EFFECT OF TESTOSTERONE ON IGF-I, AR AND MYOSTATIN GENE EXRESSION IN SPLENIUS AND SEMITENDINOSUS MUSCLES IN SHEEP

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Raluca Georgiana Mateescu

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EFFECT OF TESTOSTERONE ON IGF-I, AR AND MYOSTATIN GENE EXRESSION IN SPLENIUS AND SEMITENDINOSUS MUSCLES IN SHEEP

Raluca Georgiana Mateescu, Ph.D.

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Testosterone is known to act differentially on skeletal muscle from different regions. Two genes likely to mediate the testosterone effect are insulin-like growth factor-I (IGF-I), an important growth regulator acting in an autocrine and paracrine way, and androgen receptor (AR), as receptor density could account for differential muscle growth. Another muscle-specific gene that may play a role in differential muscle growth is myostatin, a member of the transforming growth factor-beta superfamily, shown to be a negative regulator of skeletal muscle mass. The objective of this study was to quantify and compare the expression of these three genes in two different skeletal muscles in sheep. East Friesian x Dorset sired ram lambs born from Dorset ewes were used in a 2 x 4 factorial experiment. Eighteen sets of twins were assigned to four age groups corresponding to 77, 105, 133 and 161 days of age and one individual from each set was castrated at birth. Total RNA was extracted from samples of semitendinosus (ST) and splenius (SP) muscles collected at the time of slaughter. Insulin-like growth factor I mRNA was measured using competitive reverse-transcription-polymerase chain reaction (RT-PCR). Androgen receptor and MSTN mRNA were measured by ribonuclease protection assay (RPA) with standard curves. Splenius muscle weight was greater than semitendinosus muscle weight in rams compared with wethers at 105, 133 and 161 days ($p = 0.05$, $p = 0.04$ and $p = 0.02$). The difference in IGF-I mRNA levels between the two muscles was higher in rams than in wethers at 133 and 161 days ($p < 0.05$) and the difference in AR mRNA levels was higher in rams than in wethers at 105, 133 and 161 days ($p < 0.05$), with higher

expression in the SP. No difference was found in the MSTN mRNA level between the two muscles in rams and wethers at any age. These results show that locally produced IGF-I and the regulation of AR expression are important for sexually dimorphic muscle growth patterns.

BIOGRAPHICAL SKETCH

The author was born in Bucharest, Romania on August 11, 1974. In June of 1997, the author received a Bachelor of Science in Biology at the University of Bucharest. After graduating from college the author moved to Ithaca, NY and in August 1997 started to work at the Baker Institute for Animal Health in the Canine Genetics and Reproduction Laboratory, first as a volunteer and then in November 1997 as lab technician. In spring 1999 the author was admitted to the MS/Ph.D. degree program in the Field of Animal Science. She completed the requirements for the MS degree in May 2001.

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CHAPTER I

INTRODUCTION

Male animals are generally larger than females (Glucksmann 1974) and have more muscle, especially in the neck and the forequarters. Different growth rates for individual muscles or muscle groups are of special interest for meat production as improving muscle growth of meat producing animals has been a major endeavor of farmers and agricultural scientists. Alterations in muscle growth rate are commonly attributed to changes in function and to hereditary tendencies of serial development as well as to the influence of bone growth on muscle growth (Stewart 1972, Berg & Butterfield 1976, Taylor 1980, Dickson *et al.* 1991). Besides these basic developmental changes affecting muscle growth, a variety of other factors such as sex, rate of growth, loss and gain of body weight or deposition of fat within the musculature modifies the muscle growth pattern.

Sexual dimorphism in muscle growth relates to the protein anabolic effect of testicular hormones. It was shown (Arnold *et al.* 1997) that splenius muscle of the neck in rams and in wethers implanted with testosterone was heavier and had a biphasic growth pattern when compared to the single phase of growth of the same muscle in wethers. These results confirm the hypothesis that testosterone is implicated in the increased neck muscle mass in sexually mature rams. Among serum insulin-like growth factor I (IGF-I), growth hormone (GH) and cortisol, only IGF-I concentrations increased in response to the testosterone treatment (Arnold *et al.* 1996). Subsequently, the expression of IGF-I, androgen receptor (AR) and myostatin (MSTN) genes was measured in both splenius (sexually dimorphic) and semitendinosus (not sexually

dimorphic) muscles in rams (Mateescu & Thonney 2002) and it was suggested that the increased splenius muscle mass of the neck associated with sexual maturity of rams is mediated by an increase in mRNA of the IGF-I and AR genes.

