal or greater affinity than IGF-II and also binds insulin with very low affinity (< 2% of IGF-I). The type II IGF receptor (IGF-II-R) binds IGF-II with highest affinity, binds IGF-I with much lower affinity, and does not bind insulin (Florini et al., 1996). Both IGF receptors have been found in skeletal muscle cells from humans (Shimizu et al., 1986) and rodents (Ballard et al., 1986). The IGF-I-R is, like the insulin receptor, a heterotetrameric protein complex containing a tyrosine kinase domain that mediates signal transduction (De Meyts et al., 1994). The structurally distinct IGF-II-R is a monomeric protein lacking tyrosine kinase activity and is also known as the cation independent

mannose-6-phosphate receptor. There is no known signal transduction mechanism that is initiated by this receptor. However, the IGF-II-R plays an important role for the degradation of IGF-II. Inactivation of IGF-II-R expression in mice by gene targeting resulted in fetal overgrowth, skeletal abnormalities, and perinatal death due to overexposure of fetuses to IGF-II (Wang et al., 1994; Lau et al., 1994; Ludwig et al., 1996). In most cells and tissues, the growth-promoting action of the IGFs is mediated via the IGF-I-R (Van Wyk et al., 1985; Czech, 1989) and the IGF-I-R gene is expressed by most cells in vivo as well as in culture. Inactivation of the IGF-I-R gene leads invariably to severe neonatal growth deficiencies as a consequence of hypoplasia in several tissues (Liu et al., 1993). In addition, like the IGFs, the expression of the IGF-I-R gene is modulated by a number of physiological factors, including developmental stage, nutritional status and hormones (Werner et al., 1995).

C. IGF Binding Proteins

The actions of the IGFs are modulated by a family of six high-affinity binding proteins (IGFBP-1–6 that have been identified by molecular cloning of their cDNAs from rat and human tissues (Shimasaki and Ling, 1991). These proteins bind IGF-I and -II with an affinity equal to or greater than that of the IGF-I-R (Rechler and Clemmons, 1998) and they can act in an endocrine, paracrine, or autocrine fashion. Recent reports have established the presence of an additional group of cysteine rich proteins classified as the IGFBP-related proteins (IGFBP-rps 1-10) (Baxter et al., 1998). These proteins bind IGF-I and -II with relatively low affinity and are structurally related to the IGFBP especially in the N-

terminal region (Damon et al., 1997; Baxter et al., 1998). However, they deviate from the common IGFBP structure in the midregion and C terminus (Hwa et al., 1999). The first new member of the IGFBP superfamily to be discovered was Mac25. It was provisionally assigned the name IGFBP-7 (Damon et al., 1997; Wilson et al., 1997) because it was the first protein shown to be functionally related to the IGFBP (Akaogi et al., 1996; Oh et al., 1996). It was subsequently termed IGFBP-rp-1. Specific functions of these IGFBP-rps are not completely understood.

The postulated actions of IGFBP include circulatory transport vehicles, retardation of IGF degradation, transvascular IGF movement and direct modulation of the actions of IGF at the target cell either by enhancing or blocking the IGF activity (McGuire et al., 1992; Florini et al., 1996). IGFBP compete with IGF receptors for IGF binding, and as a consequence, they can affect cell growth. Circulating IGF-I forms complexes with the IGFBP. Due to the fact that serum IGF-I levels are far above the amount necessary to stimulate cell growth in vitro, it is hypothesized that the IGFBP play a key role in regulating the action of circulating IGF-I on target tissues. The consensus is that soluble IGFBP attenuate the circulating IGF-I actions primarily by forming large molecular weight complexes that are unable to cross the capillary endothelium for the purpose of reaching target tissues. Consequently, binding of the circulating IGF-I to the IGF-I-R is inhibited preventing a signal transduction pathway (via tyrosine kinase), which could result in cell proliferation and/or differentiation. These large IGF-

I/IGFBP complexes provide a stable reservoir of circulating IGF-I and prolong the IGF-I half life by protecting it from degradation.

IGFBP-1 (Brewer et al., 1988; Julkunen et al., 1988) and IGFBP-2 (Szabo et al., 1988; Binkert et al., 1989; Brown et al., 1989) are distinct proteins whose plasma concentrations are inversely related to growth hormone (Binoux et al., 1986). IGFBP-3 is a glycoprotein whose plasma concentrations are directly related to growth hormone (GH) secretory status (Baxter and Martin, 1986). The structures of IGFBP-4 (Shimasaki et al., 1990), IGFBP-5 and IGFBP-6 (Kiefer et al., 1991) have been determined, but little is known about their regulation by GH and/or IGF-I.

D. IGFBP Structure

The six well-characterized IGFBP represent a family of related proteins whose predicted amino acid sequences share an overall homology of approximately 40%. The regions of homology are near the amino- and the carboxy-termini, whereas the central part of the proteins tend to be unique for each IGFBP. IGFBP fragments containing either the conserved N- or C-terminal regions can bind IGF but with reduced affinity. The determinants of IGF binding to IGFBP are poorly characterized in terms of important residues in the IGFBP molecule.

Hobba et al. (1996) used tyrosine iodination to implicate tyrosine (Tyr) at position 60 in the IGF-binding site of bovine IGFBP-2 (bIGFBP-2). Subsequent reports showed that mutagenic replacement of Tyr 60 with either alanine (Ala) or phenylalanine (Phe) reduced the affinity of bIGFBP-2 for IGF-I (4.0- and 8.4-fold,

respectively) and for IGF-II (3.5- and 4.0-fold, respectively). Kinetic analysis of bIGFBP-2 mutants revealed Tyr 60 (Phe bIGFBP-2 bound to the IGF-I surface 3.0-fold more slowly than bIGFBP-2 and was released 2.6-fold more rapidly than bIGFBP-2. In contrast, Tyr 60 (Ala bIGFBP-2 associated with the IGF-I surface 5.0-fold more rapidly than bIGFBP-2, but exhibited an 18.4-fold more rapid release from this surface compared with bIGFBP-2 (Hobba et al., 1998). Thus, both the aromatic nature and the hydrogen bonding potential of the tyrosyl side chain of Tyr 60 are important structural determinants of the IGF-binding site of bIGFBP-2. Current evidence suggests that for high affinity IGF binding both the N- and C- terminal regions are required (reviewed by Hwa et al., 1999).

All IGFBP share a conserved pattern of cysteine residues, which are clustered at the termini of the predicted protein sequences for mammalian species. IGFBP-1–5 possess 18 conserved cysteine residues in their peptide sequence, whereas IGFBP-6 contains 16 cysteines and IGFBP-rp-1 contains 11 or 12 conserved cysteines (reviewed by Hwa et al., 1999). Additionally, the placement of these cysteines within the protein is well conserved (Shimasaki and Ling, 1991). Structurally, the cysteines are clustered at the conserved N-terminal third (12 cysteines in IGFBP-1 to –5; 10 in IGFBP-6) and at the conserved C-terminal third (6 cysteines) of the proteins (reviewed by Hwa et al., 1999). These cysteine residues are responsible for forming intra-molecular disulfide bonds that cause the IGFBP to fold in a specific way that is necessary for IGF binding. The even number of cysteines suggests that intradomain disulfide bond formation is more likely than interdomain disulfide linkages with cysteines in the C-terminal

domain. Furthermore, evidence obtained by Newmann et al. (1998) and Forbes et al. (1998) suggests that the N-terminal and C-terminal domains are not linked by disulfide linkages. Any alteration in the number or placement of these cysteines could result in an altered molecular conformation of the IGFBP and may alter its functional properties (reviewed by Hwa et al., 1999). Thus, the number and placement of the cysteine residues in IGFBP appear to be of fundamental importance for proper functioning of the IGFBP.

The middle part of the IGFBP sequence is cysteine-free with the exception of two additional cysteines in IGFBP-4. This midregion range in size from 55 to 95 amino acid residues. The amino acid sequence for each mid segment appears to be unique to each IGFBP, with less than 15% shared similarity (reviewed by Hwa et al., 1999). The functional relevance of the differences in amino acid sequence between IGFBP is not known (Binkert et al., 1992). The belief is that this region acts structurally as a hinge between the N- and C-terminal domains.

Recently, four mechanisms have been shown to alter the affinity of the IGFBP for IGF, making the network of regulatory components in the IGF system even more complex: IGFBP proteolysis, phosphorylation, glycosylation and adherence to the cell surface or to the extracellular matrix (Clemmons, 1997). There is increasing evidence that IGFBP-specific proteases are involved in the regulation of IGFBP function and turnover. IGFBP are cleaved at specific sites by a range of proteases including prostate-specific antigen (PSA) (Cohen et al., 1992), matrix metalloproteases (Fowlkes et al., 1994), cathepsin D (Claussen et

al., 1997), thrombin (Zheng et al., 1998), and serine proteases (Clemmons, 1998). The majority of protease, phosphorylation and glycosylation sensitive sites are localized in the middle non-conserved region and not in the N- and C-terminal domains. Following limited proteolysis, IGFBP exhibit a dramatically reduced affinity for IGF and some IGFBP fragments appear to have IGF independent activity (Baxter, 2000).

E. IGF Independent Actions of IGFBP

IGF independent actions have been shown for IGFBP-1, -3 and -5, all of which have been shown to bind to cell surfaces. These three IGFBP have well-established effects that are independent of the IGF-I-R signaling. IGFBP-1 exerts these effects by signaling through alpha(5)beta(1)-integrin, whereas IGFBP-3 and -5 may have specific cell-surface receptors with serine kinase activity. It is considered that the regulation of cell sensitivity to inhibitory IGFBP signaling may play a role in the growth control of malignant cells (Baxter, 2000). These effects may be related to the putative nuclear actions of IGFBP-3 and IGFBP-5, which have been shown to be transported to the nuclei of T47D breast cancer cells (Baxter, 2000). In digitonin-permeabilized cells, where the nuclear envelope remained intact, nuclear translocation of wild-type IGFBP-3 appeared to occur by a nuclear localization sequence (NLS)-dependent pathway mediated principally by the importin beta nuclear transport factor, and requiring both ATP and GTP hydrolysis (Schedlich et al., 2000). By fusing wild-type and mutant forms of NLS sequences (IGFBP-3 [215-232] and IGFBP-5 [201-218]) to green fluorescent protein, the critical residues of the NLS necessary and sufficient for

nuclear accumulation were identified. Using a western ligand binding assay, wild-type IGFBP-3 and IGFBP-5, but not an NLS mutant form of IGFBP-3, were shown to be recognized by importin beta and the alpha/beta heterodimer and only poorly recognized by importin alpha. Together these results suggest that the NLS within the C-terminal domain of IGFBP-3 and IGFBP-5 are required for importin-beta-dependent nuclear uptake, and probably also accumulation through mediating binding to nuclear components (Schedlich et al., 2000).

Flint et al. (2000) also demonstrated that IGFBP-5 interacts with alpha(s2)-casein and that this interaction implicates it in the regulation of plasminogen activation in the mammary gland. The generation of plasmin is a key initiating event in the remodeling of the extracellular matrix during mammary involution. As such, IGFBP-5 may play a key role in coordinating cell death (apoptosis) and tissue remodeling processes. In addition, Kanatani et al. (2000) demonstrated that IGFBP-5 stimulates bone resorption by stimulation of osteoclast formation in an IGF-I-independent fashion.

IGF-independent growth inhibition by IGFBP-3 is believed to occur through IGFBP-3-specific cell surface associated proteins or receptors and involves nuclear translocation. There is some evidence that IGFBP-3 may have its own pro-apoptotic effects that are independent of its ability to modulate IGF bioavailability. IGFBP-3-mediated apoptosis is controlled by numerous cell cycle regulators in both normal and disease processes (Grimberg and Cohen, 2000). The IGF-independent action of IGFBP-3 requires interaction with cell-surface associated proteins, presumably putative IGFBP-3-specific receptors, and is

responsible for growth inhibitory action of the known growth suppressing factors such as TGF-beta, retinoic acid, and antiestrogens in breast cancer cells. Thus, IGFBP-3 appears to be a major factor in a negative control system involved in regulating human breast cancer cell growth in vitro (Oh, 1998).

In human skeletal muscle cells, Frost and Lang (1999) found that IGFBP-1, acting independently of IGF-I, inhibits protein degradation. This IGF-independent response of IGFBP-1 occurs via beta1 integrin binding and stimulation of a rapamycin-sensitive signal transduction pathway (Frost and Lang, 1999). Thus, the IGF independent effects of the IGFBP represent a new and exciting area of research to increase our understanding of the mode of action of the IGF/IGFBP axis and correlated physiological responses.

F. IGFBP Expression

IGFBP-1 (234 amino acids) is produced mainly in the liver where it is both hormonally and metabolically regulated and it may be involved in glucose homeostasis (Murphy, 1998). Insulin and corticosteroids regulate serum IGFBP-1 through transcriptional control of hepatic IGFBP-1 synthesis (Hasegawa et al., 1995). IGFBP-1 is in low concentration in serum where it has a short half-life (IGFBP-1 contains a PEST sequence often present in proteins that have a rapid turnover) and it is the predominant IGFBP in amniotic fluid where it is found in high concentration. The binding affinity of IGFBP-1 for IGF-I can be up-regulated by serine phosphorylation (Rechler and Clemmons, 1998).

IGFBP-2 (289 amino acids) is widely expressed in the fetus where its expression closely follows that of IGF-II (Wood et al., 1993; Carr et al., 1995). It

preferentially binds IGF-II and it is found in serum, milk, cerebrospinal fluid and seminal plasma. Many cell types including hepatocytes secrete this IGFBP species, and there has been no clear evidence that this protein is glycosylated (reviewed by Hwa et al., 1999). IGFBP-2 contains an arginine-glycine-asparagine motif (RGD) that is known to be involved in binding integrins (Jones et al., 1995), however, it has not been shown to bind integrin-type receptors (Ferry et al., 1999). Also, BIAcore[1] analysis of bovine IGFBP-2 identified major IGF binding site determinants in both the N- and C-terminal domains, albeit with different affinities (Carrick et al., 2001). The amino-terminal half appears to contain components responsible for fast association. In contrast, IGF binding by the C-terminal fragment results in a more stable complex (Carrick et al., 2001).

IGFBP-3 (264 amino acids) is the most abundant IGFBP species in serum and milk. It is produced mainly by non-parenchymal hepatic cells and circulates in serum, binding IGF-I or IGF-II in conjunction with an acid-labile subunit (ALS) to form a 150 kDa circulating complex at a serum concentration of about 100 nM. Free IGF-I has a half-life of approximately 8 min in serum. This can be increased to approximately 30 min if bound to IGFBP-3 and approximately 15 h in the ternary complex with IGFBP-3 and ALS (Rechler and Clemmons, 1998). The IGF can be mobilized from this complex following limited proteolysis of IGFBP-3 to yield a C-terminal fragment that remains associated with the ALS (reviewed by Hwa et al., 1999) and a 30 kDa N-terminal fragment that has reduced affinity for IGF-I (50-fold) and IGF-II (20-fold). The degree of glycosylation and sialylation at

[1] Instrument that uses affinity-based biosensors to study biomolecular interactions in real time.

at different sites affects the efficacy of proteolytic attack (Janosi et al., 1999). IGFBP-3 has a nuclear targeting sequence and recent evidence shows that, independent of the presence of IGF-I, proteolytic fragments of IGFBP-3 are translocated to the nucleus of actively dividing cells (Jacques et al., 1997; Li et al., 1997).

IGFBP-4 (237 amino acids) is produced by mandibular, calvarial, vertebral, rib and stromal osteoblasts (Malpe et al., 1997). It is found in serum and seminal plasma. Evidence in smooth muscle cells indicates that IGFBP-4 does not associate either with the cell surface or the extracellular matrix and seems to act as a scavenger of IGF and an inhibitor of IGF action (reviewed by Hwa et al., 1999). Proteolytically modified IGFBP-4 generates a 16 kDa fragment that also could be affinity cross-linked specifically to IGF-I and IGF-II, although with a 20-fold lower affinity compared with intact IGFBP-4 (Cheung et al., 1994). IGFBP-4 is N-glycosylated which occurs only on an asparagine that is part of the consensus sequence asparagine-X-serine/threonine, where X is any amino acid except proline (reviewed by Hwa et al., 1999).

IGFBP-5 (252 amino acids) preferentially binds IGF-II. It potentiates the actions of IGF-I in smooth muscle cells, fibroblasts and osteoblasts. In vitro studies show IGFBP-5 down-regulates the stimulatory effects of IGF by inhibiting their binding to the IGF-I-R (Kalus et al., 1998). IGFBP-5 also binds with high affinity to extracellular matrix components, which protect it from proteolysis but decrease its affinity for IGF-I by about 10-fold. IGFBP-5 is able to form a ternary complex with ALS but the significance of this interaction is not yet understood

(Twigg et al., 1998). IGFBP-5 has a nuclear targeting sequence and recent evidence shows that, independent of the presence of IGF-I, fluorescently labeled IGFBP-5 is translocated to the nucleus of actively dividing cells (Schedlich et al., 1998).

IGFBP-6 (216 amino acids) has a 50-fold higher affinity for IGF-II than IGF-I (Rechler and Clemmons, 1998). It is synthesized in liver and lung and is found in cerebrospinal fluid and detected by immunohistochemistry in skin, skeletal muscle, the meninges and pancreatic islets of Langerhans. This protein is O-glycosylated, although the ability to bind IGF with high affinity appears not to be influenced by that. However, O-glycosylation may have effects on other function(s) such as resistance to proteolysis (Neumann et al., 1998). Also, a highly basic heparin-binding sequence is found in the thyroglobulin type I domain of this IGFBP (reviewed by Hwa et al., 1999).

