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UTILIZATION OF SOLUTION HYBRIDIZATION TO EXAMINE DIFFERENTIAL EFFECTS OF GROWTH HORMONE AND RACTOPAMINE ON THE RELATIVE ABUNDANCE OF IGF-I mRNA IN LIVER AND SKELETAL MUSCLE OF PIGS

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Animal Science

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Date 6/14/90

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By

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A DISSERTATION

Submitted to

Michigan State University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

Department of Animal Science

1990

ABSTRACT

UTILIZATION OF SOLUTION HYBRIDIZATION TO EXAMINE DIFFERENTIAL EFFECTS OF GROWTH HORMONE AND RACTOPAMINE ON THE RELATIVE ABUNDANCE OF IGF-I mRNA IN LIVER AND SKELETAL MUSCLE OF PIGS

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It was necessary to develop a sensitive solution hybridization-nuclease protection assay to examine the effects of growth hormone and ractopamine, a phenethanolamine, on the low abundance of IGF-I mRNA in liver and skeletal muscle of market weight pigs. An antisense RNA probe was synthesized from a porcine IGF-I cDNA and utilized in a solution hybridization-nuclease protection assay to quantitate relative abundance of IGF-I mRNA. Optimum hybridization and digestion conditions were determined. Intrassay and interassay coefficients of variations were 2.8 and 2.7%, respectively. A linear hybridization response was obtained using from 50 to 150 ug of liver RNA.

In Experiment 1, barrows (average initial body weight 83.7 kg) were administered 50 ug of recombinant porcine growth hormone per kg of body weight daily i.m. for 24 days. Pigs were fed diets containing either 14% or 20%

crude protein. At the end of the experimental period, pigs were slaughtered and tissues collected for RNA analyses. Relative abundance of IGF-I mRNA was determined in liver and longissimus dorsi (LD) muscle by a sensitive solution hybridization-nuclease protection assay. Administration of growth hormone increased abundance of liver IGF-I mRNA 2.7-fold in pigs fed the 14% protein diet and 3-fold in pigs fed the 20% protein diet relative to controls. Muscle IGF-I mRNA levels in the treated pigs were only 77% and 84% of levels in control pigs fed the 14% and 20% protein diets, respectively.

In Experiment 2, barrows (average initial body weight 72 kg) were administered ractopamine at either 0 or 20 ppm in 16% crude protein diets for 28 days. IGF-I mRNA abundance was determined in liver and skeletal muscle as in Experiment 1. Ractopamine had no effect on the relative abundance of IGF-I mRNA in either tissue.

Results from these experiments indicate that abundance of IGF-I mRNA in liver and skeletal muscle of pigs can be monitored, is dependent on dietary protein, and is altered following administration of growth hormone, but not ractopamine.

ACKNOWLEDGMENTS

I would like to thank the Department of Animal Science and College of Agriculture and Natural Resources for their support during my degree. I thank Dr. W.G. Bergen for serving as my major professor and for allowing me to pursue a Ph.D. degree in his research program. Dr. Bergen's support, patience, understanding, and kindness is greatly appreciated. I wish to express my appreciation to Dr. Bill Helferich for serving on my graduate committee and for the use of his laboratory facilities and equipment. Bill has contributed much time to my research projects with guidance and helpful suggestions. Working with Bill has been a pleasure. I would like to thank Dr. D.B. Jump for serving on my graduate committee and for his many helpful suggestions. I wish to thank Dr. R.A. Merkel for serving on my graduate committee. I am thankful for having the opportunity to work with him in both research and teaching programs. Dr. Merkel's guidance and time has been greatly appreciated.

There have been a number of faculty, staff, graduate students and undergraduate students that have helped me during my degree program. I wish to acknowledge their many

contributions.

I wish to express my deepest gratitude to my wife, Brenda, for her patience and understanding during my studies. Brenda's help, support and encouragement have been major contributions to my success. I also wish to thank her for typing this dissertation.

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LITERATURE REVIEW

Introduction

Development of strategies to improve the efficiency of lean meat production from domestic livestock is a major goal of animal scientists. Such strategies have included dietary manipulation and, more recently, administration of exogenous agents (e.g., growth hormone and beta adrenergic agonists). Growth hormone and beta adrenergic agonists increase muscle growth and decrease fat deposition in meat animals. However, the extent to which these compounds alter composition is dependent upon the nutritional status of the animal. The above manipulations create useful animal models in which the cellular and molecular mechanisms involved in skeletal muscle growth can be studied. Currently, mechanisms responsible for the increased muscle growth resulting from implementation of the above strategies are unclear.

Since insulin-like growth factor (IGF-I) has been demonstrated to have positive effects on skeletal muscle growth and protein accretion in cell cultures and in vivo, this growth factor has been implicated in mediating the actions of growth hormone and beta adrenergic agonists on

muscle growth. The following sections will describe the biosynthesis of IGF-I and will review studies that have been designed to examine the role of IGF-I in skeletal muscle growth.

Insulin-like growth factor-1 Peptides, Gene Expression, and Binding Proteins

Insulin-like growth factor-I (IGF-I; also referred to as somatomedin-C) is a mitogenic single-chain polypeptide structurally similar to insulin. The mature peptide consists of 70 amino acids and has a molecular weight of approximately 7500 daltons. IGF-I is synthesized as a prepro-peptide and then processed to the mature peptide similiar to insulin (Steiner, 1978; Steiner et al., 1980; Vassilopoulou-Sellin and Phillips, 1982; Rotwein et al., 1987). The peptide consists of 4 domains (B-C-A-D) flanked by an amino-terminal peptide (signal peptide) and a carboxyl-terminal extension (E domain) (Gammeltoft, 1989). Hansen et al.(1983) and Rotwein (1986) have found from cDNA cloning studies that two different IGF-I precursor peptides exist. One contains 153 amino acids and the other contains 195 amino acids. The two mRNAs that encode these two precursor peptides encode the mature peptide sequence (70 amino acids), amino-terminal peptide (48 amino acids), and a carboxyl-terminal peptide of 19 or 61 amino acids. The smaller precursor peptide (153 amino acids) is designated IGF-IA, whereas the larger peptide (195 amino acids) is designated IGF-IB. Studies using rat and mouse cDNAs have

also revealed IGF-IA and IGF-IB precursors (Bell et al., 1986; Casella et al., 1987; Murphy et al., 1987; Roberts et al.,1987; Shimatsu and Rotwein, 1987). A cDNA encoding IGF-IA has been isolated from porcine liver (Tavakkol et al., 1988) and chicken liver (Kajimoto and Rotwein, 1989).

The 70 amino acid sequence of the mature IGF-I peptide is identical among the porcine, human, and bovine species but differs from sheep, rat, mouse, and chicken by 1, 3, 4, and 10 residues, respectively (Francis et al., 1989; Kajimoto and Rotwein, 1989). Amino acid sequences of the IGF-IA carboxyl-terminal and amino-terminal peptides are also highly conserved (Tavakkol et al., 1988: Kajimoto and Rotwein, 1989).

The two carboxyl-terminal peptide extensions of IGF-IA and IGF-IB have no amino acid homology. The synthesis of these two forms was believed to be the result of alternative processing since only one IGF-I gene has been identified in the human genome (Brissenden et al., 1984; Tricoli et al., 1984). The human and rat genes that encode IGF-I are 45 and 73 kb, respectively, and contain five exons and four introns (Rotwein et al., 1986; Shimatsu and Rotwein, 1987). Subsequently, it was found that, in rats and humans, exons 1 and 2 encode portions of the signal peptide, exons 2 and 3 encode the mature IGF-I peptide, exons 3,4, and 5 encode the carboxyl-terminal region and 3'- untranslated sequences (Shimatsu and Rotwein, 1987; Rotwein et al., 1986). IGF-IA (153 amino acids) is encoded

by exons 1,2,3, and 5, whereas IGF-IB (195 amino acids) is encoded by exons 1,2,3, and 4 (Jansen et al., 1983; Rotwein, 1986 and 1987). Tavakkol et al. (1988) have compared human, rat, and pig exons 2,3, and 5 and found that within these exons nucleotides corresponding to the leader peptide (75 nt upstream from sequences encoding the mature peptide), mature peptide, E peptide, and 3'-untranslated region (first 85 nt) were 79, 86.2, 88, and 63.5% identical.

Processing of the primary IGF-I gene transcript has been reviewed by Daughaday and Rotwein (1989). Not only does alternative mRNA splicing occur at 5'- and 3'-ends of the IGF-I gene, but there are several polyadenylation sites at the 3'-end of exon 5. As a result, several IGF-I mRNA species are generated ranging in size from 0.7 to 8.0 kb.

Traditionally it was thought that liver was the primary site for the synthesis of IGF-I (Daughaday et al., 1972), but many studies have provided evidence that IGF-I is synthesized in multiple organs and tissues. The most definitive studies first supporting this were those of Han et al. (1987), Murphy et al. (1987a) and Han et al.(1988),in which IGF-I mRNA was detected in various organs and tissues. Prior to these studies, the observation that tissue concentrations of IGF-I in several tissues increased before an increase in serum IGF-I following administration of growth hormone to rats (D'Ercole et al., 1984) led to the proposal that IGF-I may act on tissues in a paracrine or autocrine manner.

Orlowski and Chernausek (1988) found that administration of rat growth hormone to hypophysectomized rats increased liver and kidney IGF-I concentrations relative to hypophysectomized controls, but had no effect on serum concentrations. These observations suggested a discordance of serum and tissue somatomedin levels and supported a paracrine or autocrine action of IGF-I in rats administered growth hormone. However, lack of a response in serum IGF-I concentrations to growth hormone may be due in part to the short half life of IGF-I in serum from hypophysectomized rats (Zapf et al., 1986). In a recent review, Daughaday and Rotwein (1989) discuss how IGF-binding proteins may be involved in serum IGF-I stability. In hypophysectomized rats, most serum IGF-I is associated with a 35 kd protein which may potentiate IGF-I action, whereas in normal rats most serum IGF-1 is bound to a 150 kd complex which may inhibit IGF-I action (Elgin et al., 1987). Hall et al. (1988) have proposed that the smaller binding complex (also referred to as insulin-like growth factor binding protein-I or IBP-I; rev. by Holly and Wass, 1989) may serve to transport IGF-I from the larger complex in the circulation to the IGF-I target cells.

Blum et al. (1989) have concluded that the binding proteins may function as a reservoir for the continuous release of low amounts of IGF-I. Slow release of IGF-I may prevent down regulation of the IGF-I receptor and thus be a more effective mitogenic stimulus than high concentrations

of IGF-I. Six IGF-I binding proteins have been found in porcine serum and at least two forms have been found secreted by muscle cell lines (McCusker et al., 1989a and 1989b). Physiological significances of IGF-1 binding proteins are not clearly understood. Studies designed to examine their role in IGF-1 action are complicated since these binding proteins are regulated hormonally, nutritionally and developmentally (McCusker et al., 1989b).

Regulation of IGF-1 Gene Expression

Several factors may regulate IGF-I gene expression in tissues. These include tissue-specific, hormonal, developmental, and nutritional factors (fasting, protein and energy intake). Growth hormone increases liver IGF-I transcription (Mathews et al., 1986) in growth hormone-deficient mice and IGF-I mRNA abundance in many tissues (rev. by Daughaday and Rotwein, 1989). Furthermore, the multiple species of IGF-I mRNA in rat heart, kidney, lung, and liver are decreased in hypophysectomized rats and restored after growth hormone repletion (Roberts et al., 1986 and 1987). Lowe et al.(1987) found that rat IGF-I transcripts contained different alternative 5'-untranslated regions which were expressed in a tissue-specific manner and were differentially regulated by growth hormone. Growth hormone also altered the size distribution of liver IGF-I mRNA species in growth hormone-deficient rats (Matthews et al., 1986). Hepler et al. (1989) found that when growth hormone was administered to hypophysectomized

rats, various IGF-I mRNA size classes (resulting from differences in length of 3'-untranslated regions) were differentially regulated. They suggested that the greater stability and(or) more efficient utilization of polyadenylation sites may account for more rapid rates of growth hormone-stimulated induction.

Hormonal regulation of IGF-I gene expression is not limited to growth hormone. Estrogens increased IGF-I mRNA abundance in the uterus of pituitary-intact and hypophysectomized ovarectomized rats, however, estrogens had no effect on hepatic or renal IGF-1 mRNA abundance (Murphy et al., 1987b). Later it was found that estrogens actually inhibited growth hormone-stimulated hepatic IGF-I gene expression in ovariectomized, hypophysectomized rats (Murphy and Friesen, 1988) suggesting that interaction of hormones is important in regulation of IGF-I gene expression. Interactions between thyroid hormones and growth hormone-stimulated IGF-I synthesis and secretion have been observed (Wolf et al., 1989) as well as interactions between dexamethasone and growth hormone-induced IGF-I mRNA (Luo and Murphy, 1989).

IGF-I gene expression is also regulated by plane of nutrition. Fasting has been found to decrease serum IGF-I concentrations and hepatic IGF-I mRNA abundance (Clemmons et al., 1981; Maes et al., 1983; Elmer and Schalch, 1987). Protein or energy restriction produce similiar results (Prewitt et al., 1982; Isley et al., 1983 and 1984; Maes et

al., 1984; Moats-Staats et al., 1989; VandeHaar et al., 1989). The decreased IGF-I mRNA abundance associated with fasting is due at least in part to decreased transcription of the IGF-I gene (Straus and Takemoto, 1989). Reduction in IGF-I mRNA abundance in response to altered nutritional status may involve various mechanisms, such as regulation of growth hormone receptor number and post-receptor events (Maes et al., 1983; Bornfeldt et al., 1989; Maiter et al, 1989; Thissen et al., 1989). Phillips (1986) has proposed that, during restricted nutrition, the decreased growth hormone-induced generation of somatomedins results in decreased negative feedback of somatomedins on growth hormone secretion. This results in a rise in growth hormone secretion which in the presence of low concentrations of somatomedins may allow metabolic fuels to be diverted away from growth and toward vital organs (brain, heart, lungs,etc.).

IGF-I gene expression is regulated developmentally and appears to be regulated independently of growth hormone in fetal stages of growth. For example, liver IGF-I mRNA in rats increases 8.6-fold between days 11 and 13 of gestation which is prior to the ontology of growth hormone synthesis and secretion by the pituitary and before the appearance of cell surface receptors for growth hormone (Rotwein et al., 1987). Slabaugh et al. (1982), Flandez et al. (1986), Lund et al. (1986), and Hynes et al. (1987) have all detected IGF-I mRNA in fetal tissues. The developmental increase in IGF-I mRNA also appears to parallel the developmental

increase in liver immunoassayable IGF-I during gestation. However, since multiple tissues and organs synthesize IGF-I, it is difficult to determine whether the tissue concentration represents local synthesis or that from the circulation.

IGF-I was first found to be synthesized by myoblasts by Hill et al. (1984). Subsequently, IGF-I gene expression was also found to be regulated during myogenesis. IGF-I mRNA levels are low in proliferating cultured myoblasts and increase transiently by 6- to 10-fold within 48-72 hours during myogenic differentiation (Tollefsen et al., 1989). During muscle cell differentiation, there was a 2.5-fold increase in IGF-I in the culture medium, a 2-fold increase in IGF-I receptor number, and greater than a 30-fold increase in the secretion of an IGF binding protein.

Effects of IGF-I on Muscle Growth

Most of the actions of the IGF-I on muscle appear to be primarily mediated by the IGF-I receptor (Ewton et al., 1987; Kiess et al., 1987; Ballard et al., 1988). The IGF-I receptor is a ligand-activated tyrosine-specific protein kinase structurally related to the insulin receptor (Rechler and Nissley, 1986; Ullrich et al., 1986). The expression of IGF-I receptors in rat skeletal muscle is greatest in fetal and early postnatal life and then begins to gradually decline at 4 weeks of age to adult levels which is 25% of fetal levels (Alexandrides et al., 1989).

Many studies have examined the effect of IGF-I on cultured muscle cells. IGF-I has been demonstrated to accelerate amino acid uptake in cultured human skeleton myoblasts (Hill et al., 1986), L6 rat skeletal myoblasts (Ewton et al., 1987), and myotubes derived from ovine satellite cells (Roe et al., 1989). Glucose uptake was stimulated by IGF-I in cultures of L6 myoblasts (Wang et al., 1987), BC3H-1 myocytes (Farese et al., 1989) and myocytes derived from ovine satellite cells (Roe et al., 1989). Proliferation was enhanced by IGF-I in cultures of human myoblasts (Hill et al, 1986), L6 myoblasts (Ewton et al., 1987), and rat skeletal muscle satellite cells (Dodson et al., 1985; Allen, 1986). Protein synthesis was increased and degradation decreased by including IGF-I in muscle cultures of L8 myotubes (Gulve and Dice, 1989), L6 myoblasts and myotubes (Beguinot et al., 1985), and differentiated ovine satellite cells (Roe et al., 1989). Differentiation, measured by increases in creatine kinase activities, by fusion percentages, and by formation of myosin heavy chain and myomesin, was increased by IGF-I in cultures of L6 myoblasts (Ewton and Florini, 1980; Florin and Ewton, 1986; Ewton et al., 1987). In rat skeletal muscle satellite cells, IGF-I increased differentiation as indicated by myotube nuclei density (Allen, 1986). Although muscle cell fusion is cell density-dependent in culture, IGF-I-induced differentiation is not a result of greater cell density from the mitogenic effect of IGF-I. Ewton and Florini (1981) demonstrated that IGF-I stimulated

differentiation over wide ranges of final cell densities. IGF-I is unique in that, unlike most mitogens, it stimulates both proliferation and differentiation at physiological concentrations. However, Florini et al. (1986) have demonstrated that at greater (nonphysiological) concentrations, differentiation is inhibited. Mechanisms by which IGF-I induces the above effects on cultured muscle cells have been reviewed by Florini (1987) and Florini and Magri (1989). Possible signal transducers include oncogenes (Ong et al., 1987; Schneider and Olson, 1988), cytoskeletal polypeptides that are receptor tyrosine kinase substrates (Beguinot et al., 1988), and polyamines (Ewton et al., 1984; Multhauf and Lough, 1986).

The effect of IGF-I on muscle growth was also examined in isolated rat soleus muscles. IGF-I was found to enhance RNA synthesis and the rate of polypeptide chain initiation (Monier and LeMarchand-Brustel, 1984) and to increase glucose and amino acid uptake (Poggi et al., 1979; Yu and Czech, 1984).

Although IGF-I has been demonstrated to increase weight gains in hypophysectomized rats (Shoelne et al., 1982), growth hormone-deficient dwarf rats (Skottner et al., 1989), and normal rats (Hizuka et al., 1986), few studies have been designed to examine the effects of IGF-I on muscle growth in vivo. Jacob et al. (1989) were able to reduce protein breakdown in fasted rats by administering IGF-I. Pell and Bates (1989) administered IGF-I to dwarf

mice and observed increases in body weights and increases in muscle protein synthesis and synthetic capacity (measured as RNA/protein).