Regulatory factors that contribute to differential growth are most likely located in the respective tissues since alterations in circulating concentrations of hormones or growth factors as well as in blood supply cannot explain why certain tissues grow faster or slower than the body in total. Possible levels of regulation for the mediation of different muscle growth rates include:

- local synthesis of growth factors acting in a paracrine or autocrine manner;
- sensitivity of the tissue to growth regulating hormones, i.e. the density and the affinity of the respective receptors;
- local synthesis of specific binding proteins, which may increase or inhibit the action of the relevant growth factors.

At least two of these levels of regulation are associated with testosterone. Testosterone could exert an effect on muscle growth through the IGF-I axes. Locally produced IGF-I is an important growth regulator acting in an autocrine and paracrine manner (Weimann & Kiess 1990), but different muscles may posses different IGF-I sensitivities (Boge *et al.* 1995) and (or) IGF-I synthesis rates (Thissen *et al.* 1994, Frost *et al.* 1997, Pfaffl *et al.* 1998b) and, therefore, may exhibit different growth rates. Second, testosterone action is mediated by the androgen receptor (AR), which transduces the steroid signal within cells. Therefore, receptor density could account for the relatively higher sensitivity to testicular steroids in the neck muscles (Sauerwein & Meyer 1989).

In addition to the effect of testosterone on muscle growth, muscle-specific genes could be differentially expressed in certain muscles. One of these is myostatin, a gene known to inhibit muscle development.

In a previous study we showed that there is an increas in IGF-I and AR gene expression in the splenius muscle, but one question that the experiment could not address was if the increase was a result of testosterone. Therefore, the objective of this project was to analyze the role of IGF-I, AR and myostatin genes in the differential growth phenomena in response to testosterone. The approach was to quantify IGF-I, AR and myostatin mRNA expression and to assess if mRNA expression of these genes were different in splenius (sexually dimorphic) and semitendinosus (not sexually dimorphic) muscles in rams and wethers of different ages.

1.1 Characterization and expression of the IGF-I gene

The IGF-I gene is composed of at least six exons (Wong *et al.* 1989, Dickson *et al.* 1991, Froesch *et al.* 1996) and encodes a 70 amino acid polypeptide. In sheep, exon W is located upstream of exon 1 (Ohlsen *et al.* 1993). Exon W, 1 and 2 are the leader exons differentially spliced to exon 3 to produce class W, class 1 and class 2 transcripts, respectively. Exon 3 and exon 4 encode the mature IGF-I peptide, while exon 5 does not seem to be present in sheep transcripts (Wong *et al.* 1989, Dickson *et al.* 1991, Pell *et al.* 1993, Hasty *et al.* 1993, Ohlsen *et al.* 1994). The IGF-I gene organization in sheep is depicted in Figure 1.

Class W, 1, and 2 transcripts are all found in fetal and adult sheep tissues (Ohlsen *et al.* 1994). Class 1 is the most abundant of these transcripts except for liver tissue in adult animals, where class 2 transcripts are the highest. In sheep muscle, IGF-I gene expression consisted mainly of class 1 transcripts (Pell *et al.* 1993).