Using immunoprecipitation with specific antibodies and 125I-IGF-I western ligand blotting analysis, Hembree et al. (1996) showed that porcine myogenic cultures secreted IGFBP-3 (doublet band of 39 kDa and 43 kDa), IGFBP-2 (34 kDa), IGFBP-4 (30 and 24 kDa), and IGFBP-5 (30 kDa and 28 kDa). Developmental expression of the IGFBP was evaluated by Gerrard et al. (1999) using total RNA extracted from skeletal muscle and liver of 30-, 44- 59-, 68-, 75-, 89-, and 109-d pig fetuses and from adult and neonatal pigs. This group used a combination of in situ hybridization and immunocytochemistry to determine the localization of IGFBP-2, -4, and –5 mRNA and peptides in muscle samples from contralateral pelvic limbs and showed that overall muscle IGFBP gene

expression decreased ($P < 0.05$) with increasing age. Moreover, expression of liver IGFBP-2 and –5, but not IGFBP-4, was greater ($P < 0.05$) during prenatal than during postnatal periods. The majority of immunoreactive IGFBP was located in developing muscle cells, with little localized to connective tissue except at later stages of development. These data show that IGFBP-2, -4, and –5 expression is time and tissue-dependent in liver and muscle.

Western ligand blot analysis of human, bovine, ovine and porcine sera using 125 I-IGF-I as ligand reveals a pattern of different molecular weight IGFBP consisting of 39-43 kDa bands which are glycosylated variants of IGFBP-3, a 34 kDa band which is IGFBP-2, a 28 kDa and a 24 kDa band which may represent either IGFBP-1, glycosylated or non-glycosylated IGFBP-5 and glycosylated or non-glycosylated IGFBP-4 or a combination of these IGFBP (White et al., 1998). In a study by Cohick et al. (1992), the serum levels of IGFBPs in lactating cows treated with bovine somatotropin (bST) were determined. The results indicated that bovine serum contains IGFBPs with molecular weight estimates of 43 kDa, 39 kDa, 34 kDa, 29 kDa, and 24 kDa, as determined by western ligand blotting. Using specific antisera, immunoblotting showed that the 43 kDa and 39 kDa bands were IGFBP-3 and the 34 kDa band was IGFBP-2. All five forms of IGFBP were also present in afferent mammary lymph. Similarly, Skaar et al. (1991) reported that IGFBP of 25 kDa, 30 kDa, 34 kDa, 42 kDa, 46 kDa and greater than 200 kDa were present in serum and mammary secretions of late gestation and early lactation dairy cows. Approximately 77% of the circulating IGFBP activity in prepubertal heifers is associated with IGFBP-3 and the

association between these two proteins is considered to be large enough to prevent crossing of the capillary endothelium. IGFBP-2 accounts for 22% of the total IGFBP activity (Weber et al., 1999).

The precise roles of individual IGFBP are still unknown, due mainly to the great complexity of their actions and their regulation, but also to the fact that the overwhelming majority of information about the IGFBP is derived from in vitro studies. Non-physiological concentrations of the IGFs and their binding proteins as well as the lack of other components such as the IGFBP-rps may lead to artificial culture conditions, which can explain confusing and sometimes contradictory results (Murphy, 1998). Despite their common property to interact with IGF, every IGFBP is expressed in a tightly regulated time and tissue specific manner suggesting that each protein may have its own distinct functions. Several transgenic mouse models overexpressing IGFBP-1, -2, -3, or -4 have been developed. Brain abnormalities were a common feature of IGFBP-1 transgenic models. Individual strains showed alterations in glucose homeostasis, reproductive performance, and a reduction of somatic growth as the most prominent phenotypes (Schneider et al., 2000). The latter was also the main effect observed in IGFBP-2 transgenic mice (Schneider et al., 2000). The overexpression of IGFBP-3 under the control of a ubiquitous promoter resulted in selective organomegaly, whereas mammary gland-targeted expression of this protein caused an altered involution after pregnancy. Tissue-specific overexpression of IGFBP-4 resulted in hypoplasia and reduced weight of smooth

muscle-rich tissues including bladder, aorta, and stomach (Schneider et al., 2000).

IGFBP-2 has also been disrupted using gene targeting, and homozygous null IGFBP-2 mice are characterized by a decreased spleen size and an increase in circulating levels of other IGFBP. Prominent serum proteins that bind both ^{125}I-IGF-I and –II and that correspond to predicted sizes of the mouse IGFBP-3 doublet, IGFBP-4, and IGFBP-1 are all up-regulated in homozygous IGFBP-2 mutants (Pintar et al., 1996). Thus, evidence from transgenic and knockout studies suggests that an understanding of the contribution of each IGFBP, both alone and in combination with other members of this superfamily, will be necessary before the complete role of the IGF system in growth and development can be understood.

G. IGFBP Genes

IGFBP-1 through -6 are encoded by different genes and they are all different proteins having different biological roles. These genes exhibit tissue specificity in their adult expression patterns and exhibit different responses to hormonal and physiological treatments at both the transcriptional and peptide levels (Pintar et al., 1996). Interestingly, the genes for human IGFBP-1 and IGFBP-3 not only reside on the same chromosome (human chromosome 7p12-p14), but they are only 20-kb apart with transcription oriented in a tail to tail configuration (Ehrenborg et al., 1992). Human IGFBP-2 and IGFBP-5 constitute another gene pair, located 20–40-kb apart on human chromosome 2q. Based on amino acid similarity analysis, IGFBP-1 is more closely related to IGFBP-2 than

to IGFBP-3, which, in turn, is more closely related to IGFBP-5. IGFBP-4, found on chromosome 17q12-q21.1, is more closely related to IGFBP-1 and –2, whereas IGFBP-6, located on chromosome 12q13, appears to be the most divergent of the IGFBP (Reinecke and Collet, 1998). The similarity in configuration of the human IGFBP genes, especially the gene pairs, has led to the hypothesis that a tandem gene duplication and inversion occurred early in the evolution of the IGFBP.

The gene structures of human IGFBP are highly similar, although the sizes of the genes vary from 5.7-kb for IGFBP-1 to 33-kb for IGFBP-5, due to variations in the intron sizes. All of the IGFBP are encoded by four exons, with the exception of IGFBP-3, which carries an extra exon, exon 5, which is not translated. The corresponding exons among the IGFBP are approximately equivalent in size, with exon 1 less than 600-bp, exon 2 and 3 both small exons of less than 230-bp, and exon 4 more variable in size. There is a correlation between these exons and the three protein domains of the IGFBP. The N-terminal domain is encoded within exon 1 in all of the IGFBP, as is the 5'-untranslated region and a few amino acids of the midregion. Exon 2 encodes for the nonconserved midregion. Both exon 3, which ends precisely at the invariant Gln (Q) residue in the thyroglobulin domain, and exon 4 encode for the conserved C-terminal domain. The fact that the N-terminal domain is contained within one exon strongly supports the concept of an IGFBP superfamily (reviewed by Hwa at al., 1999).

H. IGFBP-2 Gene

Bourner et al. (1992) reported that Mandin-Darby Bovine Kidney (MDBK) cells secrete a 34 kDa form of IGF binding protein whose N-terminal sequence is similar to a form of IGFBP identified as IGFBP-2 purified from rat BRL-3A cells. This bovine protein was 81% identical to rat IGFBP-2 and had an 87% similarity to the human IGFBP-2 amino acid sequence. When compared with both rat IGFBP-2 and human IGFBP-1, the positions of all 18 cysteine residues were conserved. Genomic clones encoding the rat (Brown and Rechler, 1990) and human (Binkert et al., 1992) IGFBP-2 genes have been isolated and the IGFBP-2 nucleotide sequence and genomic organization have been determined. In humans, this single copy gene spans a total of more of than 32-kb of genomic sequence and it is organized in four exons with sizes of 568, 220, 141, and 496 nucleotides, respectively, which are preceded by a region that exhibits promoter activity. This region lacks TATA or CAAT consensus sequence motifs, has one major start site for transcription, and is rich in dC and dG nucleotides (Binkert et al., 1992). These characteristics are similar to those observed for the rat IGFBP-2 gene (Brown and Rechler, 1990). The intron between exons one and two contributes 27-kb to the size of the human IGFBP-2 gene. The second and third introns comprise 1.1 and 1.95 kb, respectively. When the structure of the IGFBP-2 gene is compared to that of the IGFBP-1 and IGFBP-3 genes, the exon boundaries are conserved in all three genes.

Schoen et al. (1995) isolated and characterized a 1.6-kb cDNA clone for IGFBP-2 from chick embryo. The chicken IGFBP-2 gene spans approximately

38-kb, consists of four exons, and is similarly organized to that of the rat and human. Song et al. (1996) obtained similar results for the IGFBP-2 gene in swine. The bovine IGFBP-2 complete cDNA sequence has also been reported (Bourner et al., 1992). This cDNA contains a region of 5′ untranslated sequence followed by an open reading frame encoding 317 amino acids, which is followed by 381 nucleotides of 3′ untranslated sequence and includes a poly-A tail. In addition, the Genbank database (http://www.ncbi.nlm.nih.gov/) includes unpublished data for a 954-bp genomic bovine IGFBP-2 sequence tagged site submitted in 1999 by Wu et al. from Texas A & M University (GenBank accession no. G42681).

In general, IGFBP-2 mRNA abundance in fetal tissues is found to be high in early gestation and it decreases with maturation, thus following the same pattern of expression as IGF-II. However, this pattern is reversed in the liver. The concurrent expression of both IGFBP-2 and IGF-II mRNA in the same tissues in early fetal development suggests that both proteins are synthesized together in these tissues and act by autocrine and/or paracrine mechanisms. In later gestational ages and early postnatal life, when IGF-II mRNA is expressed in decreasing levels, IGFBP-2 mRNA is present only in selected tissues. Liver is the only tissue that continues to express abundant IGFBP-2 mRNA levels, indicating that it is the major source of this IGFBP at later gestational ages and during postnatal life when the protein functions as an endocrine factor (Delhanty and Han, 1993; Wood et al., 1993; Carr et al., 1995). IGFBP-2 appears to play a key role in myogenesis (Ernst et al., 1992; Ernst et al., 1996; Fligger et al., 1998;

Gerrard et al., 1999). The level of expression of IGFBP-2 mRNA and protein was found to be high in proliferating turkey myogenic satellite cells (Ernst et al., 1996) and mouse myoblasts (Ernst et al., 1992) and to decrease gradually as differentiation progressed. This has been associated with a sequestration of IGF-I by IGFBP-2, which makes that growth factor less available to the myogenic satellite cells (Fligger et al., 1998).

I. Role of the IGF System in Animal Growth

Serum IGF-I concentrations have been shown to be related to body weight and growth rate for numerous species including cattle (Lund-Larsen et al., 1977; Bishop et al., 1989; Davis and Bishop, 1991; Davis and Simmen, 1997). For example, in a study by Anderson et al. (1988), plasma IGF-I was positively correlated with carcass protein percentage in growing beef bulls. Such observations provided the rationale for the use of IGF-I as a marker of growth for animal selection. Selection for increased rate of gain resulted in increased circulating IGF-I in pigs (Clutter et al., 1995), and selection for greater plasma IGF-I resulted in increased growth rate in mice (Baker et al., 1991). Bishop et al. (1989) reported that Angus cattle selected for high feed conversion efficiency tended to have higher serum IGF-I concentrations than cattle selected for low feed efficiency. However, selection for weaning weight did not affect plasma IGF-I in sheep (Medrano and Bradford, 1991).

Blair et al. (1988) selected mice on the basis of serum IGF-I and observed a significant increase in 6-week weights for animals with elevated serum IGF-I, but no effect on body composition (Siddiqui et al., 1990). In previous reports,

these researchers reported a heritability estimate of 0.4 for serum IGF-I levels in mice at 35 d of age and a significant positive genetic correlation between IGF-I and body weight (Blair et al., 1987). In contrast, Davis and Simmen (1997) found moderate to high negative genetic correlations between IGF-I concentration and performance traits (weaning weights, postweaning weights, and postweaning gain) in beef cattle. Additive genetic correlations demonstrated that bull calves with lower blood serum IGF-I concentrations had higher marbling scores and quality grades but also had higher backfat thickness and yield grades (Davis and Simmen, 2000). Considering these reports in cattle, it appears that selection for reduced IGF-I concentration increases not only performance traits but also degree of marbling, quality grade, external fat thickness and yield grade. These results also indicate that mice may not be a good model for studying growth regulation in agriculturally important species such as cattle. However, both positive and negative correlations between IGF-I and rate of gain have been reported in cattle (Lund-Larsen et al., 1977; Davis and Simmen, 1997; Stick et al., 1998) and conflicting relationships between carcass fat percentage and IGF-I have been observed in cattle and sheep (Anderson et al., 1988; McCann et al., 1997). Thus, the relationship between circulating IGF-I and growth potential and carcass composition remains unclear and the IGFBP may contribute to the discrepancies in the observed results.

IGF-I mediates many of the growth-promoting effects of growth hormone and regulates postnatal growth and development. At the same time, plasma IGFBP-2 and –3 are also responsive to growth hormone (Cohick et al., 1992;

Harrell et al., 1999) and may be useful indicators of rate of gain and carcass composition in livestock. Differences in these binding proteins have been reported in gilts divergently selected for gain (Clutter et al., 1995), and in lean versus obese sheep (McCann et al., 1997). Connor et al. (2000) reported that in growing Angus bulls, plasma IGF-I at the start of a 140-d postweaning growth performance test was associated with reduced postweaning average daily gain (ADG) and increased longissimus muscle area (as measured by ultrasound). Throughout the performance test period, correlations between plasma IGF-I and hip height were consistently positive. Plasma IGFBP-2 was related to ADG during the performance test, explaining nearly 30% of the variation in ADG. Plasma IGFBP-2 and –3 were not related to carcass characteristics, and IGFBP-3 was not related to growth rates. However, mean plasma IGFBP-3 content increased along with IGF-I concentrations and weight gain throughout the postweaning performance period.

Bourner et al. (1992) observed a dose-dependent increase in smooth muscle DNA synthesis in response to IGFBP-2 in the presence of IGF-I, suggesting that IGFBP-2 may potentiate smooth muscle growth. Vleurick et al. (1999) found that cows treated with bST had significantly lower serum levels of IGFBP-2 than did control cows. In this study, IGFBP-2 levels were dramatically increased at the onset of lactation and showed absence of diurnal variation. The decrease in serum IGFBP-2 concentration in bST and growth hormone releasing factor (GHRF) treated cows seems partly regulated at the level of IGFBP-2 synthesis, because IGFBP-2 mRNA abundance in the liver of bST treated cows

was only 50% that of controls, whereas the decrease in serum IGFBP-2 protein was about 30%. Because cattle exhibited little circadian variation in plasma IGFBP-2 concentrations, it can be speculated that the circulating levels of this protein could be used as a diagnostic parameter for assessing malnutrition or growth potential. If IGFBP-2 concentrations reflect GH levels, IGFBP-2 may be an interesting parameter to indirectly study GH status using a single blood sample. Consequently, low plasma IGFBP-2 might be useful in the selection of fast growing bulls at a young age.

J. Mapping and DNA Sequence Variation (Polymorphisms) of Genes within the IGF System

Due to recent advances in molecular biology, several genes within the IGF system have been mapped in cattle (Barendse et al., 1997; Kappes et al., 1997; Band et al., 2000). Polymorphisms identified within these genes could potentially be associated with variation in traits of economic importance such as growth, and therefore could be used in marker assisted selection (MAS) programs.

The IGF-I, IGF-II, GH, IGF-I-R, GH receptor (GHR), GH-releasing hormone receptor, IGFBP-3, and IGFBP-4 genes have been mapped to bovine chromosomes (BTA) 5, 29, 19, 21, 20, 4, 4 and 19, respectively. However, few genetic markers have been identified within these genes for growth traits in beef cattle. Ge et al. (2001) identified a thymine to cytosine transition polymorphism in the promoter region of the bovine IGF-I gene in Angus cattle divergently selected for serum IGF-I concentration. This marker was associated with higher weight gain during the first 20 d after weaning and the beginning of the

postweaning test, and had a slight dominance effect (BB genotype) on postweaning gain. A dinucleotide (CA) repeat polymorphism has also been identified in the 5' flanking region of the IGF-I gene in cattle and swine (Kirkpatrick, 1992) and was reported to be associated with weaning and yearling weights (Moody et al., 1994) and with birth weight (Moody et al., 1996) in beef cattle.

A polymorphic TG-repeat microsatelite is located 90bp upstream from a major transcription start site in the bovine GHR gene (Lucy et al., 1998). A shorter allele with 11 consecutive TG repeats is common in *Bos indicus* cattle, whereas longer 16 to 20 TG repeat alleles predominate in *Bos taurus* breeds (Lucy et al., 1998). Results obtained by Hale et al. (2000) indicated that the presence of the 11 TG GHR allele in Angus steers raised under commercial conditions was associated with decreased growth by an average of approximately 17 kg at weaning and approximately 23 kg at slaughter. Moisio et al. (1998) also studied the GHR gene and reported the presence of three length variants and one base substitution polymorphism in the 3' flanking region. Allele frequencies of the length variants differed between Finnish native and commercial dairy cattle breeds (Moisio et al., 1998).

Several polymorphisms have been identified in the bovine GH gene (Lucy et al., 1993; Zhang et al., 1993; Sneyers et al., 1994). Sneyers et al. (1994) reported that a restriction fragment length polymorphism (RFLP) identified using the restriction endonuclease *Taq* I was correlated with body weight at 7 and 13 mos of age in Belgian Blue bulls. Taylor et al. (1998), by interval analysis,

identified localized effects of *Bos taurus* vs. *Bos indicus* GH alleles (*Taq* I-RFLP) on subcutaneous fat and in percentage of extractable fat from the longissimus dorsi muscle the BTA 19 region that harbors the GH gene. Moreover, Grochowska et al. (2001) reported that a polymorphism in the GH gene that causes a leucine (Leu) to valine (Val) substitution (amino acid 127) affected GH peak, GH rate and serum IGF-I concentration. The Val/Val genotypes reached the highest GH peak value compared with other GH genotypes ($P < 0.01$), whereas the Leu/Leu genotypes had the highest IGF-I concentrations ($P \leq 0.05$). Moreover, the Leu/Val heterozygotes were superior to other genotypes in milk and protein yields, whereas the Leu/Leu homozygotes reached the highest fat yield ($P \geq 0.01$).

Polymorphisms have been identified at the bovine IGFBP-3 gene locus by using the restriction endonuclease *Hae* III (Maciulla et al., 1997) and by DNA sequencing (thymine to cytosine transition, Haegeman et al., 1999). However, their potential associations with economically important traits have not been determined.