Involvement of IGF-I In Mediating Growth Hormone-Induced Muscle Growth in Pigs

It has been known for some time that growth hormone can alter carcass composition and improve feed efficiency in pigs. Lee and Schaffer (1934) found that growth hormone resulted in accretion of proportionately more muscle and less fat tissue in pigs. Since then many laboratories have examined the effect of growth hormone on animal performance, carcass composition, and tissue growth. Machlin (1972), Chung et al. (1985), Campbell et al. (1989), and Smith et al. (1989) have all observed increases in muscle growth in pigs in response to administration of porcine somatotropin. The stimulatory effect of growth hormone on growth is assumed to be mediated, at least in part, by IGF-I.

Chung et al. (1985) administered 22 ug porcine growth hormone/kg body weight/day i.m. for 30 days to barrows initially weighing 32 kg. Barrows were fed a 16% crude protein corn-soy diet. Growth rate was increased 10%, feed efficiency was increased 4%, and muscle mass was increased by 6% relative to controls. These changes were accompanied by increases in serum IGF-I concentrations (55% over control concentrations three hours after injection).

Etherton et al. (1987) conducted a dose-response experiment with 0, 10, 30, 70 ug porcine growth hormone/kg

BW/day for 35 days i.m. in growing barrows and found a dose response in carcass composition changes (increased muscle and decreased fat with greater amounts of growth hormone) and serum IGF-I (greater IGF-I concentrations with greater amounts of growth hormone). Etherton et al. (1986), Buonomo et al. (1987), and Walton and Etherton (1989) also observed increases in serum IGF-I concentration following porcine growth hormone administration. Administration of 100 ug pituitary-derived porcine growth hormone/kg body weight/day to intact males and females from 60 to 90 kg body weight increased plasma IGF-I concentrations up to 2-fold relative to controls (Owens et al., 1990). Furthermore, IGF-I concentrations were less in females than males which may explain slower growth rates of female than male pigs (Campbell and Taverner, 1988; Campbell et al., 1989). However, Owens et al. (1990) did not find greater plasma IGF-I concentrations in faster growing strains than slower growing strains of male or female pigs. Such a discrepancy may be due to other factors. Recently, Mathison et al. (1989) have conducted IGF-I receptor studies on satellite cell-derived myotube membranes from two lines of rams selected for growth rate. They found that membranes derived from rams selected for greater growth rates had more IGF-I receptors than those from slower growing rams. Owens et al. (1990) and Walton and Etherton (1989) also observed greater concentrations of IGF-I binding proteins in plasma of growth hormone-treated

pigs which could partially explain the greater concentrations of plasma IGF-I since IGF-I bound to binding proteins has a longer half life than free IGF-I in pigs, sheep, and rats (Zapf et al.,1986; Francis et al., 1988: Walton et al., 1989). The above results suggest that mechanisms at the target tissue site may be more important in modulating IGF-I action than plasma IGF-I concentrations alone.

Evock et al. (1988) demonstrated with growing barrows that 70 ug/kg BW/day of pituitary-derived porcine growth hormone was equipotent to recombinant porcine growth hormone in increaseing serum IGF-I concentrations and in increasing skeletal muscle growth. Novakofski et al. (1988) also found that recombinant and pituitary-derived porcine somatotropin were equipotent in enhancing muscle growth in pigs.

Dietary protein concentration plays a major role in the growth hormone-induced muscle growth in pigs. Smith et al. (1989a) found that dietary crude protein concentration had to be greater than 14% for recombinant porcine somatotropin to increase muscle growth. Stoner et al. (1989) also found that growth performance of porcine somatotropin-treated pigs was dependent on the energy density of the diet, but that lean tissue growth was not significantly effected by energy density. Unfortunately, no measurements of IGF-I were made in these studies.

There are no reports of measurements of tissue concentrations of IGF-I (except serum IGF-I concentrations)

or IGF-I mRNA in pigs administered growth hormone. Since growth hormone receptors are present in skeletal muscle (Satyanarayama et al., 1988; Zanelli et al., 1989) and muscle cells (Adaofio and Kostyo, 1988; Lev and Holland, 1986), it seems plausible that growth hormone may act directly on muscle to increase IGF-I synthesis. This muscle IGF-I may mediate the growth hormone signal on muscle growth. Although administration of growth hormone has never been reported to decrease muscle protein degradation in vivo in fed states, it has been reported to increase protein synthesis in mice (Pell and Bates, 1989) and steers (Eisemann, 1989). If IGF-I were mediating the actions of growth hormone on muscle growth, then one would expect muscle protein degradation to be decreased as well, since IGF-I administration has been demonstrated to affect both the synthesis and degradation of muscle protein in vivo (as discussed earlier). Such discrepancy is difficult to interpret, but may be due to other factors that are induced by growth hormone which prevent degradation rates from changing.

Measurements of skeletal muscle IGF-I mRNA levels could provide clues to possible autocrine and(or) paracrine roles of IGF-I in growth hormone-induced muscle growth. Such studies have been conducted with rodents. Turner et al. (1988) implanted adult female Wistar-Furth rats with growth hormone-secreting GH3 cells and examined liver and muscle growth and IGF-I mRNA abundance 80 days later.

Compared with control normal rats, liver and gastrocnemius muscle weights were increased 314% and 55%, respectively. Respective changes in skeletal muscle and liver IGF-I mRNA abundance were 2.4 and 8-fold greater in treated rats. Increases in both liver and muscle IGF-I mRNA with concommitant increases in muscle growth suggests that IGF-I may play both endocrine and paracrine/autocrine roles in skeletal muscle growth; however, differential roles of liver IGF-I and muscle IGF-I were not distinguished in this study. Matthews et al. (1988) studied the role of IGF-I in mediating growth hormone-induced growth in transgenic mice carrying GH fusion genes. Although circulating growth hormone concentrations were greater in transgenic mice than in normal mice at birth, hepatic IGF-I mRNA concentrations and circulating IGF-I levels were not greater until 2 weeks of age. Furthermore, accelerated growth was not observed until three weeks of age, after the changes in IGF-I status. Results of this study led Matthews and his colleagues to conclude that IGF-I may be directly involved in mediating the growth hormone effect on growth.

Unfortunately, skeletal muscle IGF-I mRNA abundance was not quantitated in the above study, so it is unknown if abundance of IGF-I mRNA was altered in response to increased circulating levels of growth hormone. Isgaard et al.(1989) have demonstrated that administration of growth hormone to hypophysectomized male rats induces expression of IGF-I mRNA in skeletal muscle, but effects of similiar treatments on intact rats were not tested. Subsequently,

it was demonstrated (Isgaard et al., 1988) that single injections i.v. every 8 hours or s.c. every 12 hours (pulsatile treatment) were more effective in increasing abundance of skeletal muscle IGF-I mRNA than continuous infusions. Although growth hormone increased hepatic IGF-I mRNA levels, pulsatile treatments were no more effective than continuous infusions. However, body weight gain was 25% greater in rats subjected to the pulsatile injections which implies that growth rate may correlate better with abundance of IGF-I mRNA in muscle than in the liver. Future studies of this type need to include measurements of muscle growth so that the relationship between liver and muscle IGF-I mRNA abundance and muscle growth can be established.

Involvement of IGF-I In Mediating Beta Adrenergic Agonist-Induced Muscle Growth

Beta adrenergic agonists increase skeletal muscle growth and reduce fattening in many species (for reviews, see Hanrahan, 1987; Williams, 1987, and Thorton and Tume, 1988). Yang and McElligott (1989) have discussed studies designed to determine the mechanism of action of beta adrenergic agonists. An indirect mechanism for the anabolic action of beta adrenergic agonists has not been identified. Many studies have demonstrated that various circulating hormones are probably not involved in beta-adrenergic agonist-induced muscle hypertrophy. For

example, although plasma growth hormone concentrations increased 2- to 3-fold in sheep fed the beta adrenergic agonist, cimaterol, for six weeks (Beermann et al., 1987), cimaterol has been demonstrated to increase muscle growth in beta agonist-fed hypophysectomized rats (Thiel et al., 1987). Clenbuterol also stimulates growth in castrated and adrenalectomized rats demonstrating that effects of clenbuterol in male rats occur in the absence of a gonadal or adrenal hormones (Rothwell and Stock, 1988). Futhermore, serum from rats treated with clenbuterol and serum from normal rats had similiar effects on protein synthesis and degradation and proliferation in cultured L8 myoblasts (McElligott and Chaung, 1987). Beermann et al. (1987) also found that plasma IGF-I concentrations were depressed by 34% after administration of cimaterol. However, the IGF-I concentrations decreased during the 6 hour sample collection period. This trend was probably due to the withholding of feed during the collection period. Fasting has been demonstrated to reduce plasma IGF-I concentrations (Clemmons et al., 1981) and therefore makes the above cimaterol-induced alterations in IGF-I concentrations difficult to interpret. If one examines the IGF-I measurements at the zero-hour time, IGF-I concentration is greater in cimaterol-treated lambs than in controls. There have been no other reports of plasma IGF-I measurements in animals fed beta adrenergic agonists.

Results of studies designed to examine the effects of beta adrenergic agonists on muscle protein turnover have

been inconsistent. This has been due to the use of different agonists, doses, and routes of administration in different systems designed to quantitate changes in muscle protein synthesis and degradation. Since skeletal muscle contains $beta_1$ and $beta_2$ adrenergic receptors (Apperly et al. 1976; Stiles et al., 1984; Waldech et al., 1986; Watson-Wright and Wilkinson, 1986; Rothwell et al., 1987; Liggett et al, 1988), it is possible that beta adrenergic agonists act directly on muscle to exert their anabolic actions. The increased fractional synthesis rate of skeletal muscle protein in pigs fed ractopamine (Bergen et al., 1989; Helferich et al., 1990), in lambs fed clenbuterol (Claeys et al., 1989), and in rats fed clenbuterol (Maltin et al., 1987; MacLennan and Edwards, 1989) is consistent with reports of increased muscle protein synthesis in cultures of muscle cells containing ractopamine or clenbuterol (Anderson et al., 1990), ractopamine (Adeola et al., 1989), and cimaterol (Young et al., 1990). Furthermore, ractopamine increases the relative abundance of skeletal muscle alpha actin mRNA in pigs (Helferich et al., 1990) and myosin light chain mRNA in steers (Smith et al., 1989a) which indicates that ractopamine enhances protein synthesis pretranslationally. There have also been reports that beta adrenergic agonists reduce muscle protein degradation in vivo and in cultures of muscle cells (Forsberg and Merrill, 1986; Reeds et al. 1986; Bohorov et al., 1987; Morgan et al., 1988; Young et

al., 1990). It is plausible that IGF-I mediates the beta adrenergic agonist-induced increase in skeletal muscle protein synthesis and decreased protein degradation. IGF-I has been demonstrated to increase muscle growth by reducing protein degradation in rats (Jacob et al., 1989) and by increasing protein synthesis in dwarf mice (Pell and Bates, 1989). IGF-I has also had similiar effects in muscle cell cultures (Gulve and Dice, 1989; Beguinot et al., 1985; Roe et al., 1989). If IGF-I does mediate beta adrenergic agonist activity, then it most likely acts in a paracrine or autocrine manner rather than in an endocrine manner. If IGF-I acted in an endocrine manner in response to beta adrenergic agonists, then circulating levels of IGF-I would likely be increased. Increased serum IGF-I would result in greater anabolic activity in cultures of L8 myoblasts containing such serum. As stated earlier, McElligott and Chaung (1987) detected no such difference.

Summary of the Literature Review

The above review has described the expression of IGF-I in mammalian tissues and has summarized some of the studies that have been designed to determine the role of IGF-I in growth and development of skeletal muscle. Many of these studies have been conducted in hypophysectomized rats in order that the interaction between growth hormone and IGF-I expression can be characterized. Unfortunately, very few studies in meat-producing animals (cattle, pigs, sheep, and

poultry) have included measurements of IGF-I tissue peptide concentrations and mRNA abundance. Two animal models that are useful for studying biological mechanisms involved in growth of skeletal muscle can be created by 1) administration of growth hormone to finishing swine and 2) oral administration of beta adrenergic agonists to pigs. Both of these manipulations are associated with changes in skeletal muscle mass which are due at least in part to alterations in muscle protein synthesis or accretion (Machlin, 1972; Chung et al., 1985; Maltin et al., 1987; Evock et al., 1988; Novakofski et al., 1988; Bergen et al., 1989; Campbell et al., 1989; Claeys et al., 1989; MacLennan and Edwards, 1989; Smith et al., 1989b; Helferich et al., 1990). Since IGF-I status has been demonstrated to be altered by the above perturbations in various species, it is possible that IGF-I mediates the changes in muscle protein metabolism. IGF-I is synthesized by many tissues and has been demonstrated to play endocrine and paracrine and (or) autocrine roles in growth and development of various tissues and organs, including skeletal muscle and liver. Measurements of changes in IGF-I mRNA abundance in skeletal muscle and liver in the animal models described above could provide clues to the possible endocrine and paracrine and (or) autocrine roles of IGF-I in muscle hypertrophy in growing pigs.

CHAPTER 1

DEVELOPMENT OF A METHOD TO DETERMINE THE RELATIVE ABUNDANCE OF IGF-I mRNA IN LIVER AND SKELETAL MUSCLE OF PIGS

Abstract

To determine the relative abundance of IGF-I mRNA in liver and skeletal muscle of market weight pigs, it was necessary to develop a sensitive solution hybridization-nuclease protection assay. Utilization of Northern blotting techniques was sufficient to detect IGF-I mRNA in uterine tissue of pregnant pigs (12 days of gestation), but was not sufficient to detect differences in abundance of IGF-I mRNA in liver and skeletal muscle of market weight pigs. An antisense RNA probe was synthesized from a porcine IGF-I cDNA and utilized in a solution hybridization assay. It was necessary to purify the probe via agarose gel electrophoresis and electroelution prior to hybridization to liver and muscle RNA in order to remove traces of the DNA template. Hybridization temperature at 65^{o}C was found to reduce background relative to hybridization at 42^{o}C. Following hybridization, nuclease

S1 was utilized to digest single-stranded nonhybridized RNA. Optimum digestion conditions were 133 U of nuclease S1 per 100 ug RNA at 37°C for 1 hour. Protected fragments were then electrophoresed in 8 M urea/5% acrylamide gels under denaturing conditions. After gels were dried and subjected to autoradiography, protected fragments were excised and radioactivity was quantitated via liquid scintillation analysis. Results were corrected for nonspecific hybridization by subtracting the signal obtained with tRNA. Intrassay and interassay coefficients of variations were 2.8 and 2.7%, respectively. A linear response was obtained using from 50 to 150 ug of liver RNA. The sensitivity of this assay was sufficient to detect increases in IGF-I mRNA abundance in liver and skeletal muscle of market weight pigs treated with growth hormone.

Introduction

Since IGF-I status has been demonstrated to be altered by administration of growth hormone or beta adrenergic agonists in various species (Chung et al., 1985; Etherton et al., 1986; Beermann et al., 1987; Buonomo et al., 1987; Etherton et al., 1987; Walton and Etherton, 1989; Owens et al., 1990), it is possible that IGF-I mediates the changes in muscle protein metabolism. IGF-I is synthesized by many tissues and has been demonstrated to play endocrine and paracrine and (or) autocrine roles in growth and development of various tissues and organs, including skeletal muscle and liver. Measurements of changes in IGF-I mRNA levels in skeletal muscle and liver in the animal models described above could provide clues to the possible endocrine and paracrine and (or) autocrine roles of IGF-I in muscle hypertrophy in growing pigs. The objective of research presented in this chapter was to develop an assay that could detect any changes in abundance of IGF-I mRNA following treatment with growth hormone or beta adrenergic agonists.

Materials and Methods

Tissue Sample Collection and RNA Isolation.

Following exsanguination during slaughter, samples of liver and longissimus dorsi (LD) muscle were immediately excised from newborn piglets and market weight barrows. Samples were collected, cut into pieces approximately 8 cm^3, frozen by submersion in liquid nitrogen and stored at -80°C. Samples of frozen (-80°C) uterine tissue were obtained from Dr. Frank Simmen (University of Florida). RNA was isolated from LD muscle and liver by an acid guanidine thiocyanate phenol chloroform procedure (Chomczynski and Sacchi, 1987; see Appendix A for details). Under the conditions employed in the RNA isolation procedure (extraction at pH 5 to 6), the final RNA preparations do not contain DNA (Chomczynski and Sacchi, 1987; Wallace, 1987). Final RNA preparations were resuspended in TE-8 [10 mM Tris (Boehringer Mannheim Biochem., Indianapolis, IN), 1 mM EDTA (disodium EDTA, Fisher Scientific, Fair Lawn, NJ; pH 8.0)] and stored at -80°C. RNA solutions were scanned from 320 to 220 nm and A_{260}/A_{280} were determined to ensure that values greater than 1.8 were obtained. Concentrations of RNA in final

preparations were determined from A_{260} values. Poly(A)$^+$ RNA was isolated from total RNA by batch absorption and elution from oligo dT cellulose (Boehringer Mannheim Biochem., Indianapolis, IN) as described by Maniatis et al. (1982).

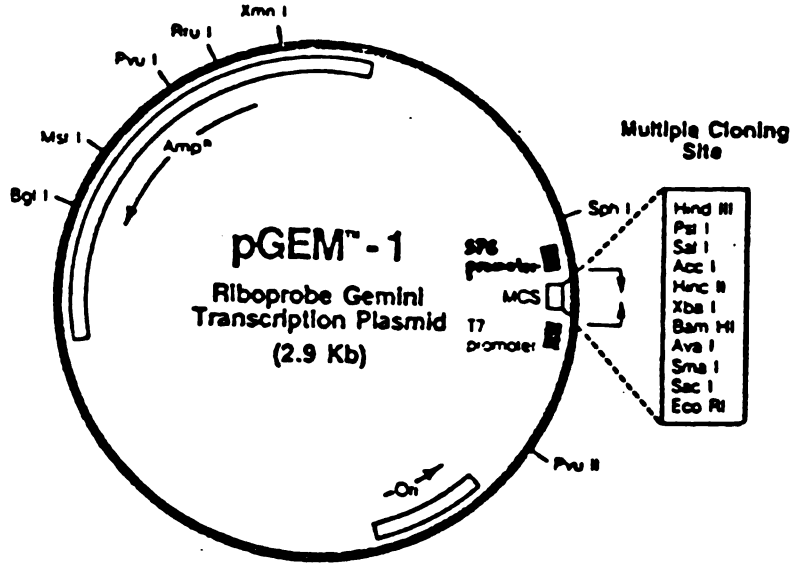
Northern Blot Analysis. Northern blot analysis was conducted as described by Jump et al. (1984) with the following modifications. Aliquots of total or Poly(A)$^+$ RNA were denatured and electrophoretically separated in 1.2% agarose gels containing 2.2 M formaldehyde. RNA was transferred to Zetabind membrane (Cuno, Inc., Meriden, CT) with 50 mM sodium phosphate buffer, pH 6.5. Blots were prehybridized in 50% formamide (Boehringer Mannheim Biochem., Indianapolis, IN), 5X SSC (25X SSC is equivalent to 3.75 M sodium chloride, 0.375 M sodium citrate), 5X Denhardt's solution [100X Denhardt's solution is equivalent to 2% bovine serum albumin (from fraction V, essentially fatty acid-free, Sigma Chem. Co., St. Louis, MO), 2% Polyvinylpyrrolidone-40 (Sigma Chem. Co., St. Louis, MO), and 2% Ficoll-400 (Sigma Chem. Co., St. Louis, MO)], 50 mM sodium phosphate buffer (pH 6.5), 1 mM EDTA (disodium EDTA, Fisher Scientific Co., Fair Lawn, NJ), and 0.5 mg tRNA/ml (Boehringer Mannheim Biochem., Indianapolis, IN) for 2 hours at 42^{o}C. The hybridization solution was identical to the prehybridization solution, except that the hybridization solution contained only 1X Denhardt's solution and contained two million cpm of probe/ml.