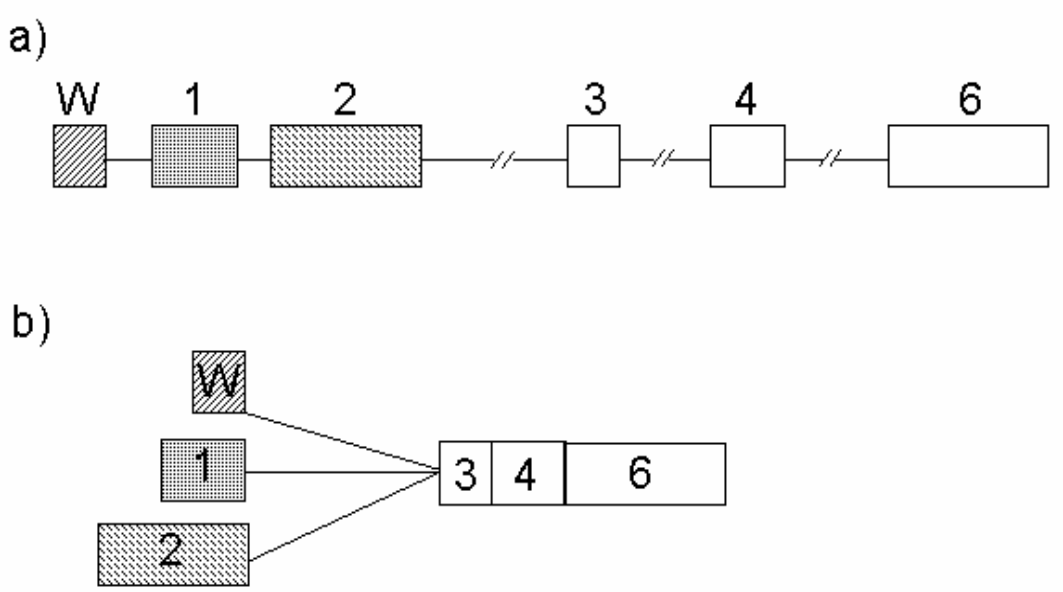


Figure 1.

a) The organization of ovine IGF-I gene. (Wong *et al.* 1989)
b) Predicted arrangement of exons in ovine IGF-I mRNA transcripts. Exons W, 1 and 2 are alternatively spliced into exon 3 and generate transcripts W, 1 and 2.

1.2 IGF-I and its biological functions

Insulin-like growth factor I, a member of the family of insulin-related peptides, is a widely distributed trophic hormone capable of mediating autocrine, paracrine or endocrine effects. Insulin-like growth factor I was originally referred to as "somatomedin" to reflect its expression in response to growth hormone (GH) stimulation and its ability to mediate some of the growth-promoting effects of GH. Insulin-like growth factor I is structurally highly related to insulin (49% peptide identity) but, unlike insulin, is not cleaved during processing (Van den Brande 1994). Insulin-like growth factor I plays important roles in both embryonic and post-natal

mammalian growth (Froesch *et al.* 1985, Jones & Clemmons 1995, Liu & Leroith 1999). Early studies compared the metabolic effect of IGF-I and insulin on muscle, including stimulation of protein metabolism, glucose transport, and glycogen and triglyceride synthesis (Hill *et al.* 1986, Harper *et al.* 1987, Roeder *et al.* 1988, Frost *et al.* 2003). More recently, research has shifted to the role of IGF-I in myogenesis and differentiation and in response to senescence, physical injury, and disease states (Florini *et al.* 1996, Le Roith *et al.* 1997, Le Roith 1999, Menetrey *et al.* 2000).

Transgenic mice overexpressing IGF-I have enhanced body growth with an increase in muscle mass (Mathews *et al.* 1988). In contrast, mice deficient in IGF-I are significantly smaller than their litter mates, have a severe muscular dystrophy, and most (> 95%) die at birth (Powell-Braxton *et al.* 1993, Liu *et al.* 1998). Specifically, the IGF-I-deficient heterozygotes were 10 to 20% smaller in total body size and in the size of individual organs, although these organs were histologically normal. Insulin-like growth factor I-deficient homozygotes were dead at birth, their body weight was < 60% of their wild type siblings, and their lungs were not inflated (Powell-Braxton *et al.* 1993). It is evident that in these animal models IGF-I plays a fundamental role in muscle maturation and in the development of muscle mass. Although 75% of circulating IGF-I is liver-derived, normal growth and development are possible even in the absence of liver IGF-I production (Yakar *et al.* 1999, Sjogren *et al.* 1999) suggesting that locally produced IGF-I is sufficient for normal growth and development. Furthermore, targeted deletion of the acid-labile subunit gene, which also results in a large (60%) reduction in circulating IGF-I levels, is associated with only minor effects on growth rate and no discernable effects on glucose metabolism (Ueki *et al.* 2000). A major question that still remains to be answered, however, is why the liver produces an output sufficient to maintain such high levels of IGF-I in the circulation.