Hypothesis and Objectives

The project reported in this dissertation includes objectives to examine variation in the IGFBP system at the DNA and protein levels using a unique Angus cattle population that has been divergently selected for serum IGF-I concentration since 1989. Information on both gene sequence variation and gene expression will help to determine if genes within the IGFBP axis are good biological indicators of growth and carcass merit in beef cattle. We hypothesize

that divergent selection for serum IGF-I concentrations has caused changes in expression of other genes in or influenced by the IGF-I system. Evaluation of correlated responses in the expression of genes in the IGFBP axis resulting from single trait selection for serum IGF-I concentration will provide insight into potential means for targeting and controlling expression of these genes. In addition, identification of DNA polymorphisms at loci for IGFBP could reveal sequence differences responsible for variation in gene expression responsible for important traits such as growth, carcass merit and beef quality. The use of molecular markers to assist the breeding process may allow genetic improvements to be achieved in a cost-effective manner and in a reasonably small number of generations. Development of genetic markers that relate to growth, carcass merit or meat quality would provide additional benefits by enabling producers to identify precise genotypes in breeding stock that are responsible for high merit in production traits. Such markers could be used as biological indicators of genetic merit in groups of animals and to support breeding decisions that would enhance these traits. In order for MAS to be successful, it is imperative that these markers be extensively evaluated in families with defined pedigrees prior to their application in populations with poorly defined genetic backgrounds.

The specific objectives for this dissertation project were to:

1. Evaluate serum IGFBP in Angus cattle divergently selected for serum IGF-I concentration and investigate potential associations between

serum IGFBP, body weight, postweaning gain and serum IGF-I concentration.

2. Identify DNA sequence variation (polymorphisms) at the bovine IGFBP-2 gene locus.
3. Evaluate IGFBP-2 polymorphisms in different breeds of cattle and in Angus cattle divergently selected for serum IGF-I concentrations for potential associations with growth, carcass merit and meat quality traits.

CHAPTER 2

EVALUATION OF SERUM INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS (IGFBP) IN ANGUS CATTLE DIVERGENTLY SELECTED FOR SERUM INSULIN-LIKE GROWTH FACTOR-I (IGF-I) CONCENTRATION

Abstract

Postweaning expression of serum insulin-like growth factor I (IGF-I) and associated serum insulin-like growth factor binding proteins (IGFBP) were investigated in 68 1992 fall-born and 84 1999 fall-born, purebred Angus cattle, selected for either high or low serum IGF-I concentrations since 1989 at The Ohio State University. Relative serum levels of individual IGFBP were determined by densitometric analysis of autoradiographs obtained by [^{125}I]IGF-I western ligand blotting. Body weight and postweaning gain data and serum IGF-I concentrations were provided by Dr. Mike Davis (The Ohio State University). A 2 x 2 factorial experimental design, which included the main effects of sex of calf and IGF-I selection line and the corresponding interaction term, was used for the analysis of variance. IGFBP species of 38-42 kDa, 34 kDa, 30 kDa and 24 kDa were identified by western ligand blotting. The 34 kDa IGFBP species was identified as bovine IGFBP-2 by immunoblot analysis. No significant effect of line was observed for any of the IGFBP ($P > 0.05$), however, a significant effect of sex was observed ($P < 0.05$). In both fall 1992 and fall 1999 calves, heifers expressed higher levels of the 34 kDa IGFBP species (i.e., IGFBP-2) than bulls ($P < 0.0005$). In fall 1992 calves, bulls expressed greater levels of both the 38-42 and 24 kDa IGFBP species than heifers ($P < 0.0001$). Also, in the fall 1992 calves, the relative level of the 38-42 kDa IGFBP species was positively

correlated with serum IGF-I concentration and the relative level of the 24 kDa IGFBP species was negatively correlated with serum IGF-I. In the fall 1999 calves, the relative level of IGFBP-2 was negatively correlated with serum IGF-I concentration at d 28 and 42 of the postweaning test period ($P < 0.01$). Also, IGFBP-2 was negatively correlated with birth weight, weaning weight, on test weight, weight at d 28, 42 and 56 of the postweaning test period, off test weight and off test hip height. No significant effect of line was observed for body weight or postweaning gain or for serum IGF-I concentration in the fall 1992 calves ($P > 0.05$). In contrast, the fall 1999 high line had higher serum IGF-I concentrations at d 28, 42 and 56 of the postweaning test period and also had higher on test weight, weight at d 28, 42 and 56 of the postweaning test period, off test weight, and off test hip height than the low line ($P < 0.05$). In both fall 1992 and fall 1999 calves, bull calves had higher serum IGF-I concentration and higher body weight, hip height and total postweaning gain than heifers ($P < 0.05$).

Introduction

Insulin-like growth factor I (IGF-I) is a single-chain 70-amino acid, basic polypeptide (Rinderknecht and Humbel, 1978a,b) that appears to mediate the growth promoting and metabolic activities of growth hormone (Van Wyk and Underwood, 1978). IGF-I plays an important physiological role in the growth and development of mammals by acting locally in specific organs or systemically through the blood (Werner et al., 1994). IGF-I serum concentration is associated with growth traits in many livestock species (Anderson et al., 1988; Graml et al., 1994) and may be useful as a physiological indicator trait in selection programs

designed to improve growth rate in cattle (Lund-Larsen et al., 1977; Bishop et al., 1989; Davis and Bishop, 1991). Native serum IGF-I molecules are not typically found in an active form, but are bound to soluble, high affinity binding proteins (IGFBP) (McGuire et al., 1992) that determine IGF-I availability and help regulate its biological activities (Rechler, 1993). Soluble IGFBP attenuate the circulating IGF-I action by forming large molecular weight complexes, which are unable to cross the capillary endothelium to reach target tissues. Consequently an IGF-I reservoir is maintained (Binoux and Hossenlopp, 1988). Binoux and Hossenlopp (1988) postulated that the 150 kDa IGFBP complex functions to create an IGF-I reservoir that can be released slowly and continuously, thus permitting efficient use of receptor sites (Blum et al., 1989). Furthermore, association with the 150 kDa IGFBP complex extends the metabolic half-life of IGF-I (Cohen and Nissley, 1976; Rechler and Clemmons, 1998). Smaller IGFBP can cross the capillary barriers and, therefore, can act as transport vehicles to the target cells and tissues (Binoux et al., 1982; Binoux and Hossenlopp, 1988). For these reasons, effects of IGFBP must be considered when viewing actions of IGF-I in biological systems. An understanding of how these processes are controlled and regulated is essential for determining IGF-I actions as an anabolic agent. The objective of this study was to investigate the effect of divergent selection for serum IGF-I concentration and sex of calf on serum IGFBP in Angus cattle and to determine if correlated responses exist among serum IGFBP, body weight and postweaning gain and serum IGF-I concentration.

Materials and Methods

IGF-I Selection Lines. A divergent selection experiment for serum IGF-I concentration in Angus cattle was initiated in 1989 at The Ohio State University. Complete protocols for the ongoing selection experiment are described elsewhere (Davis et al., 1995; Davis and Simmen, 1997). The selection criterion is the mean of serum IGF-I concentrations measured on each animal at d 28, 42 and 56 of the postweaning performance test after adjusting for age of calf and age of dam. Cows from the initial base population were randomly assigned to the selection lines. The experiment includes spring and fall replicates with approximately 50 cows per line in each replicate. Different sets of four bulls with unknown IGF-I levels were used to produce the spring 1989 and 1990 and fall 1990 calf crops. Each successive year, the four bull calves with the highest and the four bull calves with the lowest residuals for IGF-I concentration are saved for breeding within their respective selection lines. Bulls selected for breeding are used only as yearlings and then sold. Approximately eight cows are culled from each line per year based on physical unsoundness, reproductive failure, and age. Culled females are replaced with approximately eight pregnant heifers that possess the highest or lowest residuals for IGF-I concentration. All available heifers are bred and selections are made among those females that conceive.

All animals are reared under common conditions at the Eastern Ohio Resource Development Center (EORDC), Belle Valley, except during the postweaning test period. During the postweaning period, bulls and heifers are fed in different drylot locations, because no single facility is adequate to house all

animals. Bull calves are given ad libitum access to a corn-soybean meal-based diet, plus 2.3 kg/bull/d of grass hay. Heifer calves are fed a corn-soybean meal diet intended to yield postweaning gains of approximately 0.75 kg/d.

All calves are weighed at birth, weaning, beginning of the postweaning performance test, and every 28 d thereafter until conclusion of the 140 d postweaning period. In addition, calves are weighed at d 42, when one of the three blood samples is collected. Backfat thickness and ribeye area are measured on all calves at d 56 and 140 using ultrasound.

In the present study, serum from calves of the divergent selection project born in fall 1992 and in fall 1999 were used to evaluate circulating levels of the IGFBP. Also, body weight and postweaning gain data and serum IGF-I concentration from the corresponding years were used to determine (by correlation analysis) if a relationship existed between serum IGFBP and those specific traits.

Western Ligand Blotting. Relative circulating IGFBP concentrations were determined by [^{125}I]IGF-I western ligand blotting (McCusker and Clemmons, 1988; Cohick et al., 1992). Briefly, serum samples from d 28, 42 and 56 of the postweaning test period for the fall 1992 calves and from d 56 of the postweaning test period for the fall 1999 calves were diluted 1:1 with Laemmli's electrophoresis buffer (without 2-mercaptoethanol). Diluted samples (4µl) were subjected to SDS-PAGE (Laemmli, 1970) using 12% separating acrylamide gels with 4% stacking gels on a Protean® II xi electrophoresis cell (Bio-Rad Laboratories, Hercules, CA). Serum from a randomly chosen animal (unrelated

to the IGF-I selection lines) was used as a control on all gels and BenchMark™ prestained protein molecular weight standards (Invitrogen, Carlsbad, CA) were used to estimate molecular weights of IGFBP species. All samples were replicated on two different gels. Separated proteins were transferred electrophoretically onto nitrocellulose membranes using a Trans-Blot® electrophoretic transfer cell (Bio-Rad Laboratories, Hercules, CA). Following transfer, gels were stained with Coomassie® Brilliant Blue R-250 (Bio-Rad Laboratories, Hercules, CA) and membranes were stained with Ponceau S Solution (SIGMA, St. Louis, MO) to assure a complete transfer. Membranes were blocked by incubating with 1 X TTBS (20mM Tris-HCL, 500 mM NaCl, 0.1% Tween20, pH 7.4) with 1% BSA, rinsed and incubated with radiolabeled ligand (4,000 dpm/cm^2 [^{125}I]IGF-I) for 18 hr at 4°C. Blots were rinsed, dried and exposed at –70°C in cassettes with intensifying screens and Kodak X-OMAT AR film. IGFBP on ligand blots were quantified using either a scanning laser densitometer (LKB Ultroscan XL; Pharmacia-LKB, Piscataway, NJ, USA) and the LKB 2400 GelScan XL software package for the fall 1992 samples or the Quantity One® v 4.1 software package on a Fluor-S™ Multilmager System (Bio-Rad Laboratories, Hercules, CA) for the fall 1999 samples.

IGFBP-2 Immunoblotting. IGFBP-2 immunoblotting was performed according to the manufacturer's protocol (Upstate Biotechnology, Lake Placid, NY). Briefly, serum samples collected on d 56 of the postweaning test period from a fall 1999 low IGF-I line heifer calf were diluted 1:1 with Laemmli's electrophoresis buffer (without 2-mercaptoethanol). Diluted samples (4µl) were

subjected to SDS-PAGE (Laemmli, 1970) using 12% separating acrylamide gels with 4% stacking gels on a Mini-Protean® II electrophoresis cell (Bio-Rad Laboratories, Hercules, CA). Separated proteins were transferred electrophoretically onto Immun-Blot® PVDF membranes (Bio-Rad Laboratories, Hercules, CA) using a Mini Trans-Blot® electrophoretic transfer cell (Bio-Rad Laboratories, Hercules, CA). BenchMark™ prestained protein molecular weight standards (Invitrogen, Carlsbad, CA) were used to estimate molecular weights of IGFBP species. Also, 200 ng of purified human IGFBP-2 (GroPep, North Adelaide, Australia) and human IGFBP-3 (Upstate Biotechnology, Lake Placid, NY) were used as positive and negative controls, respectively. Blotted membranes were washed twice with Milli-Q water and blocked in freshly prepared PBS containing 3% nonfat dry milk (PBS-MLK) followed by an overnight incubation at 4°C with an 1:1,000 dilution of the anti-bovine IGFBP-2 antibody (Upstate Biotechnology, Lake Placid, NY) in PBS-MLK. Proteins were visualized using a goat anti-rabbit horseradish peroxidase (HRP) conjugated IgG as the secondary antibody (dilution 1:5,000) and the TMB Membrane Peroxidase Substrate System (Kirkegaard and Perry Laboratories Inc, Gaithersburg, MD).

IGF-I Radioimmunoassay (RIA). Serum IGF-I concentrations were determined by RIA in the laboratory of Dr. R.C.M. Simmen at the University of Florida, Gainesville using procedures described by Bishop et al. (1989). Briefly, following acid-ethanol extraction, each sample was diluted 1:10 in assay buffer and assayed in duplicate. Recombinant human IGF-I served as both the standard and as an iodinated tracer in the assay. Antiserum raised against

human IGF-I in rabbits (UBK487) was used at a dilution of 1:18,000. Antigen-antibody complexes were precipitated by addition of goat anti-rabbit gamma globulin and normal rabbit serum. Comparison of the assayed cattle sera to the standard displacement curves determined the serum IGF-I concentration of individual samples.

Statistical Analysis. The effects of line, sex and the line x sex interaction were considered using analysis of variance (Mixed Procedure; SAS, 1996) of data obtained by densitometric analysis of autoradiographs.

The linear model used was:

$y_{ijklmnop} = \mu + L_i + S_j + B_k + A_{(k)l} + LS_{ij} + C_m + AD_n + WA_o + e_{ijklmnop}$ *where:*

$y_{ijklmnop}$ = *p* observation pertaining to the *i*th IGF-I selection line, *j*th sex of calf, *k*th blot, (*k*) *l*th animal nested within blot, the *ij*th interaction between IGF-I selection line and sex of calf, *m*th blot background covariate, *n*th age of dam covariate and *o*th calf weaning age covariate.

μ = overall mean.

L_i = fixed effect of *i*th IGF-I selection line.

S_j = fixed effect of the *k*th sex of calf.

B_k = random effect of the *k*th blot.

$A_{(k)l}$ = random effect of *l*th animal nested within the *k*th blot.

LS_{ij} = fixed effect of the *ij*th line x sex interaction.

C_m = *m*th blot background covariate.

AD_n = *n*th age of dam covariate.

WA_o = *o*th calf weaning age covariate.

e_{abcdef} = residual error term.

The average between replications (adjusted for background) was used for the corresponding residual correlation analysis between serum IGFBP relative levels, body weight, postweaning gain and serum IGF-I concentration (Mixed Procedure; SAS, 1996). Also, the effect of line, sex and the line x sex interaction for body weight, postweaning gain and serum IGF-I concentration of calves from the IGF-I selection lines born in fall 1992 and fall 1999 were determined using age of dam and weaning age as covariates (GLM Procedure; SAS, 1996).

Results and Discussion

IGFBP Identification by [^{125}I]IGF-I Western Ligand Blotting and Immunoblotting. Blood serum from the fall 1992 and fall 1999 calves of the IGF-I selection experiment was subjected to [^{125}I]IGF-I western ligand blotting. Exposed autoradiographs revealed the presence of four distinct IGFBP bands of relative molecular mass estimates of 38-42 kDa, 34 kDa, 30 kDa, and 24 kDa as determined by comparison of migration distances of specific bands with prestained molecular weight protein standards (Figure 2.1). These results confirm previous reports in which this technique has been used successfully to determine cellular and circulating bovine IGFBPs (Conover, 1990; Cohick et al., 1992). For example, [^{125}I]IGF-I western ligand blotting performed by White et al. (1998) in human, bovine, ovine and porcine sera revealed a pattern of different molecular weight IGFBP consisting of 39-43 kDa bands, a 34 kDa band, a 28 kDa band and a 24 kDa band. Cohick et al. (1992) also used western ligand blotting and demonstrated that bovine serum and afferent mammary lymph of

lactating cows treated with bovine somatotropin (bST) contains IGFBP with molecular weight estimates of 43 kDa, 39 kDa, 34 kDa, 29 kDa, and 24 kDa. In addition, Skaar et al. (1991) reported that IGFBP of 200 kDa, 46 kDa, 42 kDa, 34 kDa, 30 kDa and 25 kDa were present in serum and mammary secretions of late gestation and early lactation dairy cows.

A commercially available anti-bovine IGFBP-2 antibody was used to determine (by immunoblotting) which band on [^{125}I]-IGF-I western ligand blots corresponds to IGFBP-2. Serum of a low IGF-I line heifer calf was used. By this approach, the 34 kDa IGFBP species was determined to be bovine IGFBP-2 (Figure 2.2).

Wood et al. (1988) demonstrated that IGFBP species associated with bands of approximate molecular masses between 38 and 42 kDa were glycosylation variants of the same gene product. This protein was classified as being the acid-stable subunit of the 150 kDa complex associated with the majority of bound IGF-I in serum (Conover et al., 1990; McGuire et al., 1992). A later report by Cohick et al. (1992), using bovine specific antisera, showed that the 43 kDa and 39 kDa IGFBP bands were glycosylation variants of IGFBP-3 and the 34 kDa band was IGFBP-2. In human, bovine, ovine and porcine sera, the 28 kDa and 24 kDa bands may represent IGFBP-1, glycosylated or non-glycosylated IGFBP-5 and glycosylated or non-glycosylated IGFBP-4 or a combination of these IGFBP (White et al., 1998). Therefore, there is some controversy with respect to the identities of the 24 kDa IGFBP and the 28-30 kDa IGFBP.

The IGFBP are expressed in almost all tissues and all of the IGFBP are expressed in the liver (primary source of circulating IGFBP) except IGFBP-5 whose expression is very low or absent in the liver (Shimasaki et al., 1991). IGFBP-5 preferentially binds IGF-II and potentiates the actions of IGF-I in smooth muscle cells, fibroblasts and osteoblasts (Kalus et al., 1998), however, it is considered to be absent in blood (Cohick and Clemmons, 1993). IGFBP-4 is found in serum and seminal plasma and does not associate either with cell surfaces or the extracellular matrix (reviewed by Hwa et al., 1999). IGFBP-1 is produced mainly in the liver and is found in low concentration in serum where it has a short-life (Murphy, 1998).