A 580-base pair porcine IGF-I cDNA subcloned into the

EcoRI site of the multiple cloning site of pGEM-1 (Promega, Madison, WI) was obtained from Dr. Frank Simmen (Figure 1.1; Tavvakol et al., 1988). The cDNA encodes a 25-amino acid leader peptide, the mature (processed) 70-amino acid porcine IGF-IA peptide, and a 35-amino acid carboxy-terminal extension peptide. The cDNA insert was excised with EcoRI (Boehringer Mannheim Biochem., Indianapolis, IN) and purified by agarose gel electrophoresis and electroelution as described by Maniatis et al. (1982). The purified cDNA was labeled with a Random Primed DNA Labeling Kit (Boehringer Mannheim Biochem., Indianapolis, IN) using [^{32}P]dCTP (3000 Ci/mmol; Amersham) to a specific activity of 4 X 10^8 cpm/ug DNA. After hybridization at 42^{o}C for 16 hours, blots were washed with 2X SSC and 0.1% SDS (Boehringer Mannheim Biochem., Indianapolis, IN) at room temperature, then washed for three cycles in 0.1X SSC and 0.1% SDS at either 55^{o} or 65^{o}C. Dried blots were subjected to autoradiography using XAR-5 film (Eastman Kodak, Rochester, NY) at -80^{o}C with two intensifying screens (DuPont, Wilmington, DE).

Synthesis of Antisense IGF-I RNA Probe. Orientation of the cDNA insert in pGEM-1 was determined by BamHI restriction enzyme digestion. There is a BamHI site in the multiple cloning sequence in pGEM-1 and a BamHI site within the cDNA insert 450 base pairs from the 5′ end of the cDNA. Following digestion, products were electrophoresed in agarose gel, stained with ethidium bromide, and visualized

Figure 1.1. Maps of the plasmid vector (top) and IGF-I cDNA insert (bottom) that were used for the synthesis of the IGF-I cDNA and RNA probes. The cDNA was subcloned into the EcoRI site of the plasmid vector, pGEM-I (Promega, Madison, WI), and generously provided by Dr. Frank Simmen (Tavakkol et al., 1988).


Xmn I
Pvu I
Pvu I
Msl I
Bgl I
Amp^R
pGEM™-1
Riboprobe Gemini
Transcription Plasmid
(2.9 Kb)
SP6 promoter
MCS
T7 promoter
ori
Pvu II
Sph I
Multiple Cloning Site
Hind III
Pst I
Sal I
Acc I
Hinc II
Xba I
Bam HI
Ava I
Sma I
Sac I
Eco RI

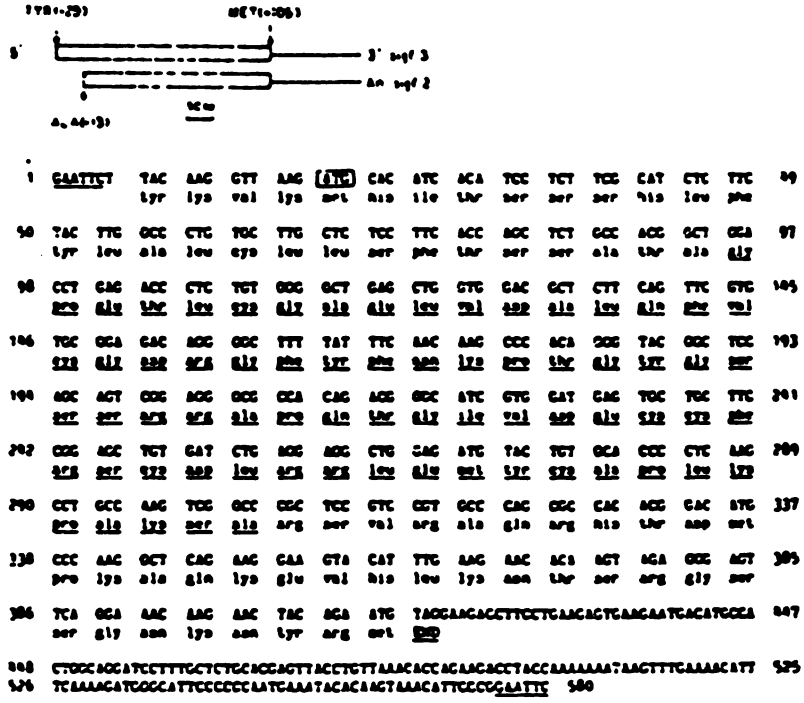


Figure 1.1

utilizing a uv light (302 nm)[30]. Orientation was determined from the sizes of the BamHI fragments that were present following digestion. It was determined that use of the SP6 promoter in pGEM-1 would generate antisense transcripts (discussed in Results and Discussion). The recombinant plasmid containing the IGF-I cDNA was linearized with PvuII and then utilized as a template in an SP6 Transcription Kit (Boehringer Mannheim Biochem., Indianapolis, IN) with [^{32}P]UTP (400 Ci/mmol; Amersham Corp., Arlington Hts., IL) for the synthesis of a [^{32}P]UTP-labeled RNA probe (see Appendix B for details). Due to the location of the PvuII site within the plasmid, 98 base pairs of plasmid sequences were also transcribed during the transcription reaction, resulting in the synthesis of a 678-nucleotide probe. The probe was purified by 1.2% agarose gel electrophoresis and electroelution (Maniatis et al., 1982) and then used in a solution hybridization assay as described by Krieg and Melton (1987) with the following modifications. RNA samples (100 ug) were annealed with two million cpm of labeled probe for 14 hours at either 42^{o} or 65^{o}C in 80% formamide, 0.4 M NaCl, 40 mM PIPES (pH 6.4; US Biochem. Corp., Cleveland, OH), and 1 mM EDTA. Hybridizations were conducted in 1.5-ml microfuge tubes. For a negative control, tRNA was included as a sample and subjected to the same hybridization conditions as the tissue RNA samples. Following hybridization, 13.3, 26.6, 133, or 266 units of nuclease S1 (Boehringer Mannheim Biochem., Indianapolis, IN) were added to each sample and the mixture was incubated

at 37°C for 1 hour in order to determine the optimum concentration of nuclease S1 for digestion of single-stranded nonhybridized labeled RNA. Samples were then extracted with phenol-chloroform. The RNA was precipitated from the aqueous phase with ethanol. RNA was electrophoresed in 1.2% agarose as described for Northern analysis above or resuspended in 90% formamide gel loading buffer, denatured at 90°C for 3 min and then loaded onto a 5% acrylamide/8M urea gel. Following electrophoresis, agarose gels were subjected to autoradiography, whereas acrylamide gels were dried and then exposed to film (XAR-5; Eastman Kodak, Rochester, NY) at -80°C with intensifying screens (DuPont, Wilmington, DE). The size of protected RNA fragments was determined by including DNA molecular weight markers in adjacent lanes. These marker lanes were cut from the gels prior to drying, stained with ethidium bromide, and visualized utilizing a uv light (302 nm) for measurements of migration distances. Protected fragments 580 base pairs in length were excised from the sample lanes of the gels and radioactivity quantitated by liquid scintillation analysis. Results were corrected for nonspecific hybridization by subtracting the values obtained from tRNA negative controls.

Results and Discussion

Results of Northern blot analyses are presented in Figure 1.2. Analysis of 40 ug of total RNA from uterine tissue of pigs at 12 days of gestation resulted in hybridization of the IGF-I cDNA probe to mRNA species of 1.2 and 8 kilobases. These results are consistent with those of Tavakkol et al. (1988). Hybridization was detectable with 23 ug of market weight pig liver and 38.3 ug of newborn pig liver, but the signal was very low relative to the background. Hybridization to 1.2 and 8 kilobase species was evident with the newborn pig liver, but not with the market weight pig liver. Due to the high background on the autoradiogram, small changes in IGF-I mRNA abundance would not be detectable with this method. A disadvantage with using poly $(A)^+$ RNA is that quantitation is not accurate due to incomplete removal of poly $(A)^-$ RNAduring the isolation procedure. Furthermore, large quantities of RNA would be necessary in order to generate sufficient quantities of Poly$(A)^+$ RNA.

The solution hybridization-nuclease protection assay descibed by Krieg and Melton (1987) provides a very sensitive method to determine the relative abundance of

Figure 1.2. Autoradiograms from Northern blot analysis of IGF-I mRNA in 40 ug of total RNA from uterine tissue of a pregnant pig (12 days of gestation), 23 ug of Poly(A)$^+$ RNA from liver of a market weight pig, and 38.3 ug of Poly(A)$^+$ RNA from liver of a newborn pig. RNA was denatured and electrophoretically separated in 1.2% agarose gels containing 2.2 M formaldehyde and then transferred to Zetabind (Cuno). Blots were prehybridized at 42^{o}C for 2 hours in 50% formamide, 5X SSC, 5X Denhardt's solution, 0.1% SDS, 1 mM EDTA, 50 mM sodium phosphate buffer (pH 6.5), and 0.5 mg/ml tRNA. RNA was hybridized with a porcine IGF-I cDNA probe at 42^{o}C for 16 hours. Composition of the hybridization solution was identical to the prehybridization solution except that the hybridization solution contained only 1X Denhardt's and contained two million cpm of probe/ml. Following hybridization, blots were washed with three cycles of 0.1X SSC and 0.1% SDS at 65^{o}C. Uterine and liver blots were exposed to film with two intensifying screens at -80 C for 3 and 7 days, respectively.

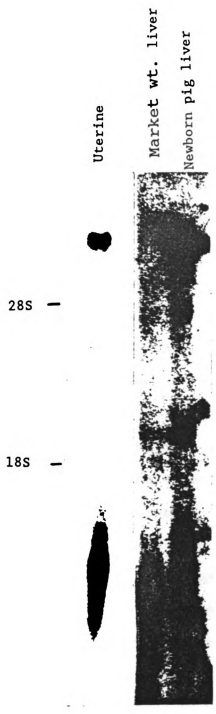


Figure 1.2

mRNAs in various tissues. The sensitivity of this method is greater than that of Northern blot or dot blot analyses with cDNA probes because RNA-RNA hybrids are much more stable than RNA-DNA hybrids (permitting hybridization under conditions of greater stingency) and the RNA can be labeled to higher specific activities (Krieg and Melton, 1987). IGF-I mRNA abundance has been determined in rat liver and muscle with this technique in numerous studies, but there are no reports in which this method has been used to determine IGF-I mRNA abundance in liver or muscle of pigs.

Orientation of the cDNA in the pGEM-1 plasmid vector was determined by BamHI restriction enzyme digestion. Digestion with BamHI resulted in a 3298 and a 147 base pair fragment (Figure 1.3) indicating that synthesis of transcripts with the SP6 promoter and SP6 polymerase would generate antisense transcripts that would be complementary to tissue IGF-I mRNA. Digestion of the recombinant plasmid DNA with EcoRI, PvuII, and SacI each resulted in expected fragment sizes. To minimize the amount of plasmid sequence that was transcribed, the recombinant plasmid was linearized with PvuII prior to transcription. The assay represented in Figure 1.4 was conducted without purifying the probe via agarose gel electrophoresis prior to hybridization to tissue RNA. This assay was performed using plasmid template DNA as a standard. Plasmid DNA containing the IGF-I cDNA insert serves as a useful standard since the antisense IGF-I RNA probe should hybridize to it in a dose-dependent manner. Presence of

Figure 1.3. Agarose gel electrophoresis of restriction enzyme digestion products. Following digestion of pGEM-1/IGF-I plasmid DNA with restriction enzymes, products were electrophoresed in 1% agarose, stained with ethidium bromide, and visualized utilizing a uv light (302 nm). Lambda and PhiX174 cut DNA were used as DNA size markers. Marker fragment sizes are indicated in base pairs (bp). The 580-bp IGF-I cDNA insert was also electrophoresed.

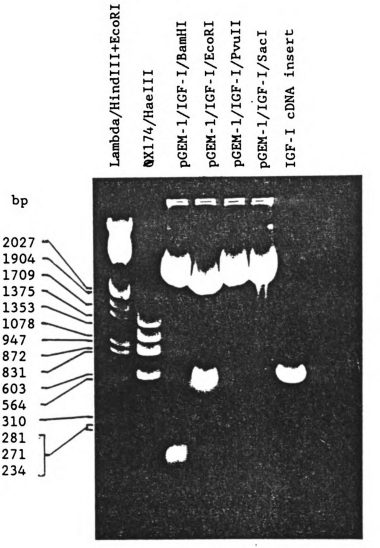

Lambda/HindIII+EcoRI
ΦX174/HaeIII
pGEM-1/IGF-I/BamHI
pGEM-1/IGF-I/EcoRI
pGEM-1/IGF-I/PvuII
pGEM-1/IGF-I/SacI
IGF-I cDNA insert
bp
2027
1904
1709
1375
1353
1078
947
872
831
603
564
310
281
271
234

Figure 1.3

Figure 1.4. Autoradiogram from a solution hybidization assay utilizing an IGF-I RNA probe contaminated with endogenous template DNA. A porcine IGF-I cDNA was utilized as a template to synthesize an antisense ^{32}P-labeled RNA probe. The probe was hybridized with 0 to 1000 pg of exogenous plasmid template DNA at 42^{o}C for 14 hours. Following hybridization, 133 units of nuclease S1 were added and samples incubated for an additional 1 hour at 37^{o}C. Protected RNA fragments were electrophoretically separated in 1.2% agarose gel containing 2.2 M formaldehyde and then subjected to autoradiography. The probe was also electrophoresed alone [Probe; 678 nucleotides (nt)in length] nuclease S1 (S1).

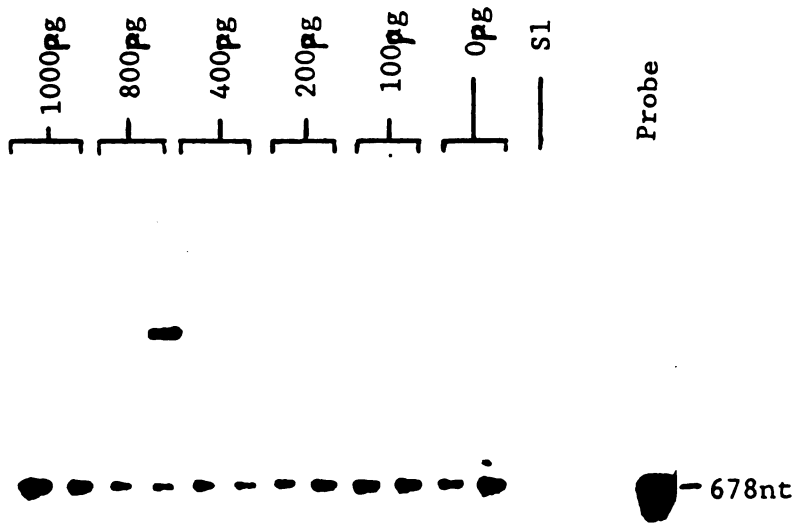


Figure 1.4

endogenous template DNA in the probe solution led to hybridization signals that appeared in reactions containing 0 ug of added DNA. The endogenous DNA rendered it impossible to accurately quantitate abundance of tissue IGF-I mRNA. Once steps were taken to remove the contaminating DNA, expected results were obtained (Figure 1.5). The probe alone appeared as a 678-nucleotide fragment and the RNA-RNA hybrids appeared as 580-base pair fragments. IGF-I mRNA was detected in porcine liver and LD muscle. The assay represented in Figure 1.5 includes electrophoresis in 8 M urea/5% acrylamide. Acrylamide was used rather than agarose in order to improve the resolution of the RNA fragments. RNA fragments less than 700 base pairs can be resolved more effectively in acrylamide than in agarose. Once the resolution was improved, hybridization to many smaller RNA species other than the 580-nucleotide mRNA was observed, indicating the need to determine optimum hybridization and nuclease digestion conditions.

Various amounts of nuclease S1 were tested to determine the amount necessary for digesting single-stranded nonhybridized labeled RNA, but an amount that would minimize degradation of RNA-RNA hybrids (Figure 1.6). When S1 was included in the assay at 266 units, the RNA hybids appeared to be partially degraded, whereas amounts less than 133 U resulted in incomplete digestion of RNA. Future assays were conducted using 133 U of nuclease S1 per

Figure 1.5. Autoradiogram from a solution hybridization assay utilizing an IGF-I RNA probe purified by gel electrophoresis. Fifty and 100 ug of RNA from liver (A) and LD muscle (C) of a fed finishing pig and from liver of a 48 hour fasted pig (B) was included in the assay. A porcine IGF-I cDNA was utilized as a template to synthesize an antisense ^{32}P-labeled RNA probe. The probe was hybridized to RNA at 42^{o}C for 14 hours. Following hybridization, 133 units of nuclease S1 were added and samples incubated for an additional 1 hour at 37^{o}C. Protected RNA fragments were electrophoretically separated in 5% acrylamide/8 M Urea gels and then subjected to autoradiography. Transfer RNA (tRNA) was included as a negative control to account for nonspecific background hybridization. The S1 nuclease-protected RNA fragments are 580 bases in length. The IGF-I probe was also included alone and is 678 bases long.

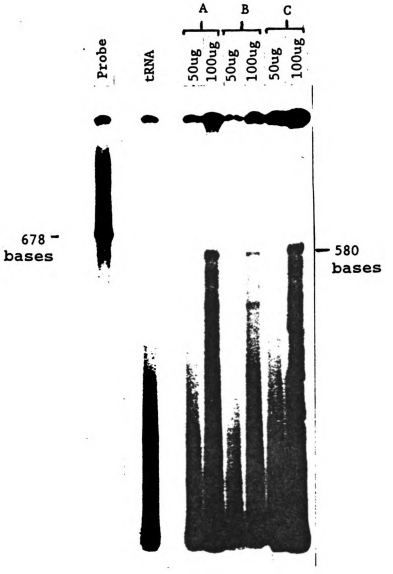

A
B
C
Probe
tRNA
50ug
100ug
50ug
100ug
50ug
100ug
678
bases
580
bases

Figure 1.5

Figure 1.6. Autoradiogram from an IGF-I solution hybridization assay in which four amounts of nuclease S1 were used following hybridization. Each hybridization reaction contained 100 ug of finishing pig liver RNA and 2 million cpm of IGF-I RNA probe. A porcine IGF-I cDNA was utilized as a template to synthesize an antisense ^{32}P-labeled RNA probe. The probe was hybridized to RNA at 42^{o}C for 14 hours. Following hybridization, 266, 133, 26.6 or 13.3 units of nuclease S1 were added and samples incubated for an additional 1 hour at 37^{o}C. Protected RNA fragments were electrophoretically separated in 5% acrylamide/8 M Urea gels and then subjected to autoradiography. The 580-base pair (bp) protected RNA fragment is shown.