Although growth factors, in general, inhibit the differentiation of skeletal muscle cells, IGF-I and IGF-II are unique in that they are the only known mitogens with opposing, time-dependent actions in skeletal myoblasts: initially, IGF-I induces proliferation and inhibits differentiation; subsequently, IGF-I promotes cell cycle exit and stimulates differentiation (Rosenthal & Cheng 1995, Adi *et al.* 2000). These opposing effects of IGF-I on myogenic differentiation are mediated, at least in part, by early inhibition and subsequent stimulation of the gene expression of myogenin (Rosenthal & Cheng 1995, Adi *et al.* 2000), a member of the MyoD family of skeletal muscle-specific transcription factors essential for myogenic differentiation (Nabeshima *et al.* 1993, Yun & Wold 1996, Samuel *et al.* 1999).

IGF-I was shown to play an important role in the growth and regeneration of peripheral nerves and skeletal muscle, and it has been investigated as a treatment for neuromuscular disorders (Cheng *et al.* 1996) and muscle regeneration following injury (Menetrey *et al.* 2000). Treatment with exogenous IGF-I protein reduces muscle degeneration and atrophy in dystrophic mice (de Luca *et al.* 1999). Insulin-like growth factor I is also a potential angiogenic factor and inhibits vessel constriction, via stimulation of nitric oxide (Walsh *et al.* 1996, Miele *et al.* 2000, Akeno *et al.* 2002).

1.2.1 IGF-I gene expression

The IGF-I gene is expressed in many tissues, but liver and to a lesser extent bone, are the primarily sources of circulating IGF-I (Jones & Clemmons 1995). Several studies showed that IGF-I can be produced by skeletal muscle, although it is only found at low levels in normal, mature animals (Isgaard 1992, McGuire *et al.* 1992, Daughaday 2000).

1.2.2 IGF-I binding proteins

In all extracellular tissues, IGF-I is bound to six well characterized binding proteins (IGFBPs) (Jones & Clemmons 1995, Firth & Baxter 2002). The IGFBPs are widely expressed in adult tissues but there is considerable variation in the type and extend of binding protein expressed. They each have different roles and their function may be cell/tissue dependent and at least some of them have IGF-I independent functions. These circulating IGFBPs act as carrier proteins, transporting IGF-I out of the circulation to the target tissues and prolonging the half-life of IGF-I by protecting it from proteolytic degradation. The IGFBPs function not only as carrier proteins for IGF-I in circulation, but also as modulators of IGF-I action (Clemmons *et al.* 1995). Most IGFBPs can either inhibit or potentiate IGF-stimulated actions, depending on cell type and incubation conditions. Evidence from in vitro studies suggests that IGFBP-2, -4, and -6 largely inhibit IGF-I action, IGFBP-5 enhances its action and IGFBP-1 and -3 can be either inhibitory or stimulatory (Murphy 1998).

More than 70% of circulating IGF-I is carried in a trimeric complex composed of IGFBP-3, the largest molecular weight IGFBP, and a liver-derived glycoprotein known as the acid-labile subunit (ALS) (Hwa *et al.* 1999). All three components of the trimeric complex are induced by growth hormone (GH) and therefore are affected by states of GH deficiency or excess (Delhanty & Baxter 1997). IGFBP-3 was the first IGFBP identified as being present in the ternary complex but recent data suggest that IGFBP-5 is also capable of forming a complex with IGF-I and ALS (Twigg *et al.* 2000).