Our results are consistent with previous reports in identifying the 34 kDa IGFBP species in bovine serum as IGFBP-2. In addition, the consensus from the literature is that the 38-43 kDa IGFBP species in bovine serum is IGFBP-3. IGFBP species of 30 kDa and 24 kDa were also identified in the present study. However, the identities of these IGFBP were not determined and there is no clear consensus in the literature regarding their specific identities.

Effect of Single Trait Selection for Serum IGF-I on Relative Circulating IGFBP Levels. IGFBP concentration was evaluated near the beginning of the IGF-I divergent selection project in serum from the fall 1992 born calves. No statistically significant differences were observed between selection lines for any of the IGFBP species in serum collected at d 28, 42 or 56 of the postweaning test period ($P > 0.05$, data not shown). At this time, the population had only undergone two years of selection and differences in serum IGF-I concentrations

between the high and low line were not significant (P > 0.05, Table 2.1). In the fall 1999 calves, differences in serum IGF-I concentrations between the high and low lines were highly significant (P < 0.0001, Table 2.2). High line calves had an average of 123 ng/mL more IGF-I than the low line calves at d 28, 42 and 56 of the postweaning test period. In the fall 1992 calves, no statistically significant effect of line was observed for body weight or postweaning gain and no significant line x sex interactions were observed (Table 2.3). However, in the fall 1999 calves, while no significant line x sex interactions were observed, the high IGF-I line had higher weight at d 28, 42 and 56 of the postweaning test period, and higher off test weight and off test hip height than the low IGF-I line (Table 2.4). Therefore, our interest was to evaluate serum IGFBP in the fall 1999 calves to see if changes in serum IGF-I concentration resulted in changes in the expression of the IGFBP. Because of the short sampling period (only 28 d) and the inconsistent time trends observed in the fall 1992 calves for individual IGFBP at d 28, 42 and 56 of the postweaning test period, only serum samples obtained at d 56 of the postweaning test period were used to further evaluate serum IGFBP concentrations.

Serum IGFBP concentration was evaluated later in the project using the fall 1999 calves. No statistically significant differences were observed between selection lines for any of the IGFBP species in serum collected at d 56 of the postweaning test period (P > 0.05, Figure 2.3). In addition, no significant line x sex interactions were observed for any of the IGFBP. These samples were collected after nine years of selection for increased or decreased serum IGF-I

and differences in IGF-I between the high and low selection lines were highly significant ($P < 0.0001$, Table 2.2). This demonstrates that even though single trait selection for high vs. low serum IGF-I concentration has been successful, this selection has not resulted in changes in the expression of the IGFBP.

Effect of Sex on Relative Circulating IGFBP Levels. A statistically significant effect of sex was observed for serum IGFBP levels in both the fall 1992 (data not shown) and fall 1999 calves (Figure 2.4). For both years, the serum levels of the 34 kDa IGFBP species (i.e., IGFBP-2) were higher in heifer calves than in bulls ($P < 0.0001$, fall 1992; $P < 0.0005$, fall 1999, Figure 2.4) and the level of the 30 kDa IGFBP species was not affected by sex ($P > 0.10$, fall 1992; $P > 0.4$, fall 1999). In fall 1992, bull calves expressed greater levels of both the 38-42 and 24 kDa IGFBP species than heifers ($P < 0.0001$, data not shown). However, these differences were not observed in the fall 1999 calves ($P > 0.05$, Figure 2.4). To our knowledge we are the first to report sex differences in the expression of specific IGFBP species in bovine serum.

Ronge and Blum (1989) used column chromatography to separate serum IGFBP complexes from lactating cows, dry cows, 100-day-old calves and growing bulls (450-500 kg). While no specific IGFBP identity was determined, these investigators observed three IGFBP fractions with a significantly different distribution between bulls, cows and calves. It is unclear whether these differences are due to gender differences or to differences in age and/or physiological status. Clapper et al. (2000) reported that in pig serum, concentrations of testosterone were negatively correlated with relative amounts

of serum IGFBP-2, but positively correlated with serum concentrations of IGF-I and estradiol-17ß ($P < 0.01$). Moreover, serum concentrations of estradiol-17ß were positively correlated with serum concentrations of IGF-I in boars ($P < 0.01$). Such results suggest that changes in serum concentration of IGF-I and relative amounts of IGFBP resulting from changes in serum concentration of estradiol-17ß and testosterone may contribute to growth differences observed among sexes. In the present study, serum IGF-I concentration was 2.7 and 3.7 times higher in males than in females for the fall 1992 and fall 1999 calves, respectively ($P < 0.0001$, Tables 2.1 and 2.2). Also, bull calves had higher weights, weight gain and off test hip height than heifers (Tables 2.3 and 2.4). In addition, a significant line x sex interaction was observed for d 56 serum IGF-I in the fall 1999 calves ($P < 0.0001$). High line bulls had the highest serum IGF-I concentration (535.5 ± 19.3 ng/mL) followed by low line bulls (329.9 ± 21.1 ng/mL), high line heifers (141.1 ± 19.3 ng/mL) and low line heifers (87.3 ± 17.2 ng/mL). Thus, differences in serum IGF-I concentration between the high and low lines were greater for bull calves than for heifers resulting in a significant line x sex interaction. In contrast, no significant line x sex interactions were observed for any of the IGFBP species or for body weight or postweaning gain in either the fall 1992 or fall 1999 calves ($P > 0.05$, data not shown).

It must be noted that sex differences in serum IGFBP expression observed in the present study may be confounded with diet and location of rearing since males and females were raised separately. The changing relationship between growth hormone (GH) and IGF-I secretion with varying

planes of nutrition suggests the central importance of nutrition-dependent changes in the somatotrophic axis in determining the growth rate of ruminants. However, Armstrong et al. (1993) reported that feed restriction for 4 d did not alter serum IGFBP in Angus and Charolais heifers. Furthermore, no differences were detected in the IGF-I clearance rate and IGF-I half-life, or in the [^{125}I]IGF-I distribution between fed and starved sheep (Hodgkinson et al., 1987). Nonetheless, Breier et al. (1986) observed that reduced feeding at both medium and low planes of nutrition in steers significantly increased mean plasma GH concentration. Also a decrease in plasma IGF-I concentration with nutritional deprivation was observed at a negative energy balance. Because circulating concentrations of IGF-I are dependent on IGFBP, changes in IGF-I during nutrient restriction may reflect changes in IGFBP. Moreover, IGFBP-3 serum concentration is directly related to GH secretory status (Baxter and Martin, 1986), whereas IGFBP-1and IGFBP-2 serum concentrations are inversely related with growth hormone (Binoux et al., 1986). Thus, the reduced feed intake of heifers from the IGF-I selection lines (as compared with the ad libitum feeding regime for bull calves) would be expected to elevate serum GH levels, which in turn would be expected to decrease circulating levels of IGFBP-2. However, in both fall 1992 and fall 1999, heifer calves had significantly higher relative circulating levels of IGFBP-2 than bulls. Thus, sex differences observed in the present study are not likely to have resulted from differences in the feeding regimes for males and females.

Residual Correlations of Serum IGFBP Levels with Body Weight, Postweaning Gain and Serum IGF-I Concentration. Residual correlation analysis was performed using data from the fall 1992 and fall 1999 calves. Results from the fall 1992 calves demonstrated that early in the selection experiment the relative serum levels of the 38-42 kDa and 24 kDa IGFBP were significantly correlated with serum IGF-I concentration ($r = 0.11$, $P < 0.04$ and -0.11, $P < 0.05$, respectively). However, residual correlations between serum IGF-I concentration and relative serum levels of the 34 kDa and the 30 kDa IGFBP species were not significant. In the fall 1999 calves, the relative level of the 34 kDa IGFBP (IGFBP-2) was negatively correlated with serum IGF-I concentration at d 28 and 42 of the postweaning test period ($P < 0.01$, Table 2.5) and its correlation with d 56 IGF-I tended toward significance ($P = 0.08$, Table 2.5). Neither, the 38-42 kDa IGFBP species, the 30 kDa IGFBP species or the 24 kDa IGFBP species were significantly correlated with serum IGF-I concentration (Table 2.5).

Under normal physiological conditions IGF-I is found predominantly as a 150 kDa complex that consists of one molecule each of IGF-I, IGFBP-3, and an acid labile subunit (ALS) which is a 85 kDa glycoprotein (Rechler, 1993; Baxter, 1994, Ooi and Boisclair, 1999). This complex functions as a storage pool of circulating IGF-I, which prolongs the IGF-I half-life. In human circulation, IGF-I and IGFBP-3 are present at equimolar concentrations (Baxter and Martin, 1986), whereas ALS circulates in excess (Baxter, 1990). Adult serum concentrations of IGFBP-3 are relatively constant and most likely account for the stability of IGF-I concentration throughout the day (Martin and Baxter, 1986). Serum also

contains lower molecular weight complexes of approximately 50 kDa which are made up of several IGFBP species that are incompletely saturated with IGF-I leaving virtually no free IGF-I in the circulation (Jones and Clemmons, 1995; Stewart and Rotwein, 1996). Therefore, under normal physiological conditions, if there is unsaturated binding capacity, IGF-I will preferentially bind to an unsaturated binding protein (Clemmons, 1998). Binding protein associated IGF-I is usually in equilibrium with the IGF-I receptor, but the extent to which IGF-I can access its receptor is influenced by the amount of high affinity carrier and the amount of IGF-I since under normal conditions, receptor number varies minimally (Clemmons, 1998).

Results of the present study determined that single trait selection for serum IGF-I has not resulted in changes in the expression of the IGFBP in serum. However, serum IGF-I concentration has changed significantly since the beginning of the divergent selection project. In 1989, the mean IGF-I concentration was 57 ± 6 ng/mL for the animals randomly assigned to the high IGF-I line and 53 ± 7 ng/mL for the animals assigned to the low line (Davis et al., 1995). In the fall 1992 calves, the serum IGF-I concentration for both the high and low line was 3.6-3.8 times higher than in 1989 and the residual correlation between the 38-42 kDa and the 24 kDa IGFBP with serum IGF-I was low. It is unknown why serum IGF-I levels increased in both lines between 1989 and 1992, however, it is possible that changes in the assay conditions contributed to this change. In 1999, the lines had significantly diverged with 3-6 times more serum IGF-I than in 1989. At the same time, the residual correlations between

the 38-42 kDa and the 24 kDa IGFBP with serum IGF-I were not significant, whereas IGFBP-2 was negatively correlated with serum IGF-I (Table 2.5). It is possible that divergent selection for serum IGF-I has caused changes in other components (e.g., growth hormone, somatostatin) of the IGF axis, which in some way is affecting the mechanism(s) by which circulating IGF-I levels are regulated in cattle. For example, growth hormone (GH) is under negative feedback control and IGF-I acts directly on the pituitary to inhibit GH secretion (Ganong, 1995). Also IGF-I stimulates the secretion of the GH-inhibiting hormone somatostatin from the hypothalamus, which inhibits the response to growth hormone releasing factor (GRF) (Ganong, 1995). At the same time, IGFBP-3 serum concentration is directly related to GH secretory status (Baxter and Martin, 1986), whereas IGFBP-1and IGFBP-2 serum concentrations are inversely related with growth hormone (Binoux et al., 1986). Armstrong et al. (1993) immunized heifers against GRF resulting in a decrease in GH, IGF-I and insulin ($P < 0.05$). In addition, a decrease in serum IGFBP-3 and an increase in IGFBP-2 was observed ($P < 0.01$).

In the fall 1992 calves, serum IGFBP levels were not significantly correlated with either body weight or average daily gain. However, in the fall 1999 calves, IGFBP-2 was found to be negatively correlated with birth weight, weaning weight, on test weight, weight at day 28, 42 and 56 of the postweaning test period, off test weight and off test hip height (Table 2.6). The residual correlation between the 30 kDa IGFBP and birth weight tended toward significance ($P = 0.06$, Table 2.6). Neither, the 38-42 kDa nor the 24 kDa IGFBP

species were significantly correlated with any of the body weight or postweaning gain traits measured in the IGF-I selection lines. At the same time, none of the IGFBP species were correlated with subcutaneous backfat or loin muscle area as measured by ultrasound at d 56 and 140 postweaning (Table 2.6). Meanwhile, all four IGFBP species were positively correlated with each other (Table 2.5).

The precise roles of individual IGFBP are still unknown, due mainly to the great complexity of their actions and their regulation, but also to the fact that the overwhelming majority of information about the IGFBP is derived from in vitro studies. For that reason, the literature is vague in terms of the function of the circulating IGFBP in livestock and the relationships between them. Our results demonstrate that IGFBP-2 is negatively correlated with body weight. In cattle, Connor et al. (2000) reported that plasma IGFBP-2 was related to average daily gain (ADG) explaining nearly 30% of the variation in that trait. However, no relationship to carcass characteristics was found. Vleurick et al. (1999) found that cows treated with recombinant bovine somatotropin (bST) had significantly lower serum levels of IGFBP-2 than did control cows. This decrease in serum IGFBP-2 concentration in bST treated cows seems partly regulated at the level of IGFBP-2 synthesis, because IGFBP-2 mRNA abundance in the liver of bST treated cows was only 50% that of controls, whereas the decrease in serum IGFBP-2 protein was about 30%. Therefore, IGFBP-2 appears to play a key role in the growth and development of mammals.

Early in this project the 38-42 kDa IGFBP species and the 24 kDa IGFBP species were correlated with serum IGF-I concentration, whereas, later in the

project, IGFBP-2 was correlated not only with serum IGF-I but also with body weights and hip height. This suggests that the mechanism by which the IGFBP regulate the actions of IGF-I in Angus cattle divergently selected for serum IGF-I is complicated and that IGFBP-2 may play a key role in this process.

Implications

This study demonstrates that single trait selection for high vs. low serum IGF-I concentration has resulted in divergence between the high and low IGF-I selection lines and also has resulted in changes in body weight and postweaning gain, but has not resulted in changes in IGFBP concentrations in serum. To our knowledge we are the first to report sex differences in bovine IGFBP-2. Circulating IGFBP-2 may play a key role as a negative regulator of postnatal growth of beef cattle.

Table 2.1. Least squares means and standard errors for serum IGF-I concentration on d 28, 42, and 56 of the postweaning test period for fall 1992 calves.

	D 28 IGF-I (ng/mL)	D 42 IGF-I (ng/mL)	D 56 IGF-I (ng/mL)
Line			
High	210.0 ± 9.1[b]	206.9 ± 8.4[b]	221.2 ± 9.4[b]
Low	198.8 ± 11.4[b]	200.0 ± 10.5[b]	205.7 ± 11.8[b]
Sex			
Bulls	299.1 ± 9.6[b]	299.1 ± 8.9[b]	313.6 ± 10.1[b]
Heifers	109.8 ± 10.9[c]	107.8 ± 10.1[c]	113.4 ± 11.4[c]

[bc]Within a column for line or sex, means not sharing a common superscript differed ($P < 0.0001$).

Table 2.2. Least squares means and standard errors for serum IGF-I concentration on d 28, 42, and 56 of the postweaning test period for fall 1999 calves.

	D 28 IGF-I (ng/mL)	D 42 IGF-I (ng/mL)	D 56 IGF-I (ng/mL)
Line			
High	303.5 ± 12.4[b]	305.6 ± 11.3[b]	336.8 ± 13.6[b]
Low	184.1 ± 12.3[c]	184.0 ± 11.1[c]	208.6 ± 13.4[c]
Sex			
Bulls	359.8 ± 13.1[b]	380.2 ± 11.9[b]	431.2 ± 14.3[b]
Heifers	127.8 ± 11.8[c]	109.4 ± 10.7[c]	114.2 ± 12.9[c]

[bc]Within a column for line or sex , means not sharing a common superscript differed ($P < 0.0001$).

Table 2.3. Least squares means and standard errors for body weight and postweaning gain (kg) measured in Angus cattle divergently selected for serum IGF-I concentration and born in fall 1992[a].

Trait	Line		Sex	
	High	Low	Bull	Heifer
Birth Weight	34.6 ± 0.6[b]	34.1 ± 0.8[b]	35.5 ± 0.6[b]	33.2 ± 0.7[c]
Weaning Weight	202.3 ± 4.3[b]	208.7 ± 5.5[b]	217.2 ± 4.6[b]	193.8 ± 5.2[c]
On test weight	227.0 ± 4.8[b]	233.1 ± 6.0[b]	244.8 ± 5.1[b]	215.3 ± 5.8[c]
Weight d 28 postweaning	270.4 ± 4.9[b]	272.0 ± 6.2[b]	298.6 ± 5.3[b]	243.8 ± 6.0[c]
Weight d 42 postweaning	287.7 ± 5.1[b]	293.3 ± 6.4[b]	330.0 ± 5.4[b]	251.0 ± 6.1[c]
Weight d 56 postweaning	300.2 ± 5.1[b]	307.9 ± 6.4[b]	351.1 ± 5.4[b]	257.1 ± 6.2[c]
Off test weight	389.3 ± 6.3[b]	389.5 ± 7.8[b]	476.9 ± 6.7[b]	302.0 ± 7.5[c]
Off test hip height, cm	116.4 ± 0.5[b]	117.4 ± 0.7[b]	120.3 ± 0.6[b]	113.5 ± 0.7[c]
Total postweaning gain	162.0 ± 2.9[b]	156.1 ± 3.6[b]	232.0 ± 3.1[b]	86.0 ± 3.5[c]

[a]No significant line x sex interaction was observed ($P > 0.05$).
[bc]Within a row for line or sex, means not sharing a common superscript differed ($P < 0.05$).

Table 2.4. Least squares means and standard errors for body weight and postweaning gain (kg) measured in Angus cattle divergently selected for serum IGF-I concentration and born in fall 1999[a].