Figure 1.7. Autoradiogram from an IGF-I solution hybridization assay in which hybridization was conducted with 100 ug of pig liver RNA at either 42 or 65 C. A porcine IGF-I cDNA was utilized as a template to synthesize an antisense ^{32}P-labeled RNA probe. The probe was hybridized to RNA at 42^{o} or 65^{o}C for 14 hours. Following hybridization, 133 units of nuclease S1 were added and samples incubated for an additional 1 hour at 37^{o}C. Protected RNA fragments were electrophoretically separated in 5% acrylamide/8 M Urea gels and then subjected to autoradiography. Transfer RNA (tRNA) was included as a negative control to account for nonspecific background hybridization. The S1 nuclease-protected RNA fragments are 580 bases in length. The IGF-I probe was also included alone and is 678 bases long.

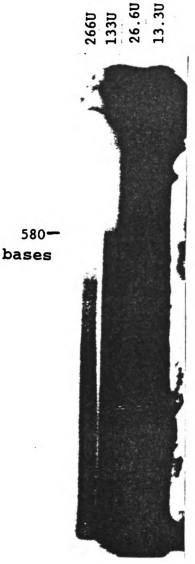


Figure 1.6

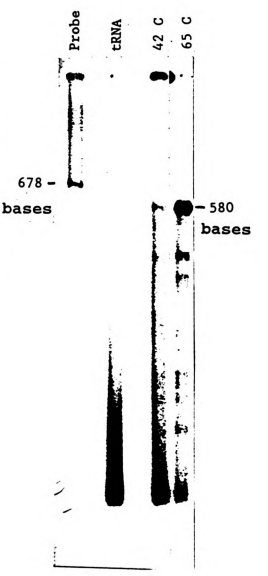


Figure 1.7

100 ug reaction. When 42° and 65°C were compared for hybridization temperatures, 65°C resulted in much less hybridization to smaller RNA fragments and greater hybridization to the 580-nucleotide species (Figure 1.7). All future assays were conducted at 65°C.

A dose-response study was conducted using 50, 75, 100, and 150 ug of liver RNA (Figure 1.8). The assay was linear within this range (Figure 1.9); therefore, future assays were conducted with 100 ug of total RNA under the optimal conditions described above. The intra- and inter-assay coefficients of variation were 2.8 and 2.7%, respectively. A detailed protocol for the assay is included in Appendix B.

The assay represented in Figure 1.8 also included RNA from liver and LD muscle of growth hormone-treated pigs (150 ug growth hormone/kg body weight daily; tissue was obtained from Dr. D. Beermann at Cornell University). After correction for nonspecific hybridization by subtraction of the signal obtained with tRNA, it was determined that growth hormone increased the abundance of IGF-I mRNA in liver and skeletal muscle by 1.4 and 1.2 times relative to controls. These results indicate that the sensitivity of the solution hybridization assay descibed above is sufficient to detect changes in IGF-I mRNA abundance in liver and skeletal muscle of finishing pigs treated with anabolic agents.

Figure 1.8. Autoradiogram from an IGF-I solution hybridization assay in which RNA from liver (LIV) and LD muscle (LD) of a control pig (C) and a growth hormone-treated pig (GH) was utilized. Fifty, 75, 100, and 150 ug of C-LIV RNA was included in the assay for examination of a dose-response relationship. A porcine IGF-I cDNA was utilized as a template to synthesize an antisense ^{32}P-labeled RNA probe. The probe was hybridized to RNA at 65^{o}C for 14 hours. Following hybridization, 133 units of nuclease S1 were added and samples incubated for an additional 1 hour at 37^{o}C. Protected RNA fragments were electrophoretically separated in 5% acrylamide/8 M Urea gels and then subjected to autoradiography. Transfer RNA (tRNA) was included as a negative control to account for nonspecific background hybridization. The S1 nuclease-protected RNA fragments are 580 bases in length. The IGF-I probe was also included alone and is 678 bases long.Transfer RNA (tRNA) was included as a negative control to account for nonspecific hybridization. The IGF-I RNA probe was included alone in the gel and is 678 nucleotides (nt) long. Protected fragments are 580 base pairs (bp) in length.

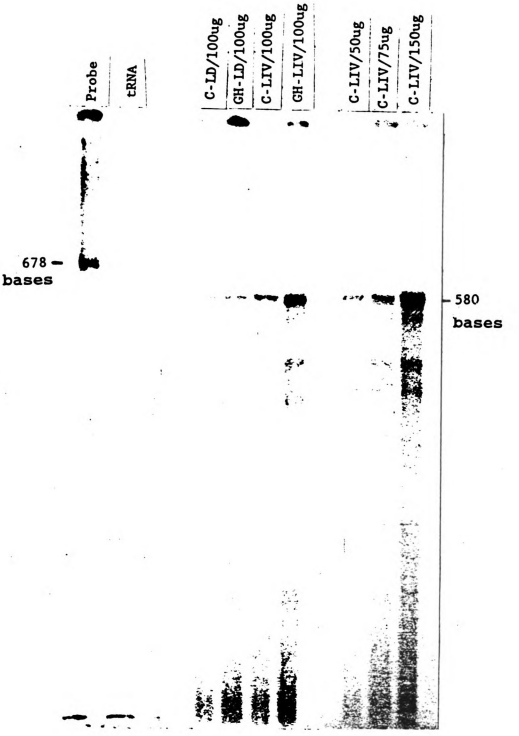

Probe
tRNA
C-LD/100ug
GH-LD/100ug
C-LIV/100ug
GH-LIV/100ug
C-LIV/50ug
C-LIV/75ug
C-LIV/150ug
678 bases
580 bases

Figure 1.8

Figure 1.9. Dose-response relationship between quantity of total RNA used and hybridization signal obtained in the solution hybridization-nuclease protection assay. A porcine IGF-I cDNA was utilized as a template to synthesize an antisense ^{32}P-labeled RNA probe. The probe was hybridized to 50, 75, 100, and 150 ug of porcine liver RNA at 65^{o}C for 14 hours. Following hybridization, 133 units of nuclease S1 were added and samples incubated for an additional 1 hour at 37^{o}C. Protected RNA fragments were electrophoretically separated in 5% acrylamide/8 M Urea gels and then subjected to autoradiography. Transfer RNA (tRNA) was included as a negative control to account for nonspecific background hybridization. The signals obtained in the 580-base pair nuclease-protected fragments were quantitated by liquid scintillation analysis. Results have been corrected for nonspecific hybridization by subtracting the signal obtained from hybridization to tRNA (y-intercept=80; slope=7.7; r squared=.978).

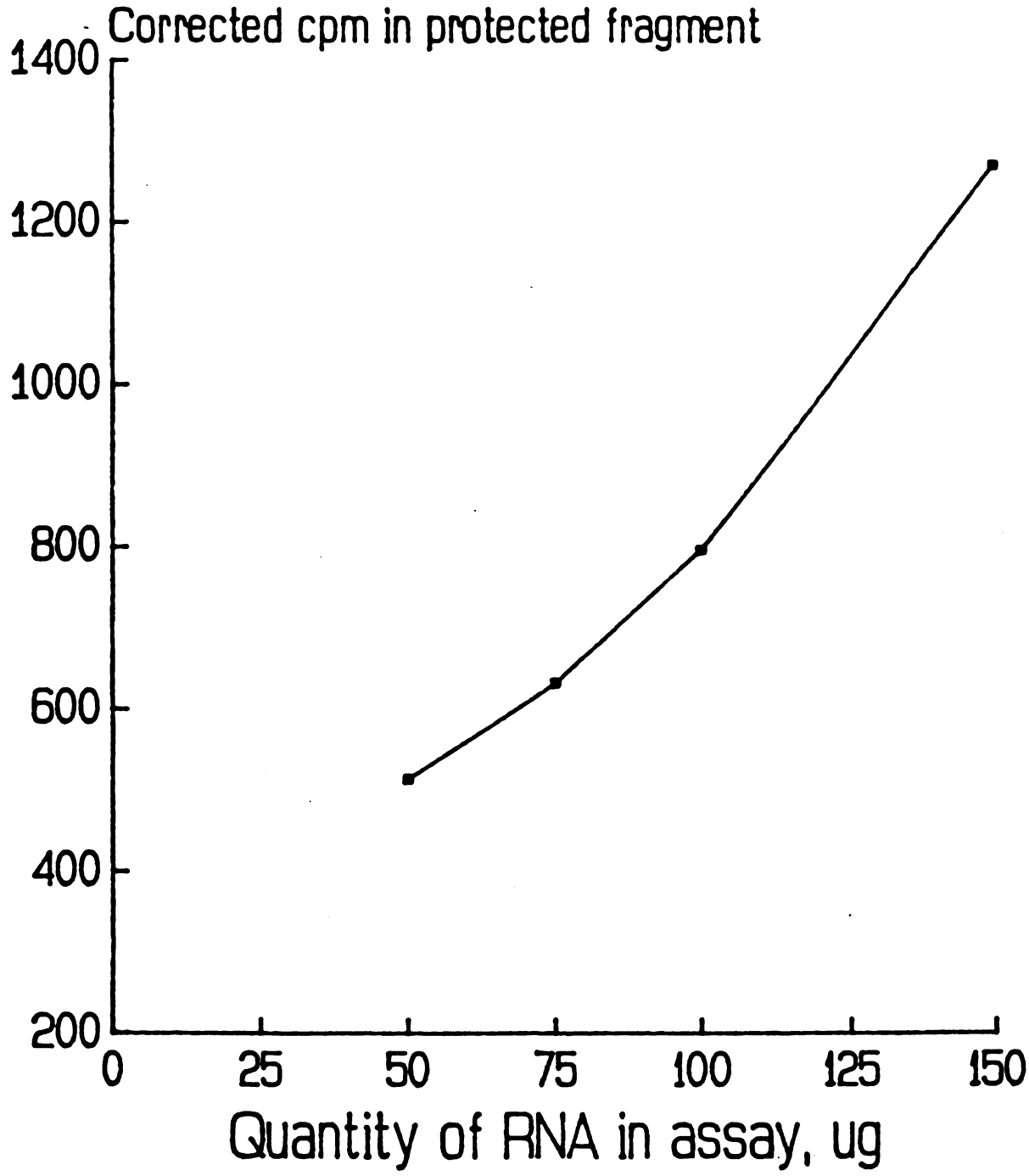

Corrected cpm in protected fragment
1400
1200
1000
800
600
400
200
0
25
50
75
100
125
150
Quantity of RNA in assay, ug

Figure 1.9

Summary

Sensitivity offered by Northern blot analyses is not sufficient to detect changes in IGF-I mRNA abundance in market weight pig liver and skeletal muscle. A sensitive solution hybridization-nuclease protection assay allowed the detection of changes in IGF-I mRNA levels in liver of market weight pigs administered growth hormone. This assay will be useful to monitor changes in abundance of IGF-I mRNA in liver and skeletal muscle of pigs administered growth hormone and beta adrenergic agonists during the finishing phase of growth.

CHAPTER 2

ADMINISTRATION OF GROWTH HORMONE TO PIGS ALTERS THE RELATIVE ABUNDANCE OF IGF-I mRNA IN LIVER AND SKELETAL MUSCLE

Abstract

Abundance of IGF-I mRNA was determined in liver and skeletal muscle of market weight crossbred barrows using a solution hybridization-nuclease protection assay. Pigs were intramuscularly administered either 50 ug recombinant porcine growth hormone (GH) per kg body weight or vehicle daily for 24 days. Pigs were fed either 14% or 20% crude protein corn-soybean meal diets. Percent muscling of carcasses from pigs administered GH was greater ($P < 0.01$) than controls. RNA was isolated from liver and longissimus dorsi muscle that was obtained during slaughter. Relative to controls, GH increased ($P < 0.05$) the abundance of liver IGF-I mRNA 2.7-fold in pigs fed the 14% protein diet and 3.0-fold in pigs fed the 20% protein diet. Muscle IGF-I mRNA abundance in GH-treated pigs was only 77% and 84% of abundance in control pigs fed the 14% and 20% protein diets, respectively ($P < 0.08$). GH increased ($P < 0.001$)

serum IGF-I concentration 1.6-fold in pigs fed the 14% protein diet and 2.0-fold in those fed the 20% protein diet. These results indicate that administration of GH to pigs influences the relative abundance of liver and skeletal muscle IGF-I mRNA and that the these alterations are dependent upon dietary protein concentration. Increased abundance of liver IGF-I mRNA and increased serum IGF-I concentrations suggests that IGF-I plays an endocrine role in mediating GH-induced muscle hypertrophy in pigs.

Introduction

Administration of porcine growth hormone to pigs increases skeletal muscle growth (Machlin, 1972; Chung et al., 1975; Etherton et al., 1987; Evock et al., 1988; Campbell et al., 1989; Smith et al., 1989a). Growth hormone-induced hypertrophy of skeletal muscle has been accompanied by increased serum or plasma insulin-like growth factor-I (IGF-I) concentrations in pigs (Chung et al., 1985; Etherton et al., 1987) and increased abundance of liver and skeletal muscle IGF-I mRNA in rats (Turner et al.,1988). Since IGF-I also increases protein synthesis when included with cultured skeletal muscle cells (Ewton and Florini, 1980; Gulve and Dice, 1989; Roe et al., 1989) and increases skeletal muscle protein synthesis when administered to dwarf mice (Pell and Bates, 1989), it is possible that IGF-I mediates the action of growth hormone on skeletal muscle growth.

Recently, Smith et al. (1989a) found that the dietary crude protein concentration had to be at least 14% in order for recombinant porcine growth hormone to increase muscle growth in finishing pigs. Dietary protein restriction (i.e., 5% crude protein in rat diets) has been demonstrated

to reduce serum or plasma IGF-I concentrations and liver IGF-I mRNA abundance in humans and rats (Prewitt et al., 1982; Isley et al., 1984; Maes et al., 1984b; Moats-Staats et al., 1989; VandeHaar et al, 1989) which suggests that dietary protein concentration may modulate the anabolic actions of growth hormone on porcine muscle growth via IGF-I.

The objectives of this study were to 1) determine whether administration of porcine growth hormone to finishing pigs increases the abundance of IGF-I mRNA in liver and skeletal muscle and 2) examine the effects of dietary protein concentrations on abundance of IGF-I mRNA.

Materials and Methods

Animals, Care and Treatments. Thirty-two crossbred barrows were divided into two blocks based upon initial body weight. Average initial body weight of pigs in the heavy and light blocks were 81.7 and 66.8 kg, respectively. Within each block, pigs were assigned to four pens so that minimal differences in body weight existed among pens. All pigs were fed a 14% crude protein corn-soybean meal diet for 11 days to allow the pigs to become acclimated to the facilities and to ensure that pigs were in good health. Temperature of the swine facility was maintained between 12° and 18°C throughout the entire experimental period. Following the preliminary feeding period, four treatments were randomly assigned to the four pens of each block so that there was a total of two pens (8 pigs) per treatment. The four treatments were: 1) somatotropin plus a 14% crude protein corn-soybean meal diet (GH14), 2) injection vehicle plus a 14% crude protein corn-soybean meal diet (CO14), 3) somatotropin plus a 20% crude protein corn-soybean meal diet (GH20), and 4) injection vehicle plus a 20% crude protein corn-soybean meal diet (CO20). Diet compositions are shown in Table 2.1. Somatotropin was a recombinant

Table 2.1. Composition and calculated analyses of diets fed to crossbred barrows

Ingredients	14% CP	20% CP
	---% of dry matter--	
Corn	80.75	63.75
Soybean meal (44% CP)	16.00	33.00
Mono-di-calcium phos.	1.00	1.00
Calcium carbonate	1.00	1.00
NaCl	0.25	0.25
Vitamin-mineral premix[a]	1.00	1.00
Calculated analysis		
Crude protein	14	20
L-Lysine	0.65	1.10
	-------kcal/kg------	
ME	3175	3157

[a]Premix provided per kg of diet: Vitamin A, 3,300 IU; Vitamin D3, 600 IU; riboflavin, 3.3 mg; nicotinic acid, 17.6 mg; d-pantothenic acid, 13.2 mg; choline, 110 mg; Vitamin B12, 19.8 ug; Zn, 74.8 mg; Fe, 9.4 mg; Mn, 37.4 mg; Cu, 9.9 mg; I, 0.5 mg; Se, 0.1 mg.

porcine growth hormone synthesized and generously supplied by the Upjohn Co. (Kalamazoo, MI). Somatotropin was mixed with sterile vehicle (2.5 mg/ml 0.03 M NaHCO3, 0.15 M NaCl, pH 9.5) daily and administered intramuscularly in the right side of the neck once daily (0900) at an amount equal to 50 ug/kg body weight/day. Pigs were weighed weekly to adjust somatotropin dosages and were administered the final injection on day 24 of the treatment period. Pigs were fed ad libitum and had access to water via automatic water dispensers. On day 25 of the treatment period, 24 hours after the last injection, pigs were weighed, transported to the Michigan State University Meat Laboratory, and slaughtered (under USDA Meat Inspection supervision) for collection of tissue samples and measurement of carcass variables. Muscle samples were collected from the left side of the carcass as described below. Percent carcass muscle was estimated using the right side of the carcass according to the NPPC (1983) equation. Longissimus dorsi (LD) muscle area was determined with a grid at the tenth rib on the right side of the carcass.

Tissue sample collection, RNA Isolation, and Analysis.

Following exsaguination, samples of LD muscle from the left side of the carcass and samples of liver were immediately excised and weighed. Samples were collected, cut into pieces approximately 8 cm^3, frozen by submersion in liquid nitrogen and stored at $-80^{o}C$. The remaining LD muscle on the left side of the carcass and the remaining liver was weighed in order that total left LD muscle and

liver weights could be calculated.

RNA was isolated from LD muscle and liver of four pigs per treatment (selected at random; 2 pigs/pen) as described in Chapter 1 and Appendix A.

Relative abundance of IGF-I mRNA in the liver and skeletal muscle RNA was quantitated using a sensitive solution hybridization-nuclease protection assay as described in Chapter 1 and Appendix B. An internal control hybridization standard (RNA isolated from liver of a somatotropin-treated pig) was used in all hybridization assays to aid in the normalization of hybridization data among assays. Data presented are normalized treatment means.

Abundance of beta-tubulin mRNA was also determined on the liver and skeletal muscle RNA samples by Northern blot analysis. Northern blot analysis was conducted as described by Jump et al. (1984) with the following modifications. Twelve ug aliquots of LD muscle RNA and 20 ug aliquots of liver RNA were denatured and electrophoretically separated in 1.2% agarose gels containing 2.2 M formaldehyde. RNA was transferred to nitrocellulose paper (Schleicher and Schuell, Keene, NH) with 10X SSC. Blots were prehybridized in 50% formamide, 5X SSC, 5X Denhardt's solution, 0.1% SDS, 1 mM EDTA, 50 mM sodium phosphate buffer (pH 6.5), and 0.5 mg/ml tRNA for 2 hours at 42^{o}C. The hybridization solution was identical to the prehybridization solution, except that the

hybridization solution contained only 1X Denhardt's solution and contained two million cpm of probe/ml. A mouse beta-tubulin cDNA (m-beta-5 tubulin) subcloned into the EcoRI site of pUC9 was obtained from Dr. Donald Cleveland at John Hopkins University (Sullivan and Cleveland, 1986). The cDNA was random primed (Boehringer Mannheim Biochemicals) using [^{32}P]dCTP (3000 Ci/mmol; Amersham Corp., Arlington Hts., IL) to a specific activity of 4 X 10^8 cpm/ug DNA. After hybridization at 42^{o}C for 16 hours, blots were washed with 2X SSC and 0.1% SDS at room temperature, then washed for three cycles in 0.2X SSC and 0.1% SDS at 55^{o}C. Under these hybridization conditions, the beta-tubulin cDNA hybridizes to mRNA species of 1800 and 2800 nucleotides, sizes consistent with beta-tubulin mRNA from other species (Lewis et al., 1985). Following autoradiography, hybridization of RNA to beta-tubulin cDNA was quantified by densitometry. Blots that contained liver RNA were stripped with 0.01X SSC and 0.5% SDS for 2 hours at 70^{o}C and then re-hybridized with a rat beta-actin cDNA probe under the same conditions as above, except that the blots were washed with 0.1X SSC and 0.1% SDS following hybridization. The beta-actin cDNA was obtained from Dr. Larry Kedes laboratory (University of Southern California School of Medicine) and has been characterized by P. Gunning (unpublished). Under the hybridization conditions employed above, the beta actin probe hybridizes to a 2100-nucleotide mRNA species. Later, blots were stained with methylene blue as described by Maniatis et al. (1982) to

visualize the 18S and 28S ribosomal bands in order to confirm that equivalent amounts of RNA were loaded onto gels and that the transfer efficiency was similar among lanes.

Blood was collected from two pigs of each treatment two hours after the final injections on day 24 of the treatment period. Serum was harvested, stored at -20°C, and analyzed for growth hormone concentration as described by Smith and Kasson (1990) and for IGF-I concentration as described by Dahl et al. (1990).

Statistical Analysis. All data were statistically analyzed using the General Linear Models Procedure of SAS (1987). A randomized complete block design was utilized. Effects of block, dietary crude protein concentration, growth hormone, and the interactions were tested using variation among pens (i.e., block X protein X growth hormone) as the error term. Due to the low number of pens per treatment and degrees of freedom for the error term, variation among pens was pooled with variation among pigs and used as the error term in cases in which the F statistic for variation for pens was less than $2F_{0.50}$ as outlined by Gill (1989); however, there is potential for bias from pooling the sum of squares (and corresponding degrees of freedoms) for pens and animals. Contrasts between means were analyzed by Bonferonni t-tests as outlined by Gill (1978).

Results and Discussion

In order to ensure that the barrows selected for the present study were healthy, all pigs were fed a conventional 14% crude protein finishing diet for 11 days prior to the treatment period. Due to the large range in initial body weights among the pigs, two blocks were formed, a heavy block which consisted of pigs with an average weight of 81.7 kg, and a light block which consisted of pigs with an average body weight of 66.8 kg. Following the 11-day preliminary feeding period, all pigs were observed to be in excellent health. Average body weights on day 1 of the treatment period for pigs selected for RNA analyses were 90.6 and 76.7 kg for the heavy and light blocks, respectively. Respective slaughter weights on day 24 of the treatment period were 109.3 and 98.7 kg. Weight gains during the treatment period, liver weights at slaughter, and various carcass measurements are presented in Table 2.2. Initial body weight (block factor in the statistical model) had no statistically significant effect on any variable, except for LD muscle weight. The mean weight of LD muscle from pigs of the heavy block was 11.4% greater ($P < 0.01$) than LD muscle from pigs of the light

Table 2.2. Effects of growth hormone for 24 days on average daily gains, liver weights, and carcass measurements of pigs fed 14% and 20% crude protein diets

	Treatment[a]				
Variable	CO14	GH14	CO20	GH20	SEM
Avg. daily gain, g/d	885	819	814	914	101
Liver weight, g[b]	1573	1875	1726	2082	127
Right LD area, cm^2	28.8	27.9	27.4	31.7	1.9
Left LD weight., g	2240	2056	2162	2355	121
Carcass muscle, %[c]	54.4	55.7	52.7	56.7	0.9

[a]CO=control; GH=growth hormone; 14=14% CP; 20=20% CP.
[b]GH effect ($P < 0.025$).
[c]GH effect ($P < 0.01$).

block. Estimated percent carcass muscle was 5% greater ($P < 0.01$) in pigs administered growth hormone than in control pigs. Greater percent carcass muscle in treated pigs is due to both an increase in muscle mass and a decrease in backfat deposition. Stimulation of muscle growth may have been greater if a larger dose of growth hormone had been administered. Several workers (reviewed by Steele et al., 1989) have conducted dose-response experiments in growing pigs and have found optimum dosages on most carcass variables from 70 to 120 ug/kg body weight/day. The improvement in percent carcass muscle, LD muscle area, and LD muscle weight between growth hormone-treated pigs and control pigs tended to be greater in the group of pigs fed the 20% protein diet (Table 2.2). This finding was not surprising since Smith et al. (1989a) reported that the dietary crude protein concentration had to be greater than 14% in order for growth hormone to improve growth and feed efficiency. Smith (1989a) observed reductions in average daily gain and increased feed to gain ratios in growth hormone-treated pigs fed diets containing only 14% crude protein. Similar trends were observed in the present study for average daily gain, LD muscle area, and LD muscle weight; however, these changes were not statistically significant in the present experiment. Newcombe et al. (1988) reported that in market weight pigs administered 3 mg of recombinant porcine growth hormone per day the LD muscle area increased linearly when dietary crude protein concentration was increased from 14% to 26%. Furthermore,

Steele et al. (1989) found that the improvement in daily gain, feed:gain and LD muscle area resulting from the administration of growth hormone to pigs fed an 11% crude protein diet was only 50% of the improvement observed in treated pigs fed diets containing at least 15% protein.

Growth hormone also increased ($P < 0.025$) mean liver weight by 20% relative to controls. Administration of growth hormone has been demonstrated to increase liver weight in pigs (Etherton et al., 1987), steers (Eisemann et al., 1989) and dwarf mice (Pell and Bates, 1989). Hypersomatotropism has been shown to increase the weights of several tissues and organs in rats (Turner et al., 1986).

Autoradiograms from the IGF-I solution hybridization assays are shown in Figure 2.1. Hybridization of the 678-nucleotide [32P]-labeled IGF-I probe to tRNA was negligible. Hybridization to liver and muscle RNA resulted in 580-base pair protected fragments. Negligible hybridization to other mRNA species occurred under the conditions of the assay. To quantitate relative adundance of IGF-I mRNA, the 580-base pair fragments were excised from the gels and subjected to liquid scintillation analysis. Relative to controls, growth hormone increased ($P < 0.05$) the abundance of liver IGF-I mRNA 2.7-fold in pigs fed the 14% protein diet and 3.0-fold in pigs fed the 20% protein diet (Table 2.3). Although the induction of IGF-I mRNA tended to be greater in pigs fed the high

Figure 2.1. Autoradiograms of IGF-I mRNA abundance in liver and LD muscle from growth hormone-treated (GH) and control (CO) pigs fed 14% crude protein (14) or 20% crude protein (20) diets. Liver and muscle RNA was isolated and analyzed via a solution hybridization-nuclease protection assay. A porcine IGF-I cDNA was utilized as a template to synthesize an antisense ^{32}P-labeled RNA probe. The probe was hybridized to 100 ug of RNA at 65^{o}C for 14 hours. Following hybridization, 133 units of nuclease S1 were added and samples incubated for an additional 1 hour at 37^{o}C. Protected RNA fragments were electrophoretically separated in 5% acrylamide/8 M Urea gels and then subjected to autoradiography. Transfer RNA (tRNA) was included as a negative control to account for nonspecific background hybridization. The S1 nuclease-protected RNA fragments are 580 base pairs (bp) in length. RNA from liver of a pig from GH20 was included in all hybridization assays as an internal standard (Std.) to aid in the normalization of data among assays. Each lane represents one pig (n=4 pigs/treatment).

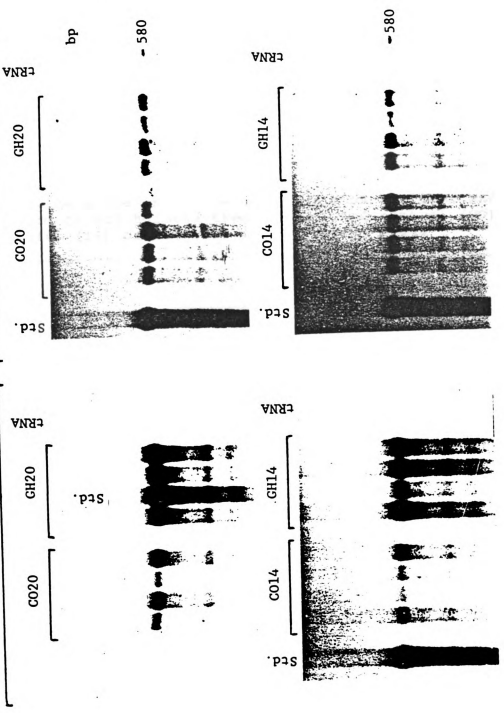


Figure 2.1

Table 2.3. Relative abundance of IGF-I mRNA in liver and LD muscle from growth hormone-treated (GH) and control (CO) pigs fed 14% (14) or 20% (20) crude protein diets[1]

	Treatment				
Variable	CO14	GH14	CO20	GH20	SEM
Liver IGF-I mRNA[a]	0.215	0.582	0.241	0.721	0.122
LD IGF-I mRNA[b]	0.304	0.234	0.276	0.230	0.029

[1]Abundance is expressed relative to an internal control hybridization standard (RNA from liver of a growth hormone-treated pig).
[a]GH effect ($P < 0.05$).
[b]GH effect ($P < 0.08$).

protein diet, statistical significance could not be detected. Protein deficient diets (5% crude protein) have been demonstrated to reduce liver IGF-I mRNA abundance in rats (Moats-Staats et al., 1989; VandeHaar et al., 1989); however, the 14% crude protein diet used in this experiment is not considered to be a protein-deficient diet for market weight pigs. A diet with less than 14% protein (e.g., 5% crude protein) presumably would have been associated with lower abundance of liver IGF-I mRNA and may have prevented the growth hormone-induced increase in IGF-I mRNA.

Muscle IGF-I mRNA abundance in growth hormone-treated pigs was only 77% and 84% of the abundance in control pigs fed the 14% and 20% protein diets, respectively (Table 2.3). Since abundance data are reported relative to total RNA, then an increase in ribosomal RNA relative to mRNA could contribute to the decrease in abundance of IGF-I mRNA. Muscle RNA content has not been quantitated in this study, but growth hormone has been demonstrated to increase muscle RNA content in steers (Eisemann et al., 1989). Despite this possibility, decreased ($P < 0.08$) abundance of IGF-I mRNA in muscle was not expected since Turner et al. (1988) observed increases in IGF-I mRNA abundance in both liver and skeletal muscle of rats implanted with growth hormone-secreting GH_3 cells. This difference could be a result of greater concentrations of serum growth hormone in rats implanted with GH_3 cells. Serum concentrations of growth hormone in these treated rats are greater than 2000 ng/ml compared to normal concentrations of 10 to 100 ng/ml

(Turner et al, 1986). Serum growth hormone was quantitated in 8 pigs (2 pigs/treatment) of the present experiment 2 hours after injection and was found to be 69.9 ng/ml in growth hormone-treated pigs relative to 4.1 ng/ml in controls (Figure 2.2). To briefly address the question of whether greater amounts of growth hormone may have increased muscle IGF-I mRNA abundance, RNA was isolated from liver and LD muscle of a market weight pig administered 150 ug growth hormone/kg body weight daily (three times more than that administered to the pigs of the present study) and a control pig from a study conducted at Cornell University (tissue was obtained from Dr. D. Beermann at Cornell University). Relative to the control, abundance of IGF-I mRNA was 1.4 times greater in liver and 1.2 times greater in LD muscle of the treated pig. Although these results suggest that larger doses of growth hormone may increase muscle IGF-I mRNA abundance, such interpretation is highly speculative due to the limited amount of data.

Turner et al. (1988) did not examine muscle IGF-I mRNA abundance until 40, 60, and 80 days following implantation of the GH_3 cells. In the present study, porcine IGF-I mRNA abundance was examined after a 24-day treatment period in tissues that were collected 24 hours after the final injection of growth hormone. Isgaard et al. (1989) conducted a time-course study in which IGF-I mRNA abundance was examined in gastrocnemius muscle of hypophysectomized

Figure 2.2. Concentrations of growth hormone (GH; open bars) and IGF-I (hatched bars) in serum from growth hormone-treated (GH) and control (C) pigs fed 14% CP (14) or 20% CP (20) diets. Each bar represents the mean of two pigs. Standard errors of individual treatment means are indicated by the error bars. Pooled standard errors of the means are 2.73 and 30.6 ng/ml for GH and IGF-I, respectively. GH effect for both serum GH and serum IGF-I ($P < 0.01$).

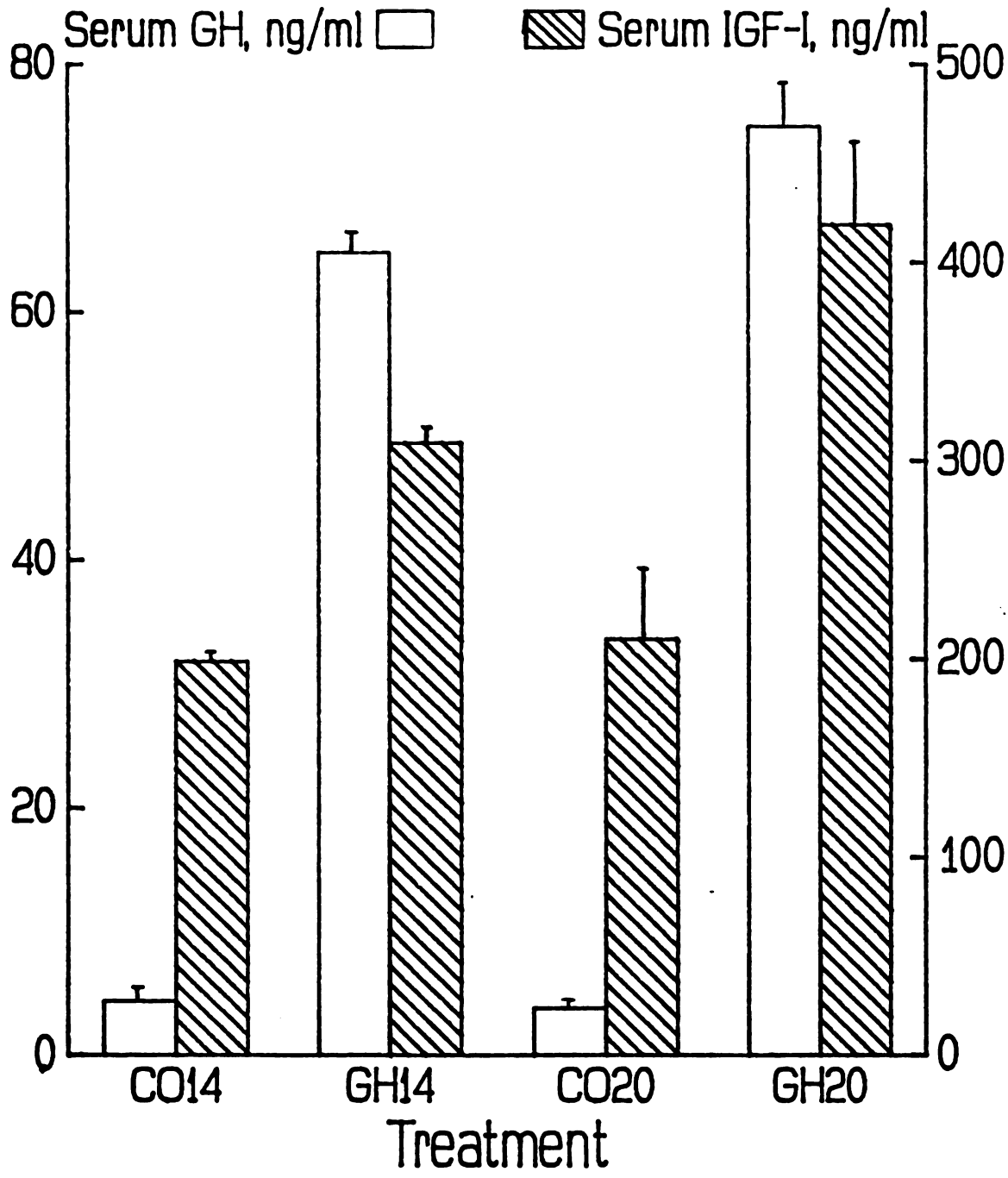

Serum GH, ng/ml
Serum IGF-I, ng/ml
80
60
40
20
0
500
400
300
200
100
0
CO14
GH14
CO20
GH20
Treatment

Figure 2.2

rats at 0, 1, 3, 6, 12, and 24 hours after a single injection of growth hormone. Maximum increases in IGF-I mRNA abundance were noted between 6 and 12 hours after injection. Abundance at 24 hours was approximately 50% of the maximum abundance. The rats of Isgaard et al. (1989) were hypophysectomized, whereas intact barrows were used in the present study. The results of these studies emphasize the need to include measurements of IGF-I mRNA abundance at several timepoints in growth hormone studies.

Growth hormone increased ($P < 0.001$) serum IGF-I concentration 1.6-fold in pigs fed the 14% protein diet and 2.0-fold in pigs fed the 20% protein diet (Figure 2.2). These increases are consistent with those observed by Etherton et al. (1987) and Owens et al. (1990) when growing pigs were administered growth hormone. This increased serum IGF-I concentration in growth hormone-treated pigs is most likely due to the increased synthesis of liver IGF-I. Although the increased abundance of liver IGF-I mRNA cannot necessarily be interpreted as an increase in IGF-I synthesis, it would seem reasonable to suggest that this increased message resulted in the increased serum IGF-I concentration. This increased serum IGF-I may act in an endocrine manner in which it is transported to the skeletal muscle where it acts to increase protein synthesis. Furthermore, the increased serum IGF-I may have been responsible for the decreased abundance of IGF-I mRNA in the LD muscle via a feedback inhibition mechanism. Increased serum IGF-I concentrations may reduce the

synthesis of muscle IGF-I by decreasing muscle IGF-I mRNA abundance. Whether these changes in IGF-I mRNA abundance are due to changes in transcription rates, processing of mRNA, or mRNA stability is unknown. Mathews et al. (1986) have reported that administration of growth hormone to growth hormone-deficient mice increases liver IGF-I transcription, but whether this occurs in nonhypophysectomized barrows is unknown. It is also unknown whether administering growth hormone to pigs alters the size distribution of liver IGF-I mRNA species as has been reported in mice (Mathews et al., 1986) and rats (Hepler et al., 1989). The solution hybridization assay utilized in this study does not permit quantitation of the various IGF-I mRNA species.