Trait	Line		Sex	
	High	Low	Bull	Heifer
Birth Weight	32.7 ± 0.8[b]	33.3 ± 0.8[b]	34.3 ± 0.8[b]	31.7 ± 0.8[c]
Weaning Weight	217.8 ± 3.8[b]	209.7 ± 3.8[b]	220.8 ± 4.0[b]	206.7 ± 3.6[c]
On test weight	236.8 ± 3.7[b]	225.9 ± 3.7[c]	238.2 ± 3.9[b]	224.5 ±3.6[c]
Weight d 28 postweaning	280.2 ± 4.1[b]	262.2 ± 4.0[c]	296.0 ± 4.3[b]	246.4 ± 3.9[c]
Weight d 42 postweaning	295.8 ± 4.2[b]	275.6 ± 4.1[c]	319.4 ± 4.4[b]	252.0 ± 4.0[c]
Weight d 56 postweaning	312.6 ± 4.7[b]	289.7 ± 4.6[c]	341.6 ± 4.9[b]	260.7 ± 4.4[c]
Off test weight	407.2 ± 5.2[b]	391.4 ± 5.1[c]	473.2 ± 5.4[b]	325.4 ± 4.9[c]
Off test hip height, cm	121.8 ± 0.5[b]	118.0 ± 0.48[c]	122.3 ± 0.5[b]	117.5 ± 0.46[c]
Total postweaning gain	170.4 ± 2.6[b]	165.6 ± 2.6[b]	235.0 ± 2.8[b]	100.9 ± 2.5[c]

[a]No significant line x sex interaction was observed (P > 0.05).
[bc]Within a row for line or sex, means not sharing a common superscript differed (P < 0.05).

Table 2.5. Residual correlation coefficients (and associated P-values) for serum IGFBP at d 56 of the postweaning test period and serum IGF-I in Angus calves divergently selected for serum IGF-I concentration and born in fall 1999.

Trait	D 42 IGF-I	D 56 IGF-I	38-42 kDa IGFBP	34 kDa IGFBP	30 kDa IGFBP	24 kDa IGFBP
D 28 IGF-I	0.71 (0.0001)	0.50 (0.0001)	-0.14 (0.22)	-0.30 (0.01)	-0.10 (0.37)	-0.15 (0.21)
D 42 IGF-I		0.69 0.0001	-0.16 (0.17)	-0.32 (0.01)	-0.16 (0.17)	-0.11 (0.33)
D 56 IGF-I			-0.14 (0.23)	-0.21 (0.08)	0.02 (0.87)	0.02 (0.88)
38-42 kDa IGFBP				0.59 (0.0001)	0.27 (0.03)	0.55 (0.0001)
34 kDa IGFBP					0.25 (0.04)	0.44 (0.0006)
30 kDa IGFBP						0.74 (0.0001)

Table 2.6. Residual correlation coefficients (and associated P-values) for serum IGFBP at d 56 of the postweaning test period and body weight and postweaning gain in Angus calves divergently selected for serum IGF-I concentration and born in fall 1999.

Trait	38-42 kDa IGFBP	34 kDa IGFBP	30 kDa IGFBP	24 kDa IGFBP
Birth Weight	-0.03 (0.80)	-0.24 (0.05)	-0.24 (0.06)	-0.11 (0.36)
Weaning Weight	-0.04 (0.77)	-0.34 (0.01)	-0.15 (0.25)	-0.17 (0.18)
On test weight	-0.003 (0.98)	-0.36 (0.01)	-0.19 (0.17)	-0.17 (0.22)
Weight d 28 postweaning	-0.02 (0.85)	-0.38 (0.01)	-0.14 (0.30)	-0.15 (0.28)
Weight d 42 postweaning	-0.03 (0.84)	-0.38 (0.01)	-0.18 (0.27)	-0.14 (0.30)
Weight d 56 postweaning	-0.01 (0.97)	-0.33 (0.02)	-0.13 (0.31)	-0.11 (0.42)
Off test weight	-0.08 (0.55)	-0.32 (0.01)	-0.14 (0.26)	-0.12 (0.36)
Off test hip height	0.04 (0.75)	-0.25 (0.04)	-0.15 (0.22)	-0.11 (0.38)
Total postweaning gain	-0.14 (0.22)	-0.14 (0.20)	-0.02 (0.85)	0.00 (0.98)
UBFD56[b]	0.13 (0.28)	-0.06 (0.60)	-0.04 (0.71)	0.08 (0.49)
ULAD56[b]	-0.003 (0.98)	-0.22 (0.80)	-0.06 (0.64)	0.04 (0.76)
UBFD140[b]	0.05 (0.68)	-0.10 (0.40)	0.03 (0.81)	0.04 (0.71)
ULAD140[b]	-0.09 (0.46)	-0.21 (0.9)	0.04 (0.97)	0.02 (0.84)

[b]UBFD56 = back fat measured by ultrasound at d 56 ; ULAD56 = loin muscle area measured by ultrasound at d 56 postweaning; UBFD140 = back fat measured by ultrasound at d 140 postweaning; ULAD140 = loin muscle area measured by ultrasound at d 140 postweaning.

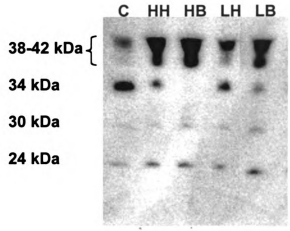


Figure 2.1. Representative [^{125}I]IGF-I western ligand blot of serum collected at d 56 of the postweaning test period from Angus calves selected for high or low serum IGF-I concentration and born in fall 1999. C, control animal; HH, high line heifer; HB, high line bull; LH, low line heifer; LB, low line bull. IGFBP species of 38-42 kDa, 34 kDa, 30 kDa and 24 kDa were identified. IGFBP sizes were determined by comparison of autoradiographs with prestained protein molecular weight standards on the membranes.

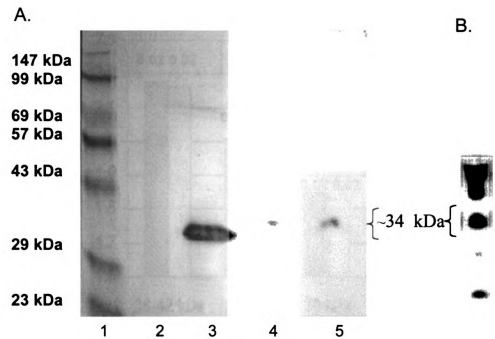


Figure 2.2. Bovine IGFBP-2 immunoblot. Panel A: Lane 1, prestained protein molecular weight standards; Lane 2, 200 ng of human IGFBP-3; Lane 3, 200 ng of human IGFBP-2; Lane 4 and 5, 2 µl of serum from a fall 1999 low IGF-I line heifer at day 56 of the postweaning test period; Panel B: [^{125}I]IGF-I western ligand blot of 2 µl of serum from the same heifer.

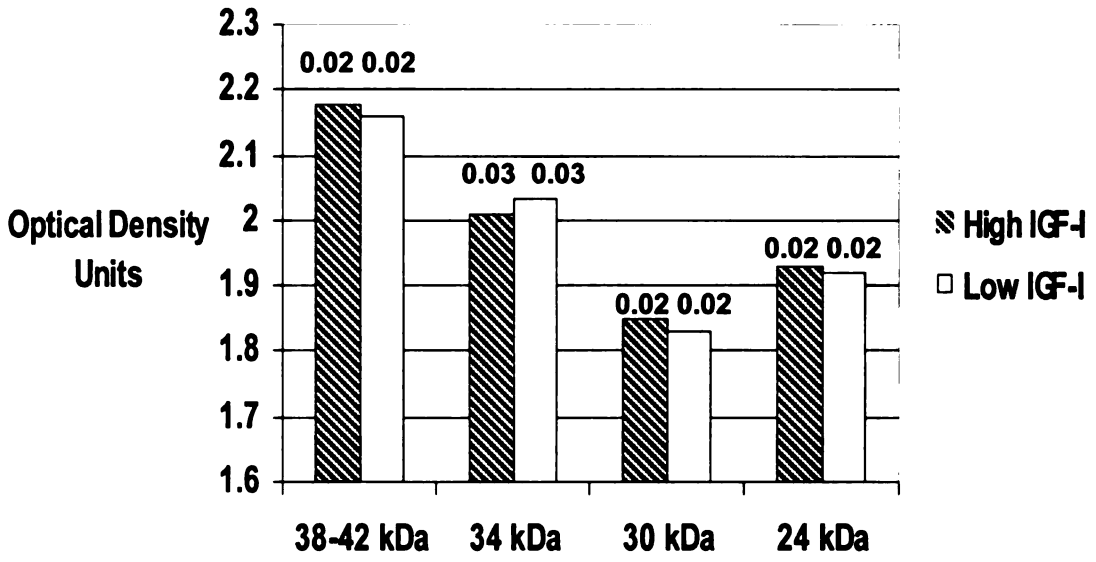


Figure 2.3. Serum IGFBP concentrations at d 56 of the postweaning test period in Angus calves selected for high (n = 40) or low (n = 44) serum IGF-I concentrations and born in fall 1999. Results of densitometric analysis of [^{125}I]IGF-I western ligand blots are shown. All samples were run in duplicate and data is presented as mean optical density units for each IGFBP. Standard errors are shown above each bar.

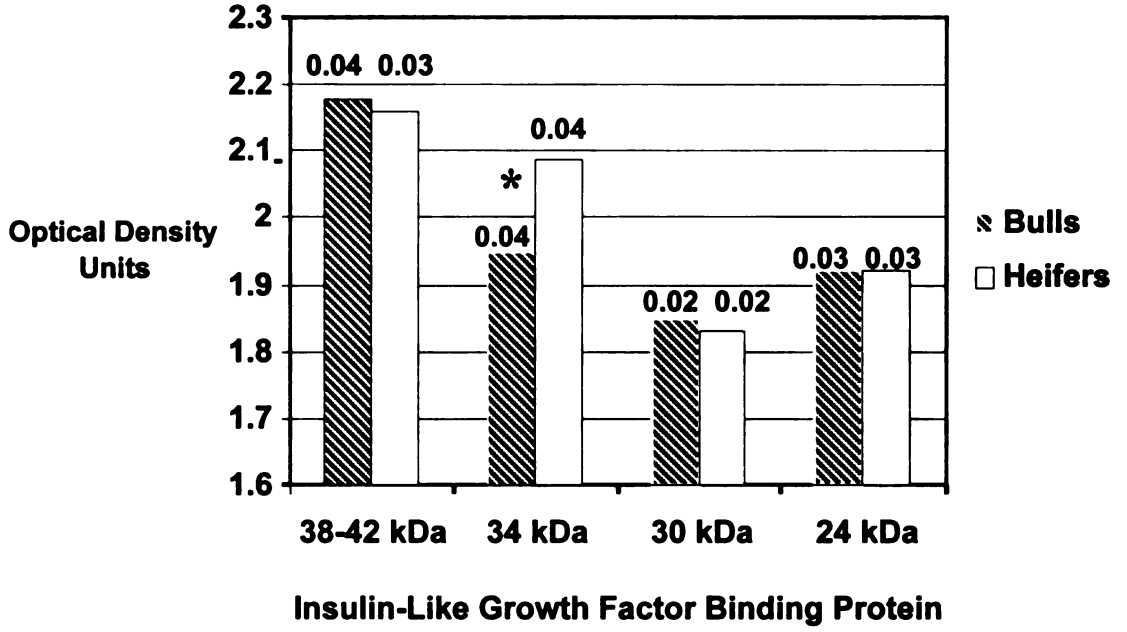


Figure 2.4. Serum IGFBP concentrations at d 56 of the postweaning test period in Angus bull (n = 39) and heifer (n = 40) calves selected for high or low serum IGF-I concentrations and born in fall 1999. Results of densitometric analysis of [^{125}I]IGF-I western ligand blots are shown. All samples were run in duplicate and data is presented as mean optical density units for each IGFBP. Standard errors are shown above each bar. Bars labeled with an asterisk (*) indicate significant differences between bull and heifer calves ($P < 0.05$).

CHAPTER 3

IDENTIFICATION AND EVALUATION OF DNA SEQUENCE VARIATION (POLYMORPHISM) AT THE INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-2 LOCUS IN BEEF CATTLE

Abstract

Insulin-like growth factor binding protein-2 (IGFBP-2) was selected as a candidate gene for growth, carcass merit and meat quality in beef cattle. Oligonucleotide primers were designed from published bovine IGFBP-2 cDNA sequence (GenBank accession no. AF074854) and were used to amplify a 1,276-bp fragment of the bovine IGFBP-2 gene by the polymerase chain reaction (PCR). Two restriction fragment length polymorphisms (PCR-RFLP) were identified in this fragment using the restriction endonucleases *Hind* III and *Nla* III. Black Angus, Hereford, Maine Anjou, MARC II, Red Angus, Salers, Shorthorn, Simmental and Tarentaise sired cattle enrolled in the Michigan Cattlemen's Association/Michigan State University (MCA/MSU) Farm-to-Fab Program, Angus cattle divergently selected for serum IGF-I concentration and Brahman cattle from a private farm in Puerto Rico were genotyped for these polymorphisms. *Hind* III PCR-RFLP alleles (designated A and B) were found to be segregating within all the genotyped cattle. The frequency of the A allele was higher than the frequency of the B allele in the Brahman (n = 8), Hereford (n = 17), and Maine Anjou (n = 11) cattle, whereas the frequency of the B allele was higher than the frequency of the A allele in the Simmental (n = 21), Tarentaise (n = 7), Salers (n = 5), Shorthorn (n = 13) and Black Angus (n = 17) cattle. Within the MARC II (n

= 8) and Red Angus (n = 1) cattle, the frequencies of the A and B alleles were equal. Genotyping of high and low IGF-I line individuals (born in spring 1995, fall 1995, fall 1997, fall 1998 and spring 1999; n = 366) from the IGF-I selection lines indicated no difference in allele frequencies between the high and low IGF-I lines or between bull and heifer calves. Analysis of variance using *Hind* III PCR-RFLP genotypes and phenotypic data from both the MCA/MSU Farm-to-Fab cattle (growth, carcass and meat quality data) and from the IGF-I selection lines (body weight and postweaning gain data and serum IGF-I concentrations) was performed to determine if an association exists between bovine IGFBP-2 genotypes and growth, carcass and meat quality traits in beef cattle. Results obtained using data from the Hereford, Shorthorn and Maine Anjou breeds of the MCA/MSU Farm-to-Fab Program (all 3 genotypes were represented only in these breeds; n = 41 animals) and from the IGF-I selection lines (n = 366) indicated that animals with the BB genotype appeared to have more desirable growth and carcass characteristics. *Nla* III PCR-RFLP alleles (designated C and D) were found to be segregating in only the Simmental, MARC II and Brahman cattle and all 3 genotypes were present only in the Brahmans.

Introduction

By definition, marker assisted selection (MAS) is the selection of specific alleles at genetic loci that are linked closely enough to loci controlling economically important traits that they can be used to identify alleles at the loci of interest. Use of MAS may increase the short term rate of genetic gain in livestock by 15 to 30% (Kashi et al., 1990), without increasing the risk involved in

breeding schemes. However, few genetic markers have been identified for growth traits in beef cattle.

The hormonal regulation of growth is multifactorial and a group of proteins known to affect animal growth and metabolism is the insulin-like growth factor (IGF) system. IGFs are associated with myogenesis in vivo and their actions are modulated by insulin-like growth factor binding proteins (IGFBP) (reviewed by Florini et al., 1996). Also, IGF independent effects have been identified for some IGFBP (Baxter, 2000). IGFBP compete with IGF receptors for IGF binding, and as a consequence, they can affect cell growth. Moreover, transgenic mouse models overexpressing IGFBP-2 resulted in a reduction of somatic growth (Schneider et al., 2000). IGFBP-2 appears to play a key role in myogenesis (Ernst et al., 1992; Ernst et al., 1996; Fligger et al., 1998; Gerrard et al., 1999). The level of expression of IGFBP-2 mRNA and protein was found to be high in proliferating turkey myogenic satellite cells (Ernst et al., 1996) and mouse myoblasts (Ernst et al., 1992) and to decrease gradually as differentiation progressed. Sequestration of IGF-I by IGFBP-2 appears to render that growth factor less available to the myogenic satellite cells (Fligger et al., 1998). Thus, IGFBP-2 may play a key role in the growth and development of livestock and it is therefore an excellent candidate gene for these traits. The objective of this study was to identify DNA sequence variation (polymorphisms) at the bovine IGFBP-2 gene locus and to evaluate IGFBP-2 polymorphisms in different breeds of cattle and in Angus cattle divergently selected for serum IGF-I concentrations for potential associations with growth, carcass merit and meat quality traits.

Materials and Methods

Michigan Cattlemen's Association/Michigan State University (MCA/MSU) Farm-to-Fab Program. Initiated in 1993 by the Purebred Council of the Michigan Cattlemen's Association, the MCA/MSU Farm-to-Fab Program was a means by which beef producers could retain ownership on a sample portion of their calf crop in order to obtain feedlot performance and carcass information. In addition to traditional measures of carcass merit, cattle were objectively evaluated for meat tenderness and fabricated into 0.64 cm trim subprimals. Briefly, one hundred and eleven steers and eight heifers from 16 consignors were delivered to the MSU Beef Cattle Teaching and Research Center on October 10 and 11, 1997. All cattle were born between January 2 and May 14, 1997. It was required that cattle be dehorned and weaned at least 21 days prior to arrival as well as properly castrated and healed. Additionally, cattle were required to have IBR, BVD, PI_3, BRSV, Haemophilus somnus and 7-Way Clostridial vaccinations two to three weeks prior to arrival. Upon arrival, all cattle received booster vaccinations for IBR, BVD and PI_3, a growth promoting implant (Implus-S® and Implus-H® for steers and heifers, respectively), treatment for internal and external parasites and an antibiotic injection (Micotil®). Calves were placed into pens of seven to nine head with the official test beginning on October 13, 1997. End point was established as 0.89 cm of backfat, measured by ultrasound, or 635.2 kg live weight, whichever occurred first. All steers were harvested and sold on a grade and yield basis. Hot carcass weights were recorded and, following a 24 hour chill, carcasses were evaluated for adjusted 12^{th} rib backfat, ribeye area, and

marbling score. Internal fat was removed on the kill floor and percent kidney, pelvic, and heart fat was determined by the difference between pre- and post-trim weights. Carcasses were fabricated to 0.64 cm trim subprimals with subprimals, bone and trimmings being weighed and documented. An 11^{th} to 12^{th} rib section was removed from each carcass, Cryovac® packaged, and aged for 14 d at 4°C prior to being de-boned and frozen. Warner Bratzler Shear Force test was conducted by thawing and cooking a 2.54 cm thick steak from each carcass to 71°C. Cooked steaks were refrigerated overnight prior to six core samples, 1.27 cm in diameter, being removed from each steak and sheared.