The role of IGF-binding proteins in regulating IGF-I action is unclear. Six IGF-binding proteins have been identified in porcine serum and at least two forms have been found secreted by muscle cell lines (McCusker et al., 1989a and 1989b). Since these binding proteins are regulated hormonally, nutritionally, and developmentally (McCusker et al., 1989b), it is possible that they play major roles in mediating the actions of IGF-I in growth hormone-induced muscle hypertrophy. Furthermore, IGF-binding proteins may serve as a mechanism by which dietary protein concentrations modulate growth hormone actions on skeletal muscle.

To determine whether the changes in IGF-I mRNA

abundance in the liver and LD muscle were a result of a specific effect of growth hormone on IGF-I mRNA or a generalized effect of growth hormone on tissue hypertrophy and increases many mRNAs, liver and LD muscle RNA were subjected to Northern blot analyses for determination of beta-tubulin and beta-actin mRNA abundance (Figure 2.3). Beta-tubulin and beta-actin are constitutive proteins that act in concert and contribute to the cytoskeletal structure of eukaryotic cells (Sullivan and Cleveland). Abundance of beta-actin mRNA was not determined in the skeletal muscle RNA due to the low abundance of beta-actin mRNA and cross-hybridization of the beta-actin cDNA probe to alpha-actin mRNA, an extremely abundant message in porcine skeletal muscle. Abundance of liver beta-tubulin and beta-actin mRNA and LD beta-tubulin mRNA was not altered by growth hormone in pigs fed the 20% protein diet; however, within the 14% protein diet, abundance of liver beta-tubulin and beta-actin mRNA was 1.9- and 1.8-fold greater ($P < 0.05$), respectively, in pigs administered growth hormone than in controls. Abundance of LD beta-actin mRNA was 1.5-fold greater ($P < 0.05$) in pigs administered growth hormone than in controls (Table 2.4). Examination of Northern blots following methylene blue staining indicated that transfer of RNA among lanes was similar (Figure 2.4). These results indicate that growth hormone may alter the abundance of several mRNAs and that this effect is dependent upon dietary protein concentration. The effect of growth hormone on muscle growth appeared to be different in pigs

Figure 2.3. Autoradiograms from Northern blot analyses of beta-tubulin and beta-actin mRNA abundance in liver and LD muscle from growth hormone-treated (GH) and control (CO) pigs fed 14% crude protein (14) or 20% crude protein (20) diets. Twelve ug of muscle RNA and 20 ug of liver RNA were denatured and electrophoretically separated in 1.2% agarose gels containing 2.2 M formaldehyde and then transferred to nitrocellulose. Blots were prehybridized at 42^{o}C for 2 hours in 50% formamide, 5X SSC, 5X Denhardt's solution, 0.1% SDS, 1 mM EDTA, 50 mM sodium phosphate buffer (pH 6.5), and 0.5 mg/ml tRNA. RNA was hybridized with beta-tubulin or beta-actin cDNA probes at 42^{o}C for 16 hours. Composition of the hybridization solution was identical to the prehybridization solution except that the hybridization solution contained only 1X Denhardt's and contained two million cpm of probe/ml. Following hybridization, blots were washed with three cycles of 0.2X SSC and 0.1% SDS at 55^{o}C and then subjected to autoradiography. Visualization of 18S and 28S RNA following methylene blue staining of the Northern blots is also shown. Each lane represents one pig (n=4 pigs/treatment).

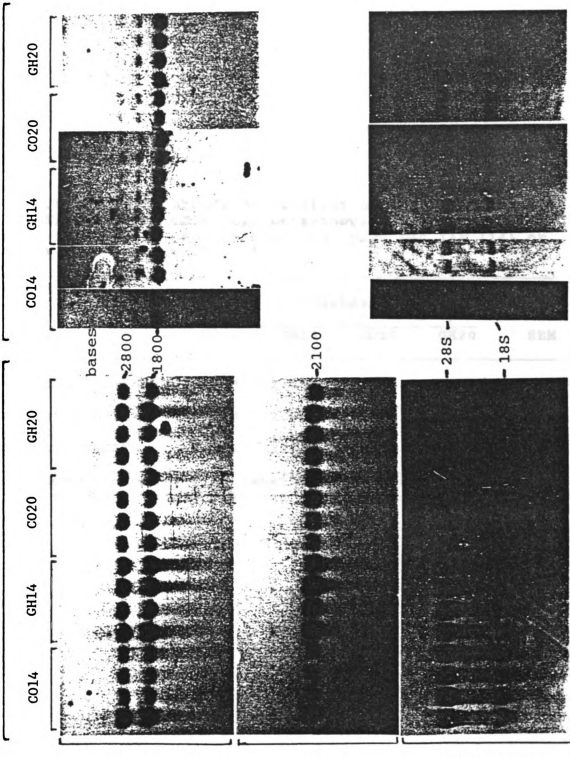

LIVER
LD MUSCLE
CO14
GH14
CO20
GH20
CO14
GH14
CO20
GH20
bases
2800
1800
2100
28S
18S
BETA-TUBULIN
BETA-ACTIN
RNA STAINED

Figure 2.3

Table 2.4. Relative abundance of liver beta-tubulin and beta-actin mRNA and LD muscle beta-tubulin mRNA in growth hormone-treated (GH) and control (CO) pigs fed 14% (14) or 20% (20) crude protein diets[1]

	Treatment				
Variable	CO14	GH14	CO20	GH20	SEM
Liver tubulin mRNA[a]	0.548	1.065	0.558	0.607	0.147
Liver actin mRNA[a]	1.280	2.282	1.076	1.120	0.174
LD tubulin mRNA[a]	1.079	1.610	1.373	1.192	0.135

[1]Abundance is expressed in densitometer units.
[a]Within the 14% protein diet, GH effect ($P < 0.05$).

fed the 14% protein diet than in pigs fed the 20% protein diet (Table 2.2) in that growth hormone tended to improve muscle growth in pigs fed the 20% protein diet, but was detrimental to muscle growth in pigs fed the 14% protein diet. Since expression of beta-tubulin is developmentally regulated in a variety of tissues, including liver and skeletal muscle (Lewis et al., 1985; Wang et al., 1986), these differential changes in muscle growth may explain the difference in beta-tubulin and beta-actin expression among the two groups of pigs. In a study by Murphy et al. (1987b) in which effects of estrogen on IGF-I expression was studied in rats, beta-actin mRNA abundance was used as a constitutive marker. Estrogen was found to increase the relative abundance of beta-actin mRNA. These studies demonstrate that perturbations in hormonal status to alter tissue growth results in changes in abundance of several mRNA species.

Summary

Administration of 50 ug recombinant porcine somatotropin/kg body weight/day to market weight pigs for 24 days increased abundance of liver IGF-I mRNA and decreased abundance of skeletal muscle IGF-I mRNA. These changes appeared to be influenced to some degree by dietary protein concentration. Greater abundance of liver IGF-I mRNA and serum IGF-I concentrations in growth hormone-treated pigs in this study would support an endocrine role for IGF-I in growth hormone-induced muscle growth. Since IGF-I has been demonstrated to increase muscle protein synthesis when administered to mice (Pell and Bates, 1989), it is possible that the increased circulating levels of IGF-I may act on the skeletal muscle to increase protein synthesis. Expression of IGF-I needs to be examined in muscle and liver at various times following administration of different doses of growth hormone so that the role of IGF-I in growth hormone-induced muscle growth can be more completely characterized.

CHAPTER 3

EFFECTS OF THE PHENETHANOLAMINE, RACTOPAMINE, ON THE RELATIVE ABUNDANCE OF IGF-I mRNA IN LIVER AND SKELETAL MUSCLE OF PIGS

Abstract

Relative abundance of liver and skeletal muscle IGF-I mRNA was determined in crossbred barrows (average body weight 93.6 kg) that were fed a 16% crude protein corn-soybean meal diet supplemented with 0 or 20 ppm of ractopamine for 28 days. Carcasses from pigs fed ractopamine had a 26% greater ($P < 0.05$) mean longissimus dorsi muscle (LD) area and a 19% greater ($P < 0.001$) mean LD muscle weight than controls. RNA was isolated from liver and LD muscle that was obtained during slaughter and used in a solution hybridization-nuclease protection assay to determine the relative adundance of IGF-I mRNA. Ractopamine had no apparent effect ($P > 0.20$) on liver or muscle IGF-I mRNA abundance. Although liver and skeletal muscle both synthesize IGF-I, ractopamine appears to have little effect on IGF-I synthesis at the mRNA level after a 28-day feeding period in market weight pigs.

Introduction

The phenethanolamine, ractopamine, increases muscle growth in market weight pigs. Bergen et al. (1989) have previously demonstrated that ractopamine increases the fractional rate of skeletal muscle protein synthesis. Increases in fractional synthesis rates of alpha-actin synthesis were accompanied by increases in the abundance of alpha-actin mRNA, indicating that ractopamine increases actin synthesis pretranslationally (Helferich et al., 1990). Insulin-like growth factor-I (IGF-I) may mediate these actions of ractopamine, since IGF-I increases protein synthesis and decreases protein degradation in cultured muscle cells (Ewton and Florini, 1980; Gulve and Dice, 1989; Roe et al., 1989), and it increases skeletal muscle protein synthesis when administered to dwarf mice (Pell and Bates, 1989). Furthermore, growth hormone-induced hypertrophy of skeletal muscle is accompanied by increases in liver and muscle IGF-I mRNA abundance in rats (Turner et al., 1988). Ractopamine may act on the liver to increase the synthesis of IGF-I. Liver IGF-I may then act on skeletal muscle in an endocrine manner to increase protein synthesis. Alternatively, ractopamine may increase

synthesis of skeletal muscle IGF-I which may act in a paracrine or autocrine manner to increase skeletal muscle growth. The objective of this study was to determine whether ractopamine increases the abundance of IGF-I mRNA in liver and skeletal muscle of pigs.

Materials and Methods

Animals, Care and Treatments. Twelve crossbred pigs with an average initial body weight of 72 kg were randomly assigned to four pens. Two dietary treatments were randomly assigned to the four pens so that there were two pens per treatment. The treatments were 1)16% crude protein corn-soy diet (Table 3.1) and 2)16% crude protein corn-soy diet supplemented with 20 ppm of the phenethanolamine, ractopamine {1-[4-hydroxyphenyl]-2-[1 methyl-3 (4-hydroxyphenyl) propylamino] ethanol; generously provided by Eli Lilly and Co., Indianapolis, IN). Pigs were fed ad libitum and had access to water via automatic water dispensers. On day 28 of the feeding period, pigs were slaughtered at the Michigan State University Meat Laboratory (under USDA Meat Inspection supervision) for collection of tissues and measurements of carcass variables. Muscle samples were collected from the left side of the carcass as described below. Longissimus dorsi (LD) muscle area was determined with a grid at the tenth rib on the right side of the carcass.

Table 3.1. Composition and calculated analyses of diet fed to barrows

Ingredients	% of dry matter
Corn	79.2
Soybean meal (48% CP)	17.9
Calcium phoshate (dibasic)	1.0
Calcium carbonate	0.9
NaCl	0.5
Vitamin-mineral premix[a]	0.5
Calculated analysis	
Crude protein	16
L-Lysine	0.70
	kcal/kg
ME	3185

aPremix provided per kg of diet: Vitamin A, 3,300 IU; Vitamin D3, 600 IU; riboflavin, 3.3 mg; nicotinic acid, 17.6 mg; d-pantothenic acid, 13.2 mg; choline, 110 mg; Vitamin B12, 19.8 ug; Zn, 74.8 mg; Fe, 9.4 mg; Mn, 37.4 mg; Cu, 9.9 mg; I, 0.5 mg; Se, 0.1 mg.

Tissue sample collection, RNA Isolation, and Analysis.

Following exsanguination, samples of LD muscle from the left side of the carcass and samples of liver were immediately excised and weighed. Samples of each were rapidly cut into pieces approximately 8 cm^3, frozen by submersion in liquid nitrogen and stored at -80°C. The remaining LD muscle on the left side of the carcass and the remaining liver was weighed in order that total left LD muscle and liver weights could be calculated.

RNA was isolated from LD muscle and liver of all pigs as described in Chapter 1 and Appendix A.

Relative abundance of IGF-I mRNA in liver and skeletal muscle RNA was quantitated using a sensitive solution hybridization-nuclease protection assay as described in Chapter 1 and Appendix B. An internal control hybridization standard (RNA isolated from liver of a somatotropin-treated pig) was used in all hybridization assays to aid in the normalization of hybridization data among assays. Data presented are normalized treatment means.

Abundance of beta-tubulin mRNA was also determined in the liver and skeletal muscle RNA samples by Northern blot analysis as described in Chapter 2. Following Northern analysis, blots were stained with methylene blue as described by Maniatis et al. (1982) to visualize the 18S and 28S ribosomal bands in order to confirm that equivalent

amounts of RNA were loaded onto gels and that the transfer efficiency was similar among lanes.

Statistical Analysis. All data were statistically analyzed using the General Linear Models Procedure of SAS (1987). Effects of ractopamine were tested using variation among pens (i.e., pens within treatment) as the error term. Due to the low number of pens per treatment and degrees of freedom for the error term, variation among pens was pooled with variation among pigs and used as the error term in cases in which the F statistic for variation for pens was less than $2F_{0.50}$ as outlined by Gill (1989); however, there is potential for bias from pooling the sum of squares (and corresponding degrees of freedoms) for pens and animals.

Results and Discussion

Administration of ractopamine for 28 days increased muscle growth as assessed by a 26% greater ($P < 0.05$) mean LD muscle area and a 19% greater ($P < 0.001$) mean LD muscle weight in barrows fed 20 ppm ractopamine than in those fed the basal control diet (Table 3.2). Accompanying the increased muscle mass in treated pigs was a 41% increase ($P < 0.01$) in average daily gain. Slaughter weights were 97.6 and 89.5 kg for the ractopamine-fed and control pigs, respectively. These changes are consistent with those observed by Bergen et al. (1989) in which market weight pigs were fed ractopamine for up to 42 days. Bergen et al. (1989) demonstrated via [^{14}C] tyrosine continuous infusions that the increased muscle mass in pigs fed ratopamine was due at least in part to an increased fractional rate of skeletal muscle protein synthesis. Furthermore, Helferich et al. (1990) has recently reported an increased fractional synthesis rate of skeletal muscle alpha actin in pigs fed ractopamine. The increase in actin synthesis was accompanied by increased abundance of skeletal muscle alpha actin mRNA indicating that ractopamine induces actin synthesis pretranslationally. Utilizing in vitro

Table 3.2. Effects of ractopamine for 28 days on skeletal muscle and body weight gains of crossbred pigs

Variable	Treatment		SEM
	Control	Ractopamine	
Right LD area, cm^2[a]	29.5	37.2	1.3
Left LD weight, g[b]	1842	2192	43.8
Average daily gain, g/d[c]	636	895	129

[a]Treatment effect ($P < 0.05$).
[b]Treatment effect ($P < 0.001$).
[c]Treatment effect ($P < 0.01$).

translation assays, Helferich et al. (1990) have also demonstrated that ractopamine induces the synthesis of other myofibrillar proteins at the pretranslational level of regulation. Skjaerlund et al. (1989) have examined abundance of skeletal muscle alpha actin mRNA in LD muscle of pigs after a 14-, 28-, and 42-day feeding period. Maximum increases in the relative abundance of alpha-actin mRNA occurred at the 28-day time point, which corresponded to the time of the maximum increase in muscle mass.

Autoradiograms from the IGF-I solution hybridization assays are shown in Figure 3.1. Hybridization of the 678-nucleotide [^{32}P]-labeled IGF-I probe to tRNA was negligible. Hybridization to liver and muscle RNA resulted in 580-base pair protected fragments. Negligible hybridization to other mRNA species occurred under the conditions of the assay. To quantitate relative adundance of IGF-I mRNA, the 580-base pair fragments were excised from the gels and subjected to liquid scintillation analysis. Ractopamine had no effect ($P > 0.20$) on IGF-I mRNA abundance in either liver or LD muscle (Table 3.3). Abundance of beta-tubulin mRNA was determined in liver and LD muscle RNA via Northern analyses and was not altered by administration of ractopamine (Figure 3.2 and Table 3.3) indicating that lack of an effect of ractopamine on message abundance was not limited to IGF-I mRNA. Staining of Northern blots with methylene blue confirmed that the transfer of RNA among all lanes was similar (Figure 3.2).

Lack of an effect of ractopamine on liver IGF-I mRNA

Figure 3.1. Autoradiograms of IGF-I mRNA abundance in liver and LD muscle from ractopamine-treated (R) and control (C) pigs. Liver and muscle RNA was isolated and analyzed via a solution hybridization-nuclease protection assay. A porcine IGF-I cDNA was utilized as a template to synthesize an antisense ^{32}P-labeled RNA probe. The probe was hybridized to 100 ug of RNA at 65^{o}C for 14 hours. Following hybridization, 133 units of nuclease S1 were added and samples incubated for an additional 1 hour at 37^{o}C. Protected RNA fragments were electrophoretically separated in 5% acrylamide/8 M Urea gels and then subjected to autoradiography. Transfer RNA (tRNA) was included as a negative control to account for nonspecific background hybridization. The S1 nuclease-protected RNA fragments are 580 bases in length. The IGF-I probe was also included alone and is 678 bases long. Each lane represents one pig (n=6 pigs/treatment).

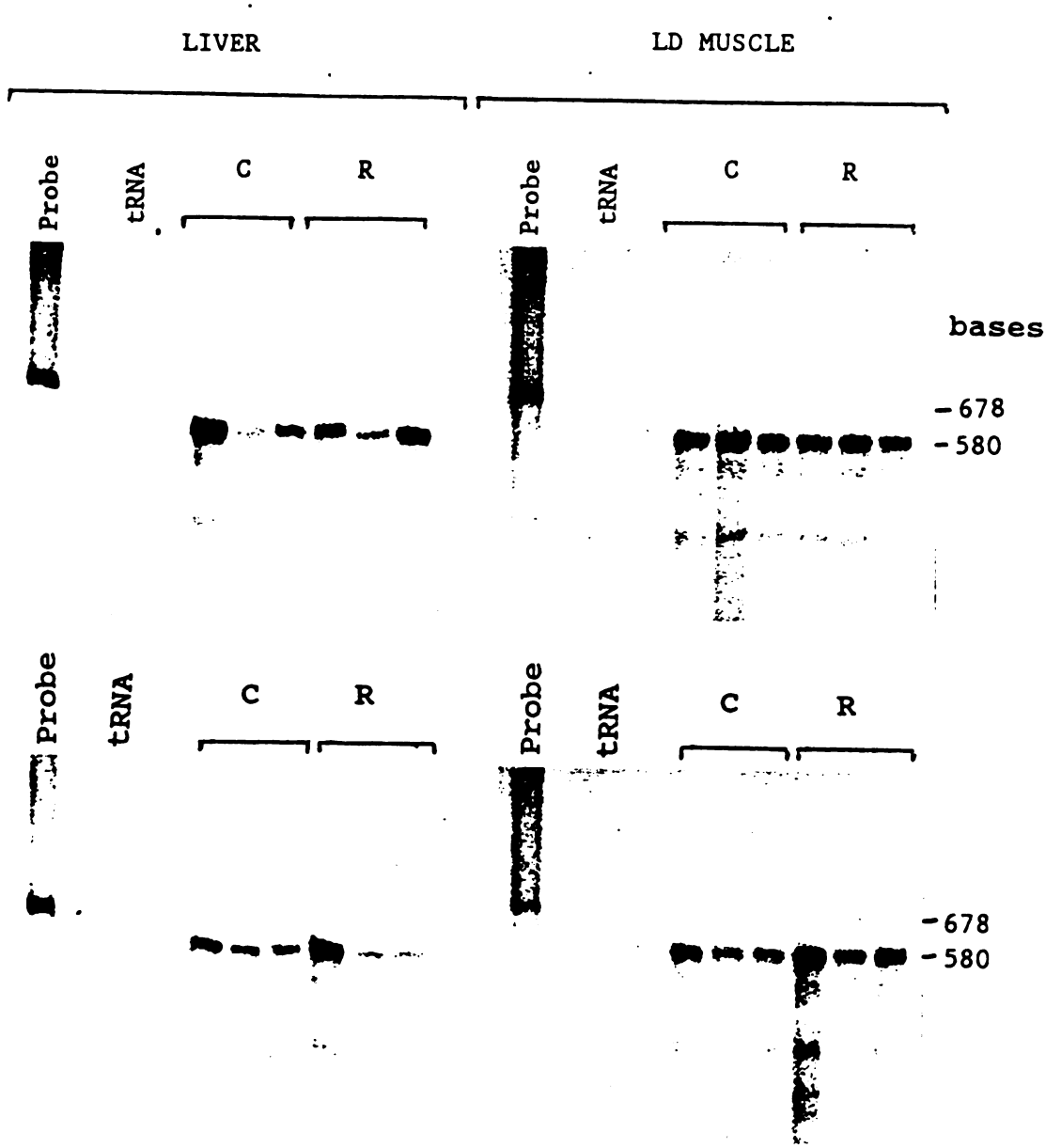

LIVER
LD MUSCLE
Probe
tRNA
C
R
Probe
tRNA
C
R
bases
-678
-580
Probe
tRNA
C
R
Probe
tRNA
C
R
-678
-580

Figure 3.1

Figure 3.2. Autoradiograms from Northern blot analyses of beta-tubulin mRNA abundance in liver and LD muscle from ractopamine (R) and control (C) pigs. Twelve ug of muscle RNA and 20 ug of liver RNA were denatured and electrophoretically separated in 1.2% agarose gels containing 2.2 M formaldehyde and then transferred to nitrocellulose. Blots were prehybridized at 42^{o}C for 2 hours in 50% formamide, 5X SSC, 5X Denhardt's solution, 0.1% SDS, 1 mM EDTA, 50 mM sodium phosphate buffer (pH 6.5), and 0.5 mg/ml tRNA. RNA was hybridized with beta-tubulin or beta-actin cDNA probes at 42^{o}C for 16 hours. Composition of the hybridization solution was identical to the prehybridization solution except that the hybridization solution contained only 1X Denhardt's and contained two million cpm of probe/ml. Following hybridization, blots were washed with three cycles of 0.2X SSC and 0.1% SDS at 55^{o}C and then subjected to autoradiography. Visualization of 18S and 28S RNA following methylene blue staining of Northern blots is also shown. Each lane represents one pig (n=6 pigs/treatment).

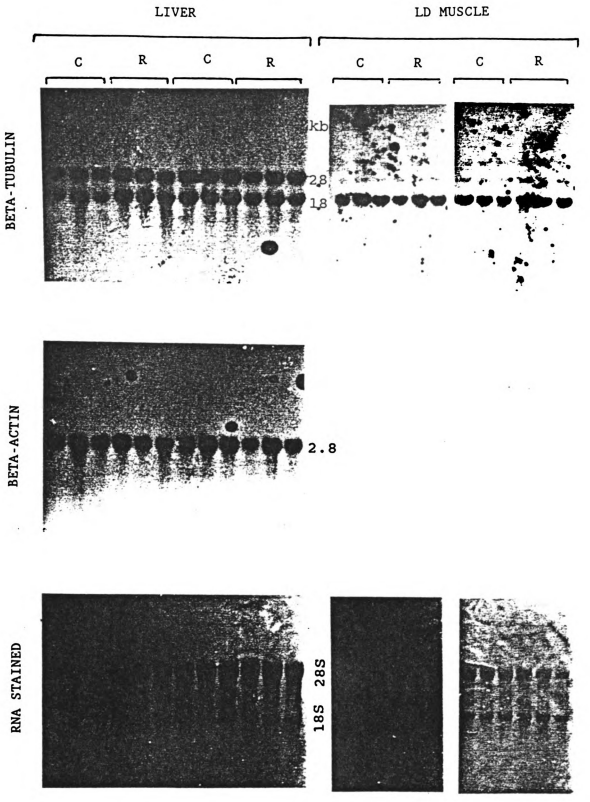

LIVER
LD MUSCLE
C
R
C
R
C
R
C
R
kb
2β
1β
BETA-TUBULIN
BETA-ACTIN
2.8
RNA STAINED
18S
28S

Figure 3.2

Table 3.3. Relative abundance of IGF-I mRNA and beta-tubulin mRNA in liver and LD muscle of ractopamine-treated (R) and control (C) pigs[1]

	Treatment		
Variable	Control	Ractopamine	SEM
Liver IGF-I mRNA	0.181	0.163	0.029
Liver beta-tubulin mRNA	0.547	0.553	0.087
Liver beta-actin mRNA	1.219	1.108	0.258
LD IGF-I mRNA	0.238	0.248	0.019
LD beta-tubulin mRNA	1.500	1.560	0.169

[1]Abundance of IGF-I mRNA is expressed relative to an internal control hybridization standard (RNA from liver of a growth hormone-treated pig); abundance of beta-actin and beta-tubulin mRNA is expressed in densitometer units.

abundance is consistent with the following observations. First, an increased abundance of liver IGF-I mRNA would likely result in greater liver IGF-I synthesis, and subsequently greater circulating concentrations of IGF-I; Beermann et al. (1987) were unable to detect any increase in plasma IGF-I concentrations in sheep fed the beta adrenergic agonist, cimaterol. Second, if circulating levels of IGF-I were elevated, then serum from treated animals would result in greater anabolic activity when included in muscle cell cultures because IGF-I increases proliferation and protein synthesis in cultures of muscle cells (Ewton and Florini, 1980; Gulve and Dice, 1989; Roe et al., 1989). McElligott and Chaung (1987) demonstrated that serum from rats treated with clenbuterol and serum from normal rats had similar effects on protein synthesis and degradation and cell proliferation in cultures of L8 myoblasts. Given these data, hepatic or circulating IGF-I does not appear to play major roles in mediating ractopamine-induced muscle hypertrophy.

Since ractopamine has been demonstrated to directly increase protein synthesis in cultures of L6 myotubes (Adeola et al., 1989; Anderson et al., 1990), beta adrenergic agonists may act directly upon the skeletal muscle in vivo to increase muscle growth. A direct action of ractopamine on muscle protein metabolism may be mediated by local synthesis of IGF-I. This locally synthesized IGF-I could then act upon the muscle to increase protein

synthesis. Administration of IGF-I to dwarf mice has been demonstrated to increase skeletal muscle protein synthesis and muscle growth (Pell and Bates, 1989). It is possible that changes in IGF-I mRNA abundance may change prior to the 28-day time point that was selected in the present study. Although abundance of IGF-I mRNA was not altered, ractopamine may alter the synthesis of IGF-I at the translational or post-translational levels. It is unknown whether ractopamine results in more translationally active IGF-I mRNA. Quantitation of tissue and serum IGF-I peptide concentrations would provide additional information; however, such measurements do not distinguish between endocrine and paracrine and(or) autocrine roles of IGF-I since the presence of muscle IGF-I peptide may be the result of local synthesis or contribution from other tissues. Changes in IGF-I receptor number and affinity in skeletal muscle and alterations in the distribution of IGF-binding proteins among tissues of pigs fed ractopamine have not been examined and may also represent mechanisms of growth regulation. These changes could occur without changes in IGF-I mRNA of peptide concentrations. Alternatively, it is possible that IGF-I does not play a role in mediating ractopamine-induced muscle hypertrophy in pigs. The actions of ractopamine may be mediated by other growth factors or involve a number of different signal transduction mechanisms.

Summary

Administration of ractopamine for 28 days to market weight pigs does not appear to have a significant effect on liver or skeletal muscle IGF-I mRNA abundance. Results of the present study do not support the hypothesis that IGF-I mediates the actions of ractopamine on skeletal muscle. If ractopamine increases skeletal muscle growth in pigs by changing liver or muscle IGF-I status after 28 days, then it must occur by mechanisms other than changes in abundance of IGF-I mRNA. Other mechanisms may include regulation at the tanslational, post-translational, receptor and post-receptor levels, or may involve changes in tissue distributions of IGF-binding proteins.

CONCLUSIONS AND RECOMMENDATIONS

Each of the two experiments that were conducted has provided us with a different model in which various biological mechanisms involved in skeletal muscle growth could be investigated. The objective of this dissertation was to determine if liver and skeletal muscle IGF-I mRNA was altered when market weight pigs were subjected to these perturbations. To accomplish this objective, it was first necessary to develop an assay with sufficient sensitivity to detect changes in abundance of market weight pig tissues in which IGF-I mRNA is in extremely low abundance, compared with tissues of younger pigs and those of rats. This is the first report in which the relative abundance of IGF-I mRNA has been quantitated in liver and skeletal muscle of market weight pigs using a solution hybridization-nuclease protection assay. Results from the experiments indicate that regulation of relative abundance of IGF-I mRNA is dependent upon dietary protein and that it is altered by administration of growth hormone to pigs. Furthermore, ractopamine-induced muscle hypertrophy was not associated with changes in abundance of IGF-I mRNA.

Mechanisms by which growth hormone or ractopamine may

induce skeletal muscle hypertrophy are shown in Figure 4.1. IGF-I may mediate the actions of growth hormone or ractopamine in an endocrine manner or in a paracrine/autocrine manner. For example, growth hormone or ractopamine may act at the liver to increase IGF-I synthesis. Increased liver IGF-I may be accomplished by increased IGF-I mRNA and subsequent IGF-I synthesis. This additional liver IGF-I may then be secreted by the liver and transported to the skeletal muscle where it may increase protein synthesis. Alternatively, growth hormone or ractopamine may act directly on the skeletal muscle (myofibers or nonmuscle cells) resulting in increased local production of IGF-I synthesis. The additional IGF-I may then act on myofibers in a paracrine or autocrine manner to increase protein synthesis.

Results of the present experiments would support an endocrine role of IGF-I in growth hormone-induced muscle growth in pigs. In the growth hormone-treated pigs, the increased abundance of liver IGF-I mRNA presumably contributed to the increased circulating concentrations of IGF-I. It is tempting to speculate that the greater circulating concentrations of IGF-I contributed to the muscle hypertrophy that was observed in the treated pigs. Lack of an increase in muscle IGF-I abundance in growth hormone-treated pigs suggests that IGF-I does not mediate growth hormone action in a paracrine and(or) autocrine manner. Furthermore, lack of responses in liver and muscle IGF-I abundance in pigs treated with ractopamine suggests

Figure 4.1. Diagram showing possible mechanisms by which IGF-I may mediate growth hormone- or ractopamine-induced skeletal muscle hypertrophy in pigs. IGF-I may mediate growth hormone or ractopamine actions by 1) acting in an endocrine manner, in which case additional liver IGF-I is transported to the skeletal muscle where it elicits its effect on muscle protein metabolism or 2) acting in a paracrine and(or) autocrine manner, in which case growth hormone or ractopamine acts directly on the muscle resulting in local production of IGF-I which effects protein metabolism.

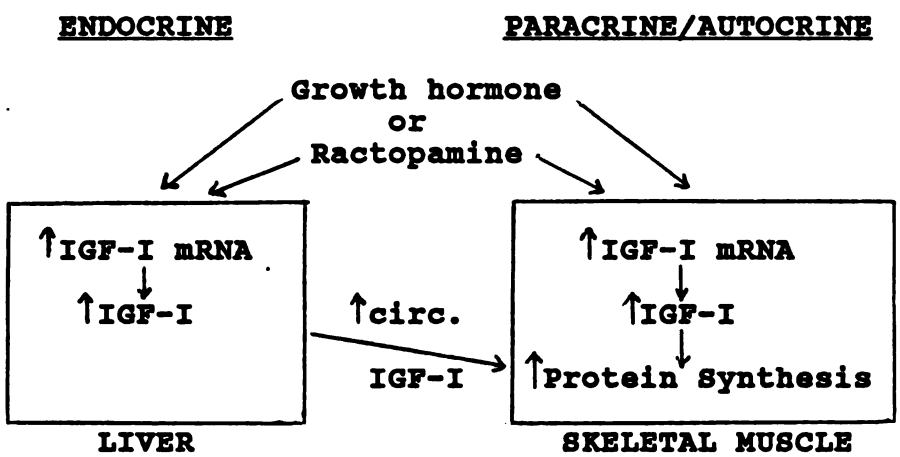

ENDOCRINE
PARACRINE/AUTOCRINE
Growth hormone
or
Ractopamine
↑IGF-I mRNA
↑IGF-I
↑circ.
IGF-I
↑IGF-I mRNA
↑IGF-I
↑Protein Synthesis
LIVER
SKELETAL MUSCLE

Figure 4.1

that IGF-I may not mediate ractopamine-induced muscle growth.

Several factors must be considered during interpretation of these results. For example, it is critical that in future studies tissues be collected at various times following the initiation of the various treatments. Selecting a single timepoint to quantitate variables does not provide a complete picture of the changes and adaptations that may occur during such treatments. Doses of pharmacological agents, composition of diets, sex, and breed all need to be considered when designing future studies. The possible interaction of circulating levels of IGF-I on tissue IGF-I synthesis suggests that several variables need to be examined, including tissue IGF-I peptide levels and circulating and tissue concentrations of IGF-I binding proteins. Future studies should be designed to investigate changes in IGF-I receptor number and affinity and alterations in post-receptor mechanisms that may be influenced by growth hormone or ractopamine. Such measurements are critical in determining the endocrine and paracrine/autocrine roles of IGF-I in muscle growth.

APPENDICES

APPENDIX A

ACID GUANIDINE PHENOL CHLOROFORM RNA EXTRACTION PROTOCOL
(modification of Chomczynski and Sacchi, 1987)

REAGENTS:

4 M Guanidine thiocyanate:

guanidine thiocyanate (Kodak)	94.60 g.
sodium citrate	0.74 g.
B-mercaptoethanol (14 M)	1.42 ml
10% Na sarcosyl	10.0 ml

Total volume of 200 ml: use sterile GD-HOH.
Adjust pH to 7.0 with 1 M HCl or 1 N NaOH.
Filter through 0.45 um millipore.
Store in sterile brown bottle at room temperature.

7 M Guanidine Hydrochloride

guanidine-HCl (Sigma, practical grade)	668.6 g.
Na-acetate (trihydrate)	2.7 g.
dithiothreitol	150.0 mg.
iodoacetamide	1.84 mg.
0.2 M EDTA (pH 8.0)	5.0 ml.

Add GD-HOH to 900 ml.
Dissolve all reagents by heating in a 50°C water bath.
When all reagents are dissolved, adjust to pH 7.0 with glacial acetic acid or 5 N NaOH.
Adjust final volume to 1 l.
Filter through 0.45 um millipore.
Store in sterile bottle in the dark at room temperature.

2 M Na-acetate, pH 5.0

Dissolve 272 g. Na-acetate (trihydrate) in 600 ml sterile GD-HOH.
Adjust to pH 5.0 with glacial acetic acid.
Adjust final volume to 1.0 l.
Filter through 0.45 um millipore.
Store at 4°C. in sterile bottle.

3 M Na-acetate, pH 5.0

Na-acetate (trihydrate)	409.8 g.
iodoacetamide	1.84 g.

Dissolve reagents in 300 ml glacial acetic acid and 300 ml sterile GD-HOH. Adjust to pH 5.0 with glacial

acetic acid. Adjust final volume to 1.0 l. Filter through 0.45 um millipore filter and store in sterile bottle at 4°C.

ETOH-Na-acetate, pH 5.0.

Absolute Ethanol	660 ml.
3 M Na-acetate, 10 nM iodoacetamide, (pH 5.0)	11 ml.

Filtration of this solution is not necessary, store in sterile bottle at -20°C.

TE-8.0:

1.0 M Tris-HCl, pH 8.0 (20°C)	10.0 ml.
0.2 M EDTA, pH 8.0	5.0 ml.

Adjust final volume to 1.0 l. with sterile GD-HOH. Store at room temperature.

10% Na-sarcosyl:

Dissolve 10 g. Na-sarcosyl (ICN) in 100 ml HOH, filter through 0.45 millipore and store at room temperature. Note: sarcosyl is a carcinogen, so take the appropriate precautions.

EXTRACTION:

Use sterile glassware and plasticware throughout the procedure.

Add 1 g frozen pig LD muscle or liver to a sterile 30 ml. Corex tube containing 10 ml 4 M guanidine thiocyanate homogenization buffer. Homogenize using a Polytron at a setting of 6 for 20 seconds or until the tissue is completely homogenized.

Add 1 ml 2 M Na acetate, pH 5, stopper, and vortex.
Add 10 ml HOH-saturated phenol, vortex.
Add 2 ml chloroform:isoamyl (24:1).
Mix vigorously, put on ice for 15 minutes.

Centrifuge at 10,000 x g for 20 minutes at 4°C.

Remove the aqueous layer with a sterile Pasteur pipet and put it into a sterile 30 ml Corex tube.

To the organic phase, add 2 ml 4 M guanidine thiocyanate and 0.2 ml 2 M Na acetate and vortex.

Add 2 ml HOH-saturated phenol and 0.4 ml chloroform:isoamyl.

Vortex and put on ice for 15 minutes.

Centrifuge at 10,000 x g for 20 minutes at 4°C.

Add this aqueous layer to the previously collected aqueous layer.

Extract the aqueous composite with 5 ml HOH-saturated phenol and 5 ml chloroform: isoamyl.

Centrifuge at 10,000 x g for 20 minutes at 4°C.

Remove the aqueous layer and put it into a sterile 30 ml Corex tube.

Add 1 volume of isopropanol to the aqueous layer, cover the tube with Parafilm and mix.

Store at -20°C for at least 2 hours to allow precipitation of RNA.

Centrifuge at 10,000 x g for 20 minutes at 4°C.

Transfer pellet to a 1.5 ml microfuge tube with 750 ul 7 M guanidine HCl. Add 75 ul 2 M Na acetate, pH 5 and 450 ul absolute ethanol and allow RNA to precipitate at -20°C for 1 hour.

Microfuge at 12,000 x g for 5 minutes. Decant and drain.

Resuspend the pellet in 300 ul of 3 M Na acetate, pH 5. Sediment and drain as described above.

Resuspend the RNA pellet in 300 ul of EtOH-Na acetate, pH 5 and sediment and drain as described above.

Resuspend the RNA pellet in 300 ul of absolute ethanol and sediment and drain as described above.

Resuspend the RNA pellet in of TE-8.0 (100 ul if LD muscle RNA or 400 ul if liver RNA).