IGF-I Selection Lines. A divergent selection experiment for serum IGF-I concentration in Angus cattle was initiated in 1989 at The Ohio State University. Complete protocols for the ongoing selection experiment are described elsewhere (Davis et al., 1995; Davis and Simmen, 1997). The selection criterion is the mean of serum IGF-I concentrations measured on each animal at d 28, 42 and 56 of the postweaning performance test after adjusting for age of calf and age of dam. Cows from the initial base population were randomly assigned to the selection lines. The experiment includes spring and fall replicates with approximately 50 cows per line in each replicate. Different sets of four bulls with unknown IGF-I levels were used to produce the spring 1989 and 1990 and fall 1990 calf crops. Each successive year, the four bull calves with the highest and the four bull calves with the lowest residuals for IGF-I concentration are saved for breeding within their respective selection lines. Bulls selected for breeding are used only as yearlings and then sold. Approximately eight cows are culled from

each line per year based on physical unsoundness, reproductive failure, and age. Culled females are replaced with approximately eight pregnant heifers that posses the highest or lowest residuals for IGF-I concentration. All available heifers are bred and selections are made among those females that conceive.

All animals are reared under common conditions at the Eastern Ohio Resource Development Center (EORDC), Belle Valley, except during the postweaning test period. During the postweaning period, bulls and heifers are fed in different drylot locations, because no single facility is adequate to house all animals. Bull calves are given ad libitum access to a corn-soybean meal-based diet, plus 2.3 kg/bull/d of grass hay. Heifer calves are fed a corn-soybean meal diet intended to yield postweaning gains of approximately 0.75 kg/d.

All calves are weighed at birth, weaning, beginning of the postweaning performance test, and every 28 d thereafter until conclusion of the 140 d postweaning period. In addition, calves are weighed at d 42, when one of the three blood samples is collected. Backfat thickness and ribeye area are measured on all calves at d 56 and 140 using ultrasound.

DNA Isolation. Blood samples from Black Angus, Hereford, Maine Anjou, MARC II, Red Angus, Salers, Shorthorn, Simmental and Tarentaise sired cattle were provided by the MCA/MSU Farm-to-Fab Program, and blood samples from Brahman cattle were provided by Agropecuarias Farms (Guanica, PR). DNA was isolated from white blood cells from a total of 108 animals using standard procedures (Sambrook et al., 1989) and DNA concentration was determined using a Du® 650 spectrophotometer (Beckman Instruments, Palo Alto, CA). In

addition, DNA from Angus cattle born in spring 1995, fall 1995, fall 1997, fall 1998, and spring 1999 as part of an ongoing serum IGF-I selection experiment initiated in 1989 at The Ohio State University was generously provided by Dr. Micheal E. Davis (n = 366).

PCR Conditions. Oligonucleotide primers used for the polymerase chain reaction (PCR) were designed from bovine IGFBP-2 cDNA sequence (GenBank accession no. AF074854) using the OLIGO® 5.0 Primer Analysis Software (NBI, Plymouth, MN). The PCR was performed in 10 μl reactions that included 1 X reaction buffer (10 X buffer contains 10 mM Tris-HCl, pH 9.0, 50 mM KCl and 0.1% Triton® X-100), 2.0 mM $MgCl_2$, 0.5 units Taq DNA polymerase (Promega Corp., Madison, WI), 200 μM each deoxynucleotide triphosphate (Gibco BRL, Gaithersburg, MD), 0.25 μM each primer, 3.7 μl distilled water and 25 ng of genomic DNA (10 ng/μl). A PTC-200 thermal cycler (MJ research, Watertown, MA) was used to amplify the desired fragments. Thermal cycling conditions included an initial denaturation at 94° C for 3 min followed by 30 cycles of 94°C for 1 min , 64°C for 1 min and 72°C for 1 min, with a final elongation at 72°C for 5 min. The PCR products were examined by electrophoresis on 1% agarose gels in 1 X TBE buffer. The gels were visualized by ethidium bromide staining and photographed using the Quantity One® v 4.1 software package on a Gel Doc 2000 imaging system (Bio-Rad Laboratories, Hercules, CA). The size of the amplified fragment was determined by comparison with a 100 bp DNA ladder (Bio-Rad Laboratories, Hercules, CA) and its identity was confirmed by DNA sequencing in the forward and reverse directions using an ABI 373 automated

sequencer and the Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA) followed by comparison of the obtained nucleotide sequence with entries in the GenBank database (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/). In order to obtain full-length sequence, the fragment was cloned into the pGem-T-Easy vector (Promega Corp., Madison, WI) and plasmid DNA was isolated with the QIAprep Spin Miniprep kit (Qiagen, Valencia, CA). Plasmid DNA was digested with the restriction endonuclease *EcoR* I (20,000 U/ml, New England Biolabs, Beverly, MA) to confirm the presence of an insert. New oligonucleotide primers were designed from previously derived sequence and were used to obtain the sequence for the middle of the fragment.

DNA Pooling Strategy for Polymorphism Identification. Several approaches were used for polymorphism identification, including restriction fragment length polymorphism (RFLP) analysis and DNA sequencing in the forward and reverse directions. Identification of polymorphisms was augmented by use of a DNA pooling strategy (Sun et al., 1998). Genomic DNA samples from up to 32 animals from different breeds of cattle (MCA/MSU Farm-to-Fab) or from each IGF-I selection line were pooled. The pooled samples were used in the PCR and the resulting product was digested with a series of restriction endonucleases that recognize four or six base palindromes. The various restriction fragments were separated according to size by gel electrophoresis using 2% agarose gels. Pooled samples containing multiple alleles showed inconsistent banding patterns relative to the size of the uncut fragment. The

presence of polymorphism was confirmed by evaluation of the individual DNA samples used to make the pools.

Statistical Analysis. A factorial experimental design was used to study the relationship between bovine IGFBP-2 genotypes and growth, carcass and meat quality traits in beef cattle. Genotype and phenotypic data (Table 3.1) from the MCA/MSU Farm-to-Fab Program cattle were analyzed using the Mixed procedure of SAS (1996).

The linear model used was:

$$y_{ijklmn} = \mu + 0_i + P_{(i)j} + G_k + SB_l + M_m + GSB_{kl} + e_{ijklmn}$$ *where:*

y_{ijklmn} = *n* observation pertaining to the *i*th owner, *j*th pen nested within the *i*th owner, *k*th IGFBP-2 genotype, *l*th sire breed, *m*th month of birth and the *kl*th interaction between genotype and sire breed.

μ = overall mean.

0_i = random effect of *i*th owner.

$P_{(i)j}$ = random effect of the *j*th pen nested within the *i*th owner.

G_k = fixed effect of the *k*th IGFBP-2 genotype.

SB_l = fixed effect of the *l*th sire breed.

M_m = fixed effect of the *m*th month of birth.

GSB_{kl} = fixed effect of the interaction between the *k*th IGFBP-2 genotype and the *l*th sire breed.

e_{ijklmn} = residual error term.

Depending on the trait under consideration, slaughter age, treatment for sickness, hot carcass weight, breed percentage, on test weight and slaughter

weight were included in the model as covariates. To avoid colinearity problems due to high correlation between traits, the Corr procedure of SAS (1996) was used to establish the degree of association between covariates.

Genotype and phenotypic data (Table 3.2) from the IGF-I selection lines were analyzed using ASREML (variance component software package, written by Arthur Gilmour of New South Wales Agriculture, Orange, Australia). The linear model used was:

$$y_{ijklmn} = \mu + G_i + S_j + Y_k + SE_l + C_m + GS_{ij} + GY_{ik} + GSE_{il} + e_{ijklmn}$$ *where:*

y_{ijklmn} = *n* observation pertaining to the *i*th IGFBP-2 genotype, *j*th sex of calf, *k*th year of birth, *l*th season of birth, *m*th calf identification (pedigree), *ij*th interaction between genotype and sex of calf, *ik*th interaction between genotype and year of birth and *il*th interaction between genotype and season of birth.

μ = overall mean.

G_i = fixed effect of the *i*th IGFBP-2 genotype.

S_j = fixed effect of the *j*th sex of calf.

Y_k = fixed effect of the *k*th year of birth.

SE_l = fixed effect of the *l*th season of birth.

C_m = random effect of the *m*th calf. $\mathbf{N\ (0,\ A\sigma^2_u)}$ where A is the additive genetic relationship matrix between animals.

GS_{ij} = fixed effect of the *ij*th interaction between genotype and sex of calf.

GY_{ik} = fixed effect of the *ik*th interaction between genotype and year of birth.

GSE_{il} = fixed effect of the *il*th interaction between genotype and season of birth.

e_{ijklmn} = residual error term. $\mathbf{NIID\ (0,\ \sigma^2_e)}$

Age of dam was used as a covariate for birth weight and both, age of dam and weaning age, were used as covariates for weaning and postweaning weights, off test hip height, total postweaning gain and postweaning serum IGF-I concentrations. The pedigree included a total of 2,357 animals.

Results and Discussion

IGFBP-2 Amplified Fragment. A 1,276-bp fragment of the bovine IGFBP-2 gene locus was amplified by the PCR using the following primers:

Primer-5': 5'-CGG GAG AAG GTC ACG GAG CAG- 3'

Primer-3': 5'-GGA AGG CGC ATG GTG GAG- 3'

DNA sequence comparison with the available bovine IGFBP-2 cDNA sequence (GenBank accession no. AF074854) revealed that this fragment is comprised of 195-bp pertaining to exon 2 (E value = 3×10^{-46}, Blast Search, NCBI) and 59-bp pertaining to exon 3 (E value = 7×10^{-24}, Blast Search, NCBI). These two exon fragments are separated by an 1,022-bp intron. The complete nucleotide sequence for this fragment is presented in Figure 3.1. The following internal primers were designed and used to complete the sequence of the middle portion of the fragment::

Primer-5": 5'-TTT CTG ATT CCG CTG CTA AC-3'

Primer-3': 5'-CCC AAC CAT CAT TCC CTT CC-3'

To our knowledge we are the first to report the sequence of bovine IGFBP-2 intron 2. Exon numbers were assigned based on the genomic organization published for swine IGFBP-2 (Song et al., 1996). Also, this fragment was compared with an unpublished 954 bp genomic bovine IGFBP-2 sequence

tagged site (STS) submitted by Wu et al. from Texas A & M University in 1999 (GenBank accession no. G42681). However, no similarity was found between our sequence and this STS.

PCR-RFLP at the Bovine IGFBP-2 Locus. Two polymorphisms were detected in the 1,276-bp fragment of the bovine IGFBP-2 gene by using the restriction endonucleases (RE) *Hind* III (Figure 3.2) and *Nla* III (Figure 3.3). Alleles identified following *Hind* III digestion (designated A and B*)* were found to be segregating in all breeds evaluated, as well as in the IGF-I selection lines. Sequencing analysis revealed that a thymine↔cytosine transition located 246-bp downstream of exon 2 is responsible for this polymorphism (Figure 3.4).

The allele frequencies (*Hind* III PCR-RFLP) for the MCA/MSU Farm-to-Fab cattle and for Brahman cattle are presented on Table 3.3. The frequency of the A allele was found to be higher than the frequency of the B allele in the Brahman (n = 8), Hereford (n = 17), and Maine Anjou (n = 11) cattle, whereas the frequency of the B allele was higher in the Black Angus (n = 17), Salers (n = 5), Shorthorn (n = 13), Simmental (n = 21) and Tarentaise (n = 7) cattle (Table 3.3). Within the MARC II (n = 8) and Red Angus (n = 1) cattle, the frequencies of the A and B alleles were equal. Allele frequencies for the IGF-I selection lines are presented in Table 3.4. The frequency of the B allele was higher than the frequency of the A allele in both the high and low IGF-I lines for animals born in spring 1995, fall 1995, fall 1997, fall 1998, and spring 1999. However, no difference in allele frequencies was observed between the high (0.32 A/0.68 B) and low IGF-I lines (0.30 A/0.70 B) or between bull (0.31 A/0.69 B) and heifer

calves (0.32 A/0.68 B) (Table 3.5). Because the frequency of the B allele was higher in both this Angus cattle population and the Angus cattle from the MCA/MSU Farm-to-Fab Program, selection may be operating in favor of the B allele within the Angus breed. Small changes in allele frequencies were observed across years and seasons of birth within the IGF-I selection lines. However, these fluctuations are likely due to random genetic drift.

Alleles identified following *Nla* III digestion (designated C and D) were segregating only in the Brahman, Simmental and MARC II animals (Table 3.6). The frequency of the C allele was higher than the frequency of the D allele in these cattle and all three genotypes were observed only in the Brahman breed. This polymorphism was not segregating in the IGF-I selection lines. These results demonstrate that the two IGFBP-2 PCR-RFLP identified in the present study are not in linkage disequilibrium as might be expected for the relatively short DNA fragment.

DNA sequence analysis revealed seven *Nla* III recognition sites within the amplified IGFBP-2 fragment and two of these sites were found to be polymorphic. The first polymorphic recognition site is located 334-bp downstream of exon 2 and is characterized by a thymine↔cytosine transition (Figures 3.5.A and 3.5.B). The second polymorphic site is characterized by a cytosine↔thymine-guanine-guanine (TGG) substitution that can be found 348 to 349-bp downstream of exon 2 (Figures 3.5.A and 3.5.B). Since the two polymorphic sites are in such close proximity, the C allele as detected by agarose gel electrophoresis following *Nla* III digestion, included the palindromic

sequence recognized by the enzyme at either one of the polymorphic sites or at both sites (Figure 3.5.A), whereas the D allele has neither recognition site (Figure 3.5.B). The absence of the TGG trinucleotide is associated with the presence of an additional cytosine located 5-bp before the TGG insertion site. The presence of either of the C allele variants that includes a *Nla* III recognition site in the first polymorphic location in heterozygotes results in a C/T double peak as shown in Figure 3.5.B. The presence of either of the C allele variants that includes a C↔TGG substitution in the heterozygotes causes the sequence to be uninterpretable as shown by the double peaks and ambiguous base calls (Figure 3.5.B). While assignment of genotypes based on migration of *Nla* III fragments in agarose gels provides an accurate determination of the presence of D alleles, it is impossible to distinguish C allele variants using this approach. Thus, the *Nla* III genotypes are likely to be more complex than those shown in Table 3.6. DNA sequencing or a more sensitive assay would be needed to more thoroughly characterize this variation.

Evidence of an Association between IGFBP-2 Hind III *PCR-RFLP Genotypes with Growth and Carcass Traits in Beef Cattle*. A statistically significant effect of IGFBP-2 genotype ($P < 0.05$) was found for days on feed, hot carcass weight, dressing percentage, trimmed hot carcass weight, cold carcass weight, ribeye area, and yield grade in the MCA/MSU Farm-to-Fab Cattle (Table 3.7). Only data from the Hereford, Shorthorn and Maine Anjou breeds was included in the analysis of variance because all three genotypes (AA, AB, BB) were represented only in those breeds ($n = 41$ animals). Animals with the BB

genotype had fewer days on feed, higher hot carcass weight and higher dressing percentage than those with the AB genotype ($P < 0.05$). No significant differences were observed between AA and BB individuals or between AA and AB individuals for these traits ($P > 0.05$). For trimmed hot carcass weight and cold carcass weight, the AA and BB genotypes represented the animals with higher weights in relation to the heterozygous (AB) animals ($P < 0.05$). The BB individuals had higher ribeye areas and better USDA yield grades than the AA and AB animals ($P < 0.05$).

In Angus cattle divergently selected for serum IGF-I concentration, analysis of variance indicated that animals with the BB genotype had higher birth weights than AA and AB animals (29.7 ± 1.0 kg vs. 28.3 ± 1.0 kg and 28.3 ± 1.2 kg, respectively, $P < 0.05$, $n = 366$). Therefore, by taking into consideration the results obtained in both cattle populations, animals with the BB genotype appear to have more desirable growth and carcass characteristics. While these results are intriguing, it must be acknowledged that the datasets are relatively small and evaluation of IGFBP-2 genotypes in a larger population is warranted.

Few genetic markers have been identified for growth, carcass merit or meat quality traits in beef cattle. In terms of IGFBP genes, polymorphisms have previously been identified only at the bovine IGFBP-3 locus (Maciulla et al., 1997; Haegeman et al., 1999). However, the potential associations between these polymorphisms and economically important traits have not been determined. In the present study, IGFBP-2 was selected as a candidate gene for economically important beef traits. In general, IGFBP-2 mRNA abundance in fetal tissues is

found to be high in early gestation and it decreases with maturation, thus following the same pattern of expression as IGF-II (Delhanty and Han, 1993; Wood et al., 1993; Carr et al., 1995). IGFBP-2 expression during early fetal development suggests that this protein act by autocrine and/or paracrine mechanisms. The liver is the major source of this IGFBP at later gestational ages and during postnatal life when the protein functions as an endocrine factor (Delhanty and Han, 1993; Wood et al., 1993; Carr et al., 1995). Also, transgenic mouse models overexpressing IGFBP-2 resulted in a reduction of somatic growth (Schneider et al., 2000) and IGFBP-2 knockout mice are characterized by a reduction in spleen size (Pintar et al., 1996). Therefore, DNA sequence variation at the bovine IGFBP-2 locus was hypothesized to be associated with potential changes in gene expression and function which could be responsible for variation in traits of economic importance.

A candidate gene approach has been used successfully to identify markers associated with growth. For example, Ge et al. (2001) identified a thymine↔cytosine transition polymorphism in the promoter region of the bovine IGF-I gene in Angus cattle divergently selected for serum IGF-I concentration. This marker was associated with higher weight gain during the first 20 days after weaning and the beginning of the postweaning test, and had a slight dominance effect (BB genotype) on postweaning gain. A dinucleotide (CA) repeat polymorphism has also been identified in the 5' flanking region of the IGF-I gene in cattle and swine (Kirkpatrick, 1992) and was reported to be associated with weaning and yearling weights (Moody et al., 1994) and with birth weight (Moody

et al., 1996) in beef cattle. Another candidate gene that has been extensively studied is the growth hormone receptor (GHR). Results obtained by Hale et al. (2000) indicated that the presence of an 11 TG repeat microsatellite allele at the GHR locus in Angus steers raised under commercial conditions decreased their growth by an average of approximately 17 kg at weaning and approximately 23 kg at slaughter. Moisio et al. (1998) also studied the GHR gene and reported the presence of three length variants and one base substitution polymorphism in the 3' flanking region. Allele frequencies of the length variants differed between Finnish native and commercial dairy cattle breeds (Moisio et al., 1998). Moreover, several polymorphisms have been identified in the bovine growth hormone (GH) gene (Lucy et al., 1993; Zhang et al., 1993; Sneyers et al., 1994). Sneyers et al. (1994) reported that a RFLP identified using the restriction endonuclease *Taq* I was correlated with body weight at 7 and 13 months of age in Belgian Blue bulls. Taylor et al. (1998), by interval analysis, localized effects of *Bos taurus* vs. *Bos indicus* GH alleles (*Taq* I-RFLP) on subcutaneous fat and in percentage of extractable fat from the longissimus dorsi muscle to the bovine chromosome 19 region that harbors the GH gene.

Estimation of Genetic Parameters for Weights, Gains and Serum IGF-I Concentration. Because a subset of animals from the IGF-I selection lines was used for the genotype/phenotype association analysis in this study, genetic parameters were estimated to confirm that this subset was representative of the larger population. Heritability estimates (h^2) were obtained using pedigree information from the IGF-I selection lines pertaining to the period between 1989

and 2000 and phenotypic data from calves born in spring 1995, fall 1995, fall 1997, fall 1998 and spring 1999 (n = 366). Traits such as birth weight, on test weight, on test hip height, off test weight, total postweaning gain, and d 28 IGF-I serum concentration were found to be moderately heritable (Table 3.8). In addition, weight at day 28, 42 and 56 of the postweaning test period, serum IGF-I concentration at d 42 and 56 of the postweaning test period and the mean serum IGF-I concentration had a relatively high h^2 (Table 3.8). Davis and Simmen (1997) reported similar results using data obtained from 1989 to 1994 of the IGF-I selection project. These investigators found that serum IGF-I concentration and performance traits in beef cattle were moderately to highly heritable, however, a direct h^2 estimate of 0.34 ± 0.11 was observed for weaning weight. Discrepancies between the present study and that report in relation to the h^2 estimate for weaning weight may be due to differences in the total number of animals included in the analysis (731 observations, Davis and Simmen, 1997; 366 observations, present study). Estimates of h^2 obtained by Davis and Simmen (1997, 2000) and in the present study indicate that it is possible to change IGF-I concentration in beef cattle via selection.

In the present study, a 1,276-bp fragment spanning the intron between exons 2 and 3 of the bovine IGFBP-2 gene was amplified by the PCR. To our knowledge we are the first to report bovine IGFBP-2 intron 2 sequence. In addition, two polymorphisms were identified in this fragment and these polymorphisms were found to be segregating in different breeds and cattle

populations. One of these polymorphsisms was found to be associated with growth and carcass merit traits.

Implications

These results indicate that an association may exist between alleles of the IGFBP-2 gene and growth and carcass traits in beef cattle. This further supports the idea that IGFBP-2 is an important candidate gene. Validation of these results in additional populations may lead to the incorporation of IGFBP-2 genotypes into MAS programs.

Table 3.1. Growth, carcass and meat quality traits measured by the MCA/MSU Farm-to-Fab program.

Birth weight	Final weight per day of age
Days on feed	Average daily gain
Slaughter weight	Slaughter weight (4% shrink)
Hot carcass weight	Dressing percentage
Trimmed hot carcass weight	Cold carcass weight
Adjusted backfat	Kidney, pelvic and heart fat
Rib-eye area	U.S.D.A yield grade
Marbling score	U.S.D.A quality grade
Average shear force value	Portions of the round
Portions of the chuck	*Knuckle*
Chuck roll	*Inside round*
Clod	*Gooseneck*
Brisket	Portions of the loin
Portions of the rib	*Strip loin*
Lip-on ribeye	*Top butt*
Back ribs	*Flap*
Skirts	*Ball tip*
Bone	*Tri tip*
Fat	*Tenderloin*
Total retail weight	*Flank Steak*
Lean	
Cap and wedge meat	
50 % lean	
80 % lean	

Table 3.2. Weight, gain and IGF-I traits measured for the IGF-I selection project.

Birth weight	Off test weight
Weaning weight	Off test hip height
On test weight	D 28 IGF-I
Weight at d 28 postweaning	D 42 IGF-I
Weight at d 42 postweaning	D 56 IGF-I
Weight at d 56 postweaning	Mean IGF-I
Total postweaning gain	

Table 3.3. Genotype and allele frequencies for a *Hind* III PCR-RFLP at the bovine IGFBP-2 locus in beef cattle.

Breed	No. of animals	Genotype frequency	Allele frequency
Angus	17	0.00 AA 0.29 AB 0.71 BB	0.15 A 0.85 B
Brahman	8	0.62 AA 0.12 AB 0.26 BB	0.69 A 0.31 B
Hereford	17	0.41 AA 0.35 AB 0.24 BB	0.59 A 0.41 B
Maine Anjou	11	0.18 AA 0.73 AB 0.09 BB	0.54 A 0.46 B
MARC II[a]	8	0.00 AA 1.00 AB 0.00 BB	0.50 A 0.50 B
Salers	5	0.00 AA 0.60 AB 0.40 BB	0.30 A 0.70 B
Shorthorn	13	0.15 AA 0.31 AB 0.54 BB	0.31 A 0.69 B
Simmental	21	0.00 AA 0.29 AB 0.71 BB	0.14 A 0.86 B
Red Angus	1	0.00 AA 1.00 AB 0.00 BB	0.50 A 0.50 B
Tarentaise	7	0.00 AA 0.14 AB 0.86 BB	0.07 A 0.93 B

[a]MARC II cattle = 1/4 Angus, 1/4 Gelbvieh, 1/4 Hereford and 1/4 Simmental.

Table 3.4. Genotype and allele frequencies for a *Hind* III PCR-RFLP at the bovine IGFBP-2 locus in Angus cattle divergently selected for serum IGF-I concentration.

Season & Line	No. of animals	Genotype frequency	Allele frequency
Spring 1995			
High	31	0.06 AA	0.26 A
		0.39 AB	0.74 B
		0.55 BB	
Low	30	0.10 AA	0.28 A
		0.57 AB	0.72 B
		0.33 BB	
Fall 1995			
High	43	0.05 AA	0.27 A
		0.44 AB	0.73 B
		0.51 BB	
Low	35	0.09 AA	0.39 A
		0.60 AB	0.61 B
		0.31 BB	
Fall 1997			
High	40	0.12 AA	0.31 A
		0.38 AB	0.69 B
		0.50 BB	
Low	33	0.09 AA	0.26 A
		0.33 AB	0.74 B
		0.58 BB	
Fall 1998			
High	46	0.11 AA	0.30 A
		0.37 AB	0.70 B
		0.52 BB	
Low	22	0.04 AA	0.36 A
		0.64 AB	0.64 B
		0.32 BB	
Spring 1999			
High	42	0.19 AA	0.44 A
		0.50 AB	0.56 B
		0.31 BB	
Low	44	0.05 AA	0.22 A
		0.34 AB	0.78 B
		0.61 BB	

Table 3.5. Genotype and allele frequencies for a *Hind* III PCR-RFLP at the bovine IGFBP-2 locus in bulls and heifers divergently selected for serum IGF-I concentration.

Season & Sex	No. of animals	Genotype frequency	Allele frequency
Spring 1995			
Bull	33	0.07 AA 0.54 AB 0.39 BB	0.33 A 0.67 B
Heifer	28	0.11 AA 0.39 AB 0.50 BB	0.31 A 0.69 B
Fall 1995			
Bull	34	0.00 AA 0.58 AB 0.42 BB	0.29 A 0.71 B
Heifer	44	0.12 AA 0.45 AB 0.43 BB	0.34 A 0.66 B
Fall 1997			
Bull	36	0.08 AA 0.39 AB 0.53 BB	0.28 A 0.72 B
Heifer	37	0.14 AA 0.32 AB 0.54 BB	0.30 A 0.70 B
Fall 1998			
Bull	30	0.07 AA 0.40 AB 0.53 BB	0.27 A 0.73 B
Heifer	38	0.10 AA 0.50 AB 0.40 BB	0.35 A 0.65 B
Spring 1999			
Bull	38	0.16 AA 0.39 AB 0.45 BB	0.36 A 0.64 B
Heifer	48	0.08 AA 0.44 AB 0.48 BB	0.30 A 0.70 B

Table 3.6. Genotype and allele frequecies for a *Nla* III PCR-RFLP at the bovine IGFBP-2 locus in beef cattle[a].

Breed	No. of animals	Genotype frequency	Allele frequency
Angus	17	1.00 CC 0.00 CD 0.00 DD	1.00 C 0.00 D
Brahman	8	0.50 CC 0.25 CD 0.25 DD	0.62 C 0.38 D
Hereford	17	1.00 CC 0.00 CD 0.00 DD	1.00 C 0.00 D
Maine Anjou	11	1.00 CC 0.00 CD 0.00 DD	1.00 C 0.00 D
MARC II[b]	8	0.75 CC 0.25 CD 0.00 DD	0.88 C 0.12 D
Salers	5	1.00 CC 0.00 CD 0.00 DD	1.00 C 0.00 D
Shorthorn	13	1.00 CC 0.00 CD 0.00 DD	1.00 C 0.00 D
Simmental	21	0.95 CC 0.05 CD 0.00 DD	0.98C 0.02 D
Red Angus	1	1.00 CC 0.00 CD 0.00 DD	1.00 C 0.00 D
Tarantaise	7	1.00 CC 0.00 CD 0.00 DD	1.00 C 0.00 D

[a]This polymorphism is not segregating in Angus cattle from the IGF-I selection lines (all animals are CC genotype).
[b]MARC II cattle = 1/4 Angus, 1/4 Gelbvieh, 1/4 Hereford and 1/4 Simmental.

Table 3.7. Relationship of bovine IGFBP-2 *Hind* III PCR-RFLP genotypes with growth and carcass traits in MCA/MSU Farm-to-Fab Cattle[a].

Trait	Genotype		
	AA	AB	BB
DOF	216.4[cd]	220.9[c]	207.0[d]
HCW, kg	353.8[cd]	345.2[c]	356.6[d]
DP	62.0[cd]	60.2[c]	62.4[d]
THCW, kg	346.0[c]	335.4[d]	348.3[c]
CCW, kg	344.8[c]	334.2[d]	345.7[c]
REA, cm^2	33.0[c]	32.2[c]	36.3[d]
YG	2.81[c]	2.7[c]	2.12[d]

[a]Only data from the Hereford, Shorthorn and Maine Anjou breeds were included in the analysis because all three genotypes (AA, AB, BB) were observed only in these breeds (n = 41).

[b]DOF = days on feed, HCW = hot carcass weight, DP = dressing percentage, THCW = trimmed hot carcass weight, CCW = cold carcass weight, RA = ribeye area, YG = USDA yield grade.

[c,d] Within a row, means lacking a common superscript letter differ ($P < 0.05$).

Table 3.8. Heritability (h^2) estimates for weight, gain and IGF-I traits measured in the IGF-I selection lines.

Trait	h^2
Birth weight (BW)	0.41 ± 0.11
Weaning weight (WW)	0.12 ± 0.10
On test weight	0.38 ± 0.14
Weight at d 28 postweaning (WD 28)	0.46 ± 0.13
Weight at d 42 postweaning (WD 42)	0.47 ± 0.13
Weight at d 56 postweaning (WD 56)	0.51 ± 0.13
Off test hip height	0.39 ± 0.13
Off test weight	0.39 ± 0.14
Total postweaning gain	0.29 ± 0.12
D 28 IGF-I (IGF-I 28)	0.23 ± 0.11
D 42 IGF-I (IGF-I 42	0.63 ± 0.11
D 56 IGF-I (IGF-I 56)	0.52 ± 0.11
Mean IGF-I	0.62 ± 0.12

5′-**CGGGAGAAGGTCACGGAGCAGCTTTGACTTGAGCTCCACCACGCCCCTTCCAGTCG**
GGAGAAGGTCACGGAGCAGCTCCACCATGCGCCTTCCGGTCGGGAGAAGGTCACGGAGC
AGCACCGGCAGATGGGCAAGGGTGGCAAACATCACCTCGGCCTGGAGGAGCCCAAGAAG
CTGCGGCCGCCACCTGCCAGGGTCAGAAAGGGTCGGGTCTGGCTGGAGGGGTGGGAGGA
CGGTCAAAGCGAGGGTCCCGGCAGCAGGGGTCCGGAGCACTGCCGTGAGATCCATGTCC
TGTGTGCCTGCGGGCAGAGTTCTTTCTGATTCCGCTGCTAACATTCAGTGTTTTTTCGG
GCAGGTCAGTGTGTTTTTGAGGGCCTCAGCATCTTTGGCTGTGAGATGGGCTGGACGTA
TGGGTTCTTGAAGTCCCTCGCTTCTAAAGCCTCCACATTTCTGCTCGGCTTCATTGCTG
TTGTTTAGTTGCTAAGATGTGTCTGACTCTTTTGTGACCCCGTGGACTGTAGCCCGTCA
TGCTGCTCTGCTCCACGATTCTCCAGGCAAGAATACTGGAGTGGGTTTCCATTTCCTGC
TCCAGGGGATCTTCCCTGATCAGGAAGTGACCTGCATCTCCTGCACTGGCAGGCAGATT
CTTTACTGCTGAGCTACTGCGGAAGCACTGCTTGGCTTTGAGATATAGCACCGCCCCCC
CGCAAAAAAAAAAAAAGGCTACAGTCCATGGAATCGAACGAGTCAGACACAGCTTAGCA
ACTACGTCCCCACCACTACCACTTGAACTTGCCCACTCTTGTTGCAATACACTACCCTC
ACTGAGCCACCCCTCTTCTCTTTCCAGCTCAGCTCTGCTGTCTTGGCCCTTGGATGGGT
TTATGGAAGGGAATGATGGTTGGGCCACTGGATGGGGTAGAGATACTTGAATTCACTGT
TTGCAAGTAGATCCCATTTTGACCCCTGAAAGGAGGGAGGAGAGGAGGGAAGACTCACC
TGCAGGCTGACTTTCTCTTGGAGGGGGGCGGGGCCTGGTGGTAAGGCAGTGAGCTTGCT
GGGCTGGGTGTCGGAAGTTCCTGGTTCCGCCAGCTTGCTGTGTGGTCTTGGGAAAGTGG
TGTTCTGCACACGGGCTTCTTTCTGAAGCTGTGCCGGGGTTGGCGTTGCGTGAACCCGT
TCTCACTCTGAGTGTCCTCTCTGTGTGTCTGTGCCTGCAG**ACCCCCTGCCAGCAGGAAT**
TGGACCAGGTCCTGGAGCGGATCTCCACCATGCGCCTTCC-3′

Figure 3.1. Nucleotide sequence of an 1,276-bp fragment of the bovine IGFBP-2 gene locus. A total of 254 bp of exon sequence (195-bp exon 2 plus 59-bp exon 3, shown in bold type) is separated by an 1,022-bp intron.

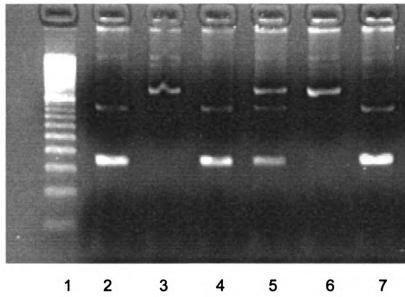


Figure 3.2. *Hind* III PCR-RFLP at the bovine IGFBP-2 locus in beef cattle. Lane 1, 100 bp PCR ruler; Lanes 2, 4 and 7, BB genotype; Lanes 3 and 6, AA genotype; Lane 5, AB genotype.

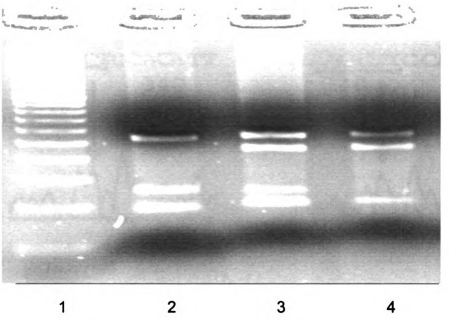
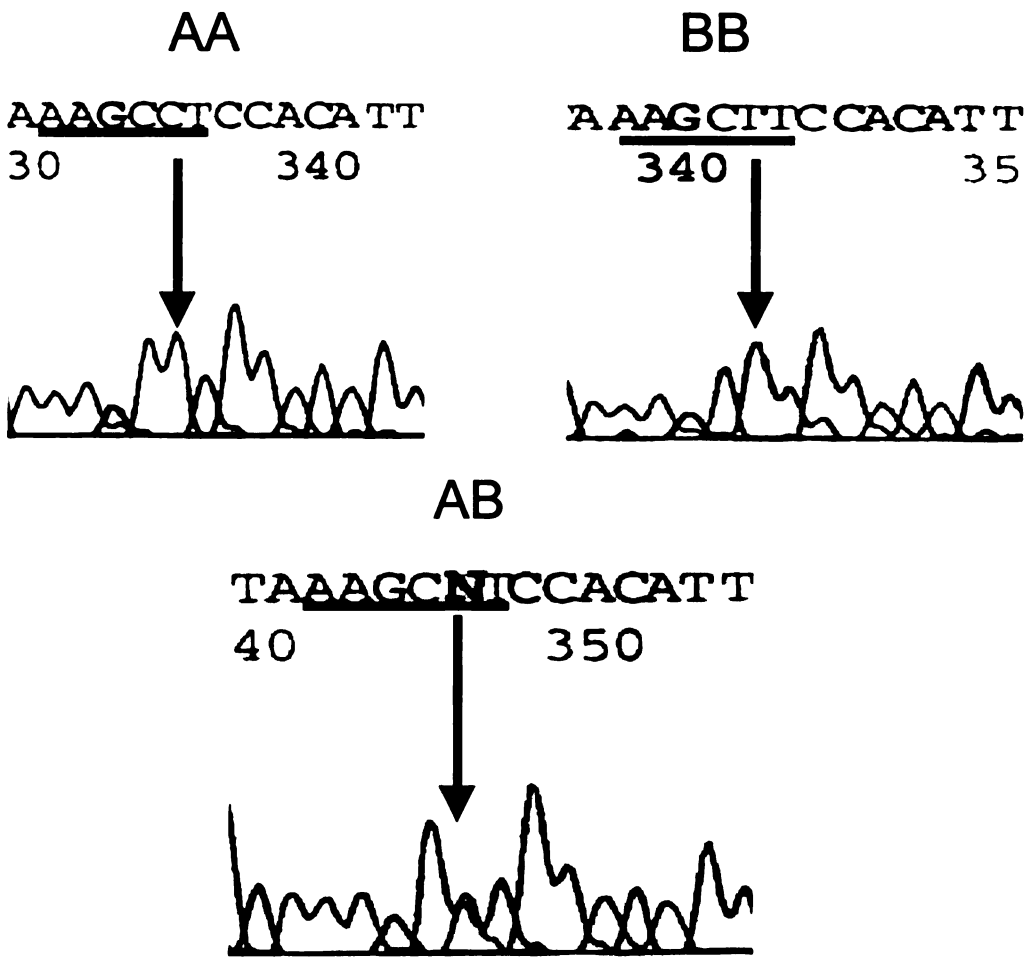


Figure 3.3. *Nla* III PCR-RFLP at the bovine IGFBP-2 locus in beef cattle. Lane 1, 100 bp PCR ruler; Lane 2, CC genotype; Lane 3, CD genotype; Lane 4, DD genotype.