Dilute 5 ul of RNA solution to 1 ml with TE-8 and scan in a spectrophotometer from 320 to 220 nm. Record the optical density at 260 and 280 nm. The ratio of 260/280 should be greater than 1.8.

RNA concentration of solution is equal to the optical density at 260 nm multiplied by 200 (dilution factor) and divided by 25 (RNA extinction coefficient). DNA contamination is not a problem with this extraction procedure because extractions are carried out at pH 5 to 6 (Chomczynski and Sacchi, 1987; Wallace, 1987).

RNA samples should be labeled and stored at -80^{o}C.

APPENDIX B

IGF-1 SOLUTION HYBRIDIZATION-NUCLEASE PROTECTION ASSAY

REAGENTS AND SOLUTIONS:

Pvu II, 24 U/ul (BMB) with buffer M. Store at -20°C.

Chloroform:Isoamyl alcohol, 24:1. Store at RT.

Phenol:Chloroform:Isoamyl, 1:1. Store at 4°C.

Ethanol, absolute. Store at -20°C.

Transcription Kit (BMB). Store at -20°C.

TE-8, filter and autoclave. Store at -20°C.

tRNA,10 ug/ul. Store at -20°C.

5 M NH4Ac, filter and autoclave. Store at RT.

4 M NaCl, filter and autoclave. Store at RT.

RNA denaturing solution (for Northern gel):

20 ul 10X MAE, pH 7
70 ul deionized formaldehyde
200 ul deionized formamide
Make up fresh.

4X RNA loading buffer: 50% glycerol

0.4% bromophenol blue
0.4% xylene cyanol
1 mM EDTA
Store at RT.

Agarose/formaldehyde mini gel (Northern):

10 ml 10X MAE, pH 7
1.2 g agarose (low EEO)
72.4 ml HOH
Melt agarose in HOH and MAE in microwave on high setting for 2.5-3 min. Cool to approx 60°C, add 17.6 ml deionized formaldehyde and pour gel.

Mini gel electrode buffer:

25 ml 10X MAE, pH 7
180 ml HOH
45 ml deionized formaldehyde

Hybridization solution:

100 ul 10 X hybridization buffer
100 ul HOH
800 ul formamide
Make up fresh.

10X hybridization buffer:
400 mM PIPES, pH 6.4
4 N NaCl
10 mM EDTA
12.09 g PIPES in 50 ml HOH, adjust pH to 6.4 with 5 N NaOH. Add 23.38 g NaCl and 5 ml 0.2 M EDTA. Bring up to 100 ml with HOH. Filter, autoclave, store at RT.

SI solution:

367.5 ul 4 N NaCl (0.28 M)
87.5 ul 3 M NaAc, pH 4.5 (50 mM)
236.3 ul 100 mM $ZnSO_4$ (4.5 mM)
5.0 ul Nuclease SI (400 U/ul from BMB; final conc. is 381 U/ml or 133.36 U/.35ml)
4.546 mlHOH
5.25 ml total volume

Proteinase K, 25 mg/ml HOH. Store at -20°C.

10% SDS. Store at RT.

Denaturing loading buffer:

2 mg bromophenol blue (0.02%)
2 mg xylene cyanol (0.02%)
9 ml formamide (90%)
1 ml 10X TBE (1X)
Store at RT.

5% Polyacrylamide/8 M urea gel:

48 g urea
4.75 g acrylamide
0.25 g bis
40 ml HOH
10 ml 10X TBE
Heat on stir plate gently (#2) until urea is in solution (about 30 min.). Filter through 0.45 um filter unit, bring up to 100 ml vol. with HOH. Degas for 20-30 min. Add 0.5 ml of 10% APS and 50 ul TEMED and pour gel immediately with 10 ml pipet and pipet-aid. Allow gel to polymerize for 1.5 h. Flush wells with 1X TBE and pre-run for 1.5-2 h. at 380 V (constant voltage). Use 1X TBE as electrode buffer.

PROTOCOL:

Linearization of pGEM-1/IGF-1 plasmid DNA:

Maps of the plasmid vector and cDNA insert that are utilized in the following assay are shown in Figure B.1.

The following is combined in each of 2 microfuge tubes;

24.5 ul HOH
3.0 ul buffer M
0.5 ul DNA (2.75 ug)
2.0 ul Pvu II (24 U)

30.0 ul total vol.

Incubate at 37°C for 2.5-3 h (vortex after 1 h).

Bring volume up to 100 ul with TE-8 in each tube.

Extract with phenol/chloroform/isoamyl alcohol and then with chloroform/isoamyl alcohol (referred to as PIC/IC extract) and ppt. DNA from aqueous phase with 5 ul 4 M NaCl and 200 ul EtOH.

Allow DNA to ppt. for 30 min at -20°C.

Centrifuge for 8 min and dry pellets in Speed-Vac.

Synthesis of riboprobe (using BMB SP6/T7 Transcription Kit):

To each tube of dried linearized DNA add:

12 ul 32P-UTP (10 uCi/ul; 400 Ci/mmol; final conc. of UTP=15 uM)
3 ul ATP, CTP, GTP mixture (1:1:1, i.e. 1 ul of each nt, 10 mmol/l; final conc. of each=500 uM)
Resuspend pellet, then add:
2 ul 10X buffer
3 ul RNase inhibitor (55 U/ul; not kit's, purchased separately)
1 ul SP6 Polymerase (10 U/ul)

Vortex well and incubate at 37°C for 1 h (vortex after 30 min).

After 1 h, add 2 ul DNase I (20-30 U) and 1 ul RNase inhibitor (55 U) to each tube, vortex, and incubate an additional 15 min at 37°C.

Add 76 ul TE-8 to bring volume of each tube to 100 ul and then PIC/IC extract.

Then add: 10 ul tRNA (10 ug/ul)

27 ul 5 M NH4Ac (1/4 vol.)
330 ul EtOH (3 vol.)

Allow RNA to ppt. for 30 min at -20°C (during ppt'n, pour mini 1.2% agarose-formaldehyde gel).

Centrifuge 8 min, resuspend pellets in 100 ul TE-8, and re-ppt. with 25 ul 5 M NH4Ac and 300 ul EtOH at -20°C for 30 min.

After centrifugation for 8 min, resuspend each pellet in 11 ul TE-8.

Add 29 ul RNA denaturing solution and heat to 65°C for 10 min.

Add 10 ul of 4X RNA loading buffer and load wide well of mini 1.2% agarose-formaldehyde gel (Northern).

Electrophorese for 1.5 h at 65 V.

Briefly blot gel dry, wrap in saran wrap and autoradiograph for 10 min.

Riboprobe band should appear midway between the tracking dyes.

Cut out riboprobe band from the gel and electroelute RNA using 1 ml TE-8 for 1.5 h at 80 V in fresh electrode buffer.

After squeezing the gel out of the dialysis tubing, remove TE-8 and place into a microfuge tube.

Centrifuge for 1 min to pellet any residual agarose.

Remove TE-8 and divide among 2 or 3 tubes and then ppt. the RNA; e.g., if 265 ul/tube, then add 5 ul tRNA, 68 ul 5 M NH4Ac (1/4 vol), and 810 ul EtOH (3 vol) and allow RNA to ppt. for 30 min at -20°C.

Centrifuge 8 min.

Resuspend pellets in a total of 200 ul of TE-8 (combined) and re-ppt. with 50 ul 5 M NH4Ac and 600 ul EtOH for 30 min at -20°C.

Centrifuge 8 min and resuspend the pellet in 200 ul of hybridization solution.

Quantitate 0.5 ul by liquid scintillation by ppt'n. of 0.5 ul with 1 ml of 10% TCA/10 mM Na pyrophosphate containing 25 ul tRNA and filtration through a glass fiber filter.

Solution hybridization:

To 100 ug of total RNA dried in microfuge tubes, add 2 million cpm of probe and hybridization solution for a total volume of 30 ul. Also include a tube containing 10 ug (1 ul) of tRNA plus probe and hybridization solution to serve as a negative control.

Heat each tube to 90°C in a heating block for 10 min.

Place each tube directly into a 65°C shaking water bath for 14-16 hours.

Nuclease digestion and electrophoresis:

Centrifuge all tubes briefly to spin down any condensation.

Add 350 ul S1 solution to each tube, vortex, and incubate 1 h at 37°C (during incubation, prepare denaturing acrylamide/urea gel sol'n, filter, and degas).

Centrifuge briefly and add 3 ul Proteinase K (25 mg/ml) and 20 ul of 10% SDS.

Vortex and incubate an additional 15 min at 37°C (during incubation pour acrylamide/urea gel).

PIC/IC extract and then ppt. RNA with 5 ul tRNA, 105 ul 5 M NH4Ac, and at least 1 ml EtOH for 30 min at -20°C.

Centrifuge 8 min.

Resuspend pellets in 100 ul TE-8 and re-ppt. RNA with 25 ul 5 M NH4Ac and 300 ul EtOH for 30 min at -20°C (start pre-running gel at this time).

Centrifuge 8 min and then dry pellets in Speed-Vac.

Resuspend pellets in 20 ul denaturing loading buffer and heat to 90°C for 2-3 min.

Two ug (8 ul) of DNA mw marker III (BMB) should be dried down, resuspended in loading buffer and heated for 2-3 min, too.

3900 cpm of probe alone should also be resuspended in 20 ul loading buffer and heated.

Cool all samples on ice and then load 5% Polyacrylamide/8 M Urea gel.

Gel should be pre-run for 1-1.5 h prior to loading.

After loading gel, electrophorese for 2.5 h at 380 V (constant voltage; current will be approx. 30-40 mA). The first tracking dye will run off the gel; the second tracking dye will end up at the bottom of the gel at completion.

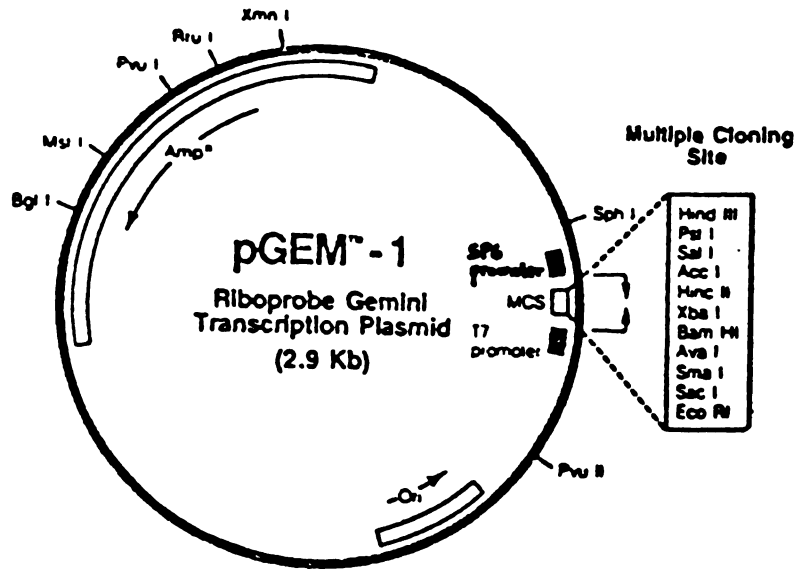
Cut off marker lane, stain with EtBr for 2 h, and then photograph.

Dry remaining gel in gel dryer for 1.5 h at 60°C on PAGE cycle.

Wrap gel in saran wrap and autoradiograph.

A dose-response curve is shown in Figure B.2. The solution hybridization-nuclease protection assay was conducted using 50, 75, 100, and 150 ug of liver RNA. Values were corrected for nonspecific hybridization by subtracting the hybridization signal obtained with tRNA (y-intercept=80; slope=7.7; r square=.978). Intra- and inter-assay coefficients of variation using 100 ug of total RNA are 2.8 and 2.7%, respectively, in this assay.

Figure B.1. Maps of the plasmid vector (top) and IGF-I cDNA insert (bottom) that were used to synthesize the IGF-I RNA probe. The cDNA was subcloned into the EcoRI site of the plasmid vector, pGEM-I (Promega, Madison, WI), and generously provided by Dr. Frank Simmen (Tavakkol et al., 1988).


Xmn I
Nru I
Pvu I
Msl I
Amp^R
Bgl I
pGEM™-1
Riboprobe Gemini
Transcription Plasmid
(2.9 Kb)
SP6 promoter
MCS
T7 promoter
Sph I
Multiple Cloning Site
Hind III
Pst I
Sal I
Acc I
Hinc II
Xba I
Bam HI
Ava I
Sma I
Sac I
Eco RI
Pvu II
ori

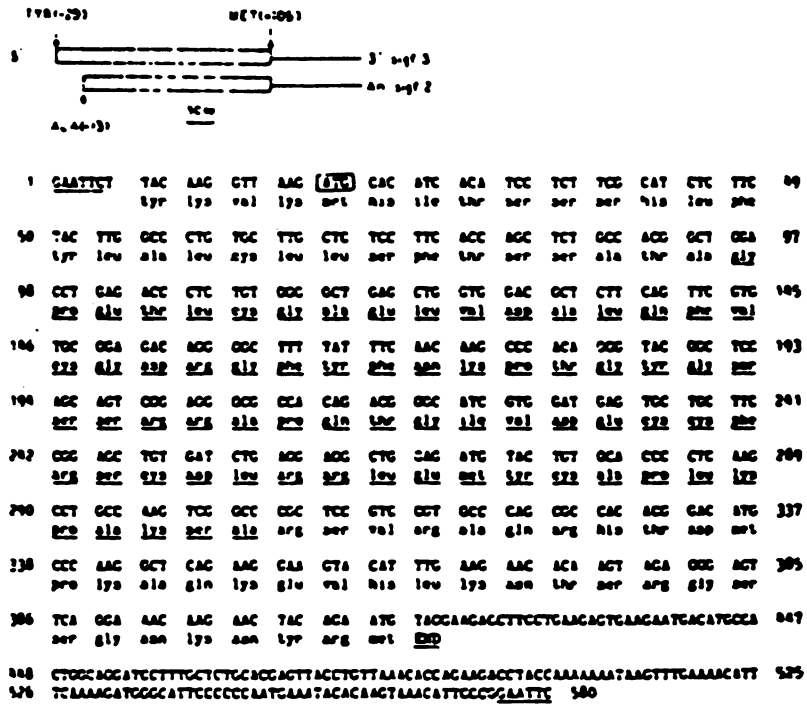


Figure B.1.

Figure B.2. Dose-response relationship between quantity of total RNA used and hybridization signal obtained in the solution hybridization-nuclease protection assay. A porcine IGF-I cDNA was utilized as a template to synthesize an antisense ^{32}P-labeled RNA probe. The probe was hybridized to 50, 75, 100, and 150 ug of porcine liver RNA at 65^{o}C for 14 hours. Following hybridization, 133 units of nuclease S1 were added and samples incubated for an additional 1 hour at 37^{o}C. Protected RNA fragments were electrophoretically separated in 5% acrylamide/8 M Urea gels and then subjected to autoradiography. Transfer RNA (tRNA) was included as a negative control to account for nonspecific background hybridization. The signals obtained in the 580-base pair nuclease-protected fragments were quantitated by liquid scintillation analysis. Results have been corrected for nonspecific hybridization by subtracting the signal obtained from hybridization to tRNA (y-intercept=80; slope=7.7; r squared=.978).

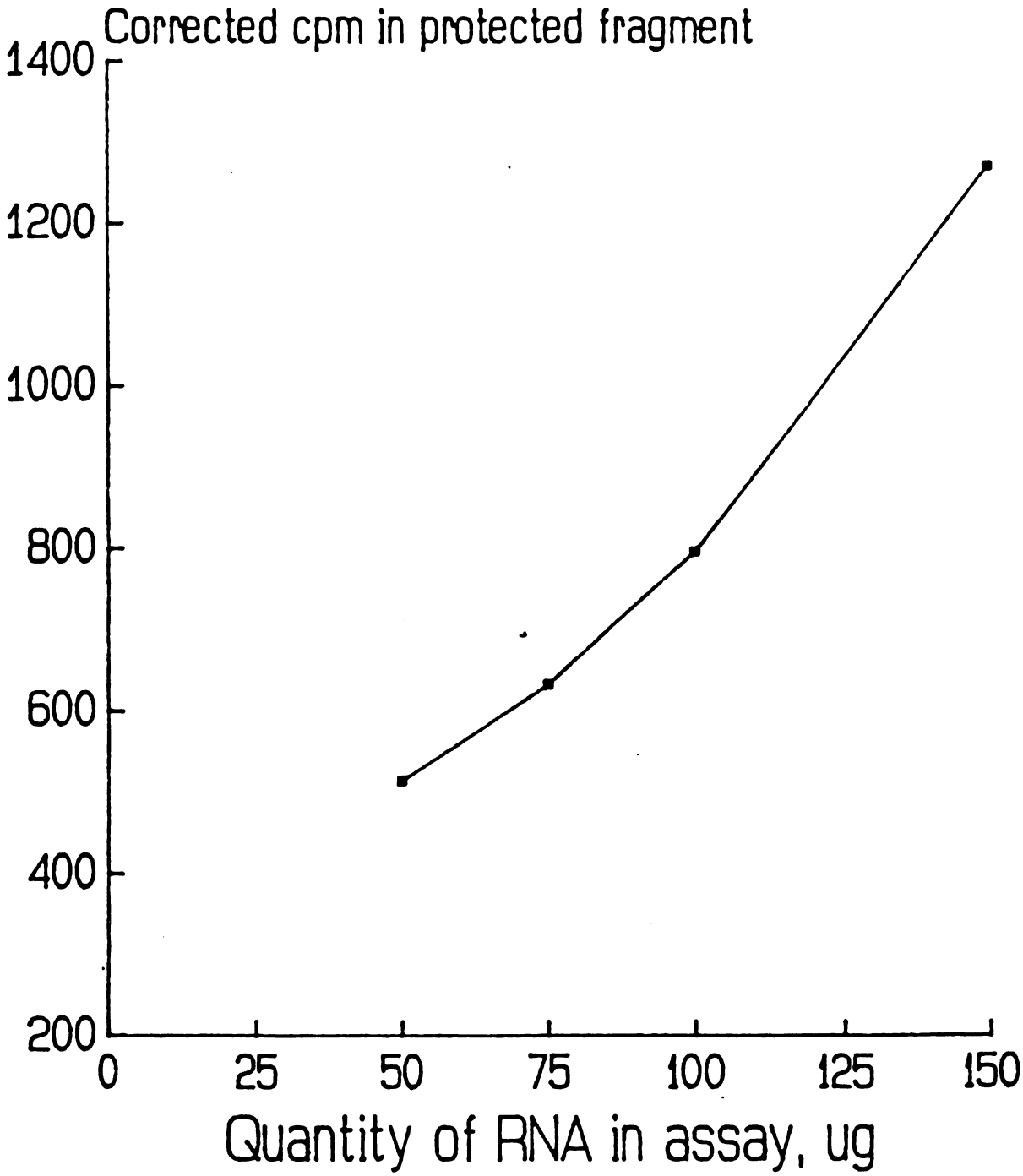

Corrected cpm in protected fragment
1400
1200
1000
800
600
400
200
0
25
50
75
100
125
150
Quantity of RNA in assay, ug

Figure B.2.

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