Hind III recognition site = AAGCTT

Figure 3.4. DNA sequencing electrophoretograms showing the AA, AB and BB bovine IGFBP-2 *Hind* III PCR-RFLP genotypes. Genomic DNA was amplified by the PCR using oligonucleotide primers specific for IGFBP-2 and PCR products were sequenced using the same primers. The restriction endonuclease (RE) recognition site is underlined and the position where a thymine↔cytosine transition occurs is represented by an arrow. The AA genotype represents animals that do not have the RE recognition site in either allele, whereas the BB genotype has the site in both alleles. For heterozygous individuals, the RE recognition site is present in one allele but absent in the other (C/T double peak, designated N by the ABI base calling software).

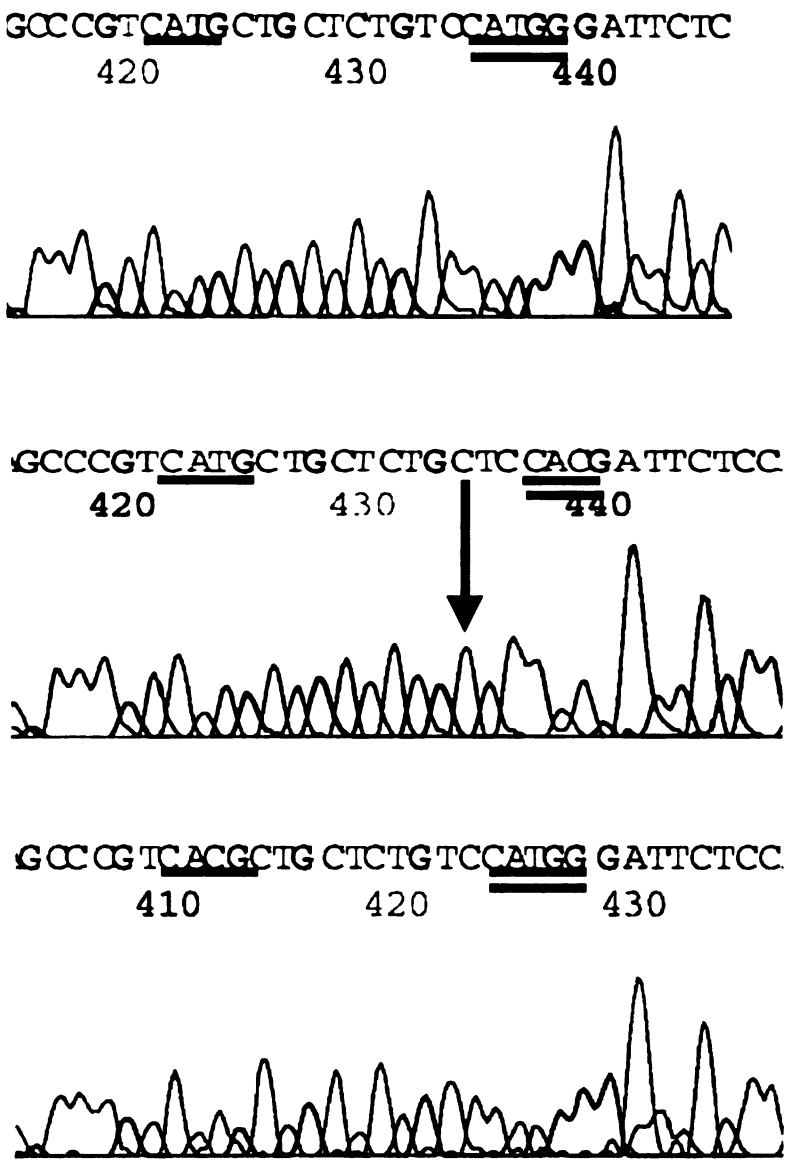


Figure 3.5.A. DNA sequencing electrophoretograms showing three sequence variants of the IGFBP-2 *Nla* III PCR-RFLP C allele. Fragments obtained from amplification of genomic DNA were cloned into the pGem-T-Easy vector and clones were sequenced to identify allele varaints. The restriction endonuclease (RE) recognition sites (CATG) are underlined. The first polymorphic site is characterized by a thymine↔cytosine transition, whereas for the second polymorphic site (shown by double underlines) a cytosine↔thymine-guanine-guanine (TGG) substitution occurs. The absence of the TGG trinucleotide was associated with the presence of an additional cytosine, which is shown by an arrow. The C allele as detected by agarose gel electrophoresis following Nla III digestion, included either one of the recognition sites or both as shown.

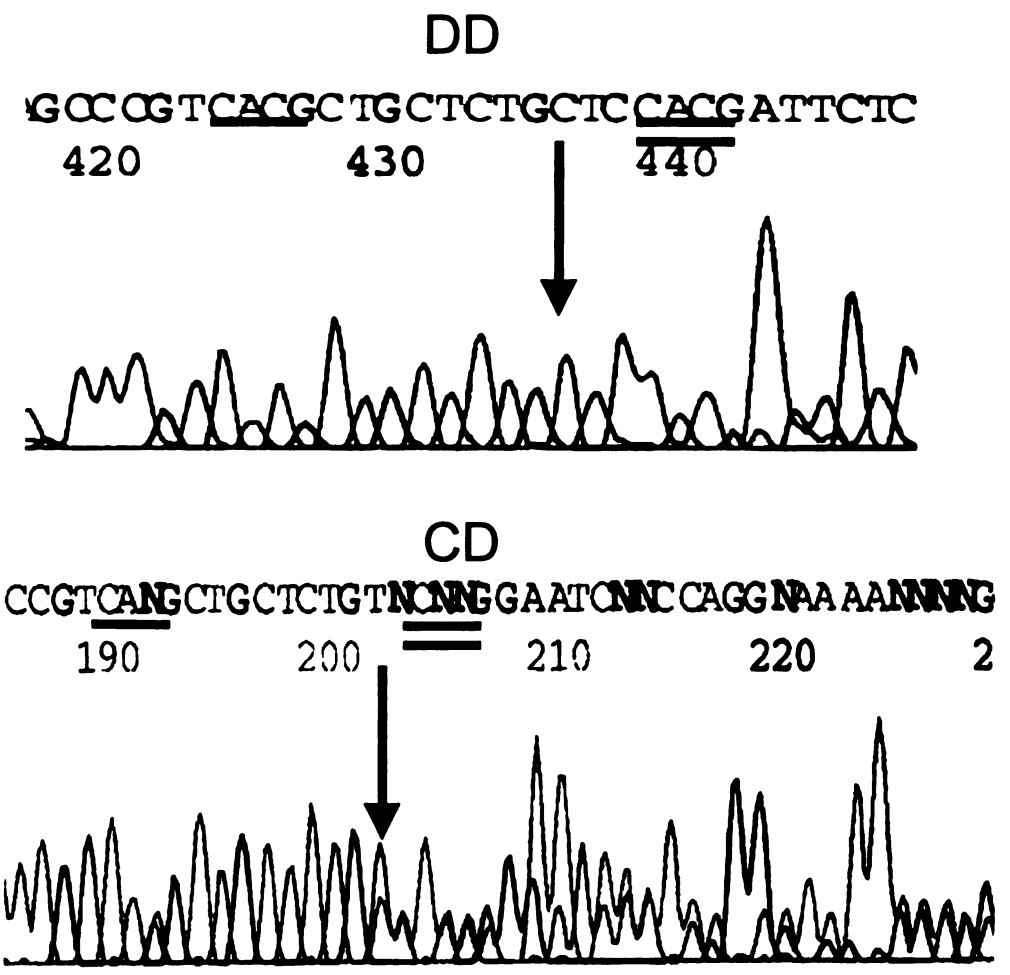


Figure 3.5.B. DNA sequencing electrophoretograms showing the DD and CD IGFBP-2 *Nla* III PCR-RFLP genotypes. Genomic DNA was amplified by the PCR using oligonucleotide primers specific for IGFBP-2 and PCR products were sequenced using the same primers. The restriction endonuclease (RE) recognition sites (CATG) are underlined. The first polymorphic site is characterized by a thymine↔cytosine transition, whereas for the second polymorphic site (shown by double underlines) a cytosine↔thymine-guanine-guanine (TGG) substitution occurs. The absence of the TGG trinucleotide is associated with the presence of an additional cytosine, which is shown by an arrow. The D allele does not have either of the *Nla* III recognition sites. The presence of either of the C allele variants that includes a *Nla* III recognition site at the first polymorphic location in heterozygotes results in a C/T double peak as shown. The presence of either of the C allele variants that includes a C↔TGG substitution in heterozygotes causes the sequence to be uninterpretable as shown by the double peaks and ambiguous base calls.

CHAPTER 4

SUMMARY AND RECOMENDATIONS FOR FUTURE RESEARCH

Consumers of animal products demand high quality at a low price. Thus, enhancement of production efficiency is a major concern for producers of food animals. The growth phenomenon is the essential process in the livestock and meat industry, both from the standpoint of animal growth and in production of most feedstuffs that animals convert to meat. An increase in our understanding of gene expression and regulation during the growth process will make it possible to develop new selection strategies to increase the rate of genetic improvement as we continually strive to improve the efficiency of meat animal production. Moreover, polymorphisms identified within these genes could potentially be associated with variation in traits of economic importance in beef cattle and therefore could be used in marker assisted selection (MAS) programs. Such programs may enhance rates of genetic change via reduced generation intervals, increased selection differentials, and/or increased accuracy of selection.

It is known that the insulin-like growth factor (IGF) system is intimately involved in growth and cellular metabolism during all stages of development. However, despite advances in basic knowledge of the IGF system, relatively little is known about its expression, regulation and effects on livestock growth. Both positive and negative correlations between circulating IGF-I and rate of gain have been reported in cattle (Lund-Larsen et al., 1977; Davis and Simmen, 1997; Stick et al., 1998) and conflicting relationships between carcass fat percentage and IGF-I have been observed in cattle and sheep (Anderson et al., 1988; McCann et

al., 1997). Thus, the relationship between circulating IGF-I and growth potential and carcass composition remains unclear and the insulin-like growth factor binding proteins (IGFBP) may contribute to the discrepancies in the observed results. Therefore, the specific objectives for this dissertation project were to:

1. Evaluate serum IGFBP in Angus cattle divergently selected for serum IGF-I concentration and to investigate potential associations between serum IGFBP, body weight, postweaning gain and serum IGF-I concentration.
2. Identify DNA sequence variation (polymorphisms) at the bovine IGFBP-2 gene locus.
3. Evaluate IGFBP-2 polymorphisms in different breeds of cattle and in Angus cattle divergently selected for serum IGF-I concentrations for potential associations with growth, carcass merit and meat quality traits.

In the present study, postweaning expression of serum IGF-I and associated serum insulin-like growth factor binding proteins (IGFBP) were investigated in 68 1992 fall-born and 84 1999 fall-born, purebred Angus cattle, selected for either high or low serum IGF-I concentrations since 1989 at The Ohio State University. Our results demonstrate that single trait selection for high vs. low serum IGF-I concentration has resulted in divergence between the high and low IGF-I selection lines and also has resulted in changes in weight and gain traits, but has not resulted in changes in expression of IGFBP in serum. Sex differences in specific circulating IGFBP species were observed. In both the fall 1992 and fall 1999 calves, heifers expressed higher levels of the 34 kDa IGFBP

species (i.e., IGFBP-2) than bulls ($P < 0.0005$). In addition, in the fall 1992 calves, bulls expressed greater levels of both the 38-42 and 24 kDa IGFBP species than heifers ($P < 0.0001$).

In the fall 1992 calves, the relative serum level of the 38-42 kDa IGFBP species was positively correlated with serum IGF-I concentration and the relative level of the 24 kDa IGFBP species was negatively correlated with serum IGF-I. In the fall 1999 calves, serum IGFBP-2 was negatively correlated with weights, gains and serum IGF-I concentration. This suggests that the mechanism by which the IGFBP regulate the actions of IGF-I in Angus cattle divergently selected for serum IGF-I is complicated. These correlations can be further evaluated within lines and sexes, which could provide additional information concerning the relationship of serum IGFBP, economically important beef traits, and serum IGF-I concentration.

Growth hormone (GH) is under negative feedback control and IGF-I acts directly on the pituitary to inhibit GH secretion (Ganong, 1995). At the same time, IGFBP-3 serum concentration is directly related to GH secretory status (Baxter and Martin, 1986), whereas IGFBP-1 and IGFBP-2 serum concentrations are inversely related with growth hormone (Binoux et al., 1986). Thus, future research should include an investigation of the effect of divergent selection for serum IGF-I on growth hormone serum levels within this unique cattle population. Also, potential correlations between relative levels of specific serum IGFBP with serum GH levels both early in the selection project (fall 1992) and after several years of selection (fall 1999) should be established.

Subcutaneous fat, skeletal muscle and liver samples were collected from spring 2000 high (n = 14) and low (n = 11) IGF-I bulls not retained for breeding. These tissues can be used to evaluate mRNA abundance of genes within the IGF system (e.g., GH receptor, IGF-I, IGF-I receptor, and IGFBPs). Such analysis will provide evidence of how endocrine, paracrine and autocrine components of the IGF system are operating in relation to divergent selection for serum IGF-I. In addition, liver, fat and skeletal muscle tissue biopsies from high and low line animals should be collected during the postweaning performance test so that mRNA abundance can be examined at the same time that serum IGF-I levels are determined. Because subcutaneous fat, skeletal muscle and liver samples were not collected from animals early in the selection project, expression of IGF system genes before and after the lines have diverged cannot be evaluated. While serum IGF-I concentrations are significantly different between the high and low selection lines, it is unknown whether IGF-I expression in these lines is regulated at the transcriptional, translational or post-translational level. Since most of the IGF-I in the circulation is produced by the liver (Baxter, 1986), evaluation of liver mRNA abundance will provide evidence as to whether or not there is transcriptional regulation of IGF-I in the high and low IGF-I selection lines.

Insulin-like growth factor binding protein-2 (IGFBP-2) was selected as a candidate gene for growth, carcass merit and meat quality in beef cattle. A 1,276-bp fragment of this gene, which spans the intron between exons 2 and 3, was amplified by the PCR and two polymorphisms (RFLP) were identified. A

polymorphism identified using the restriction endonuclease *Hind* III was found to be segregating in different breeds and in different cattle populations and was found to be associated with growth and carcass merit traits. However, it must be acknowledged that the datasets used in the present study were relatively small and evaluation of IGFBP-2 genotypes in a larger population is warranted. Validation of these results in additional populations may lead to the incorporation of IGFBP-2 genotypes into MAS programs. A second polymorphism was identified using the restriction endonuclease *Nla* III. Sequence analysis of the *Nla* III alleles revealed the presence of three variants for one of the RFLP alleles. Future research to better characterize variation at the bovine IGFBP-2 locus should include development of a rapid means for distinguishing *Nla* III allele variants and use of haplotypes of all polymorphism for genotype/phenotype association studies.

The IGFBP-2 *Hind* III polymorphism was used to genotype animals from the IGF-I selection lines and no differences were observed in allele frequencies between lines or between sexes. Because this polymorphism is segregating in the IGF-I selection lines, a genotype/phenotype association study will also be performed using *Hind* III PCR-RFLP genotypes and relative serum IGFBP levels as determined by western ligand blotting in order to determine if specific genotypes are associated with differences in expression of specific IGFBP.

Previous results obtained by Davis and Simmen (1997, 2000) indicated that it is possible to change IGF-I concentration in beef cattle via selection. These results were confirmed in the present study by evaluating selection

response in the animals from the IGF-I selection lines for which IGFBP-2 genotypes were determined. The analysis was conducted across years and seasons of birth and included pedigree information from 1989 to 2000.

IGFBP-2 has not been mapped in cattle and placing this gene on either the bovine physical map or the bovine genetic linkage map will facilitate its incorporation into quantitative trait loci (QTL) analyses. Four sires from the United States Department of Agriculture Agricultural Research Service Meat Animal Research Center reference family have been genotyped for the *Hind* III PCR-RFLP, and one of the sires was found to be heterozygous. Offspring from the backcross reference family will be genotyped in order to place IGFBP-2 on the genetic linkage map.

In the present dissertation project, postweaning expression of serum IGF-I and serum IGFBP and potential associations with growth and carcass traits in beef cattle were investigated. Also, bovine IGFBP-2 was selected as a candidate gene for traits of economic importance in beef cattle. Our results indicate that an association may exist between economically important beef traits and alleles of the IGFBP-2 gene, as well as between these traits and serum IGFBP-2 concentrations. This work supports IGFBP-2 as an important candidate gene for beef cattle growth and further investigation of this gene is warranted.

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