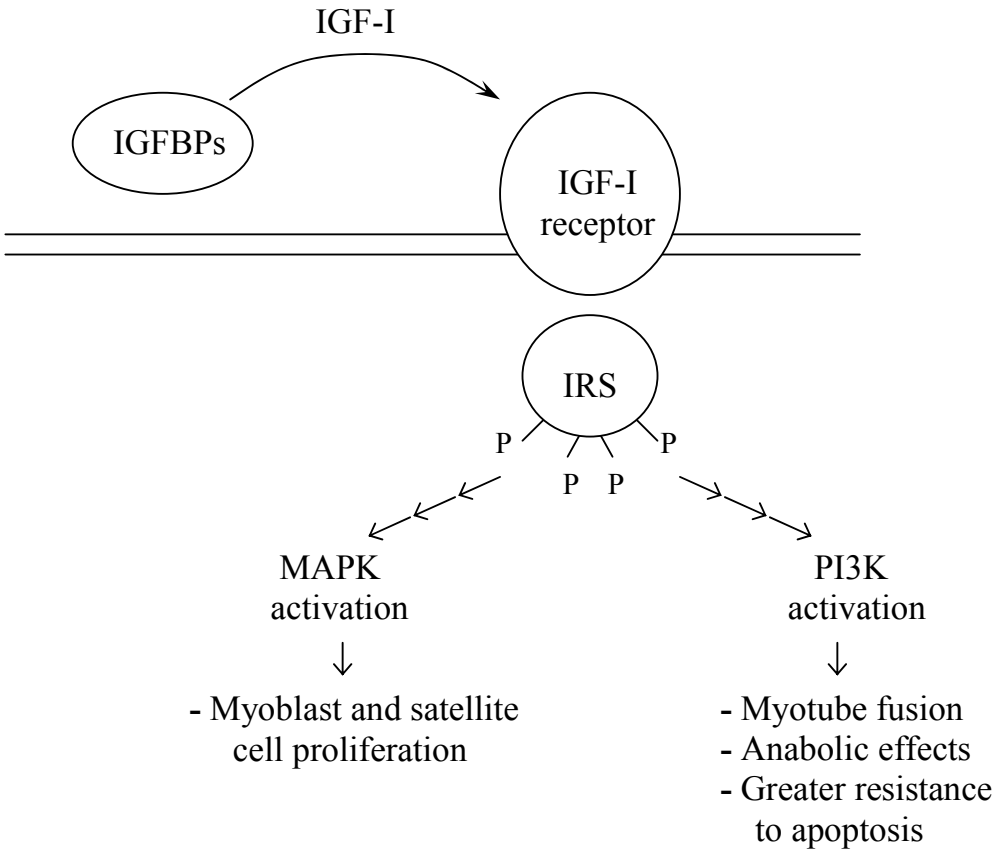
1.2.3 IGF-I receptor

While IGF-I can bind under certain conditions to the IGF-II receptor, the insulin receptor and hybrid receptors, the actions of IGF-I as a potent mitogen, anti-apoptotic factor and modulator of differentiation are mediated mainly through the IGF-I receptor (Jones & Clemmons 1995). Gene knockout studies revealed that the IGF-I receptor mediates the cellular responses of both IGF-I and IGF-II. The IGF-I receptor is a member of the tyrosine kinase growth factor receptor family that becomes activated upon ligand binding. Binding of IGF-I to the IGF-I receptor activates the intracellular tyrosine kinase domain, resulting in receptor autophosphorylation. This autophosphorylation allows enhanced binding of the insulin receptor substrate (IRS) proteins-1 and -2 and their subsequent tyrosine phosphorylation by the IGF-I receptor (Butler *et al.* 1998). IRS-1 signaling is necessary to the developmental myogenic effects of IGF-I. In mice with transgenic deletion of both IRS-1 alleles, constitutive overexpression of IGF-I results in rescue of normal organ development (e.g., kidney and heart), but is unable to produce normal growth of gastrocnemius muscle (Pete *et al.* 1999). Phosphotyrosines on IRS proteins act as docking sites for proteins containing Src homology-2 (SH2) domain-containing proteins which in the end results in downstream activation of the mitogen-activated protein kinases (MAPKs) and activation of phosphatidylinositol 3-kinase (PI3K) (De Meyts *et al.* 1994, De Meyts 2002).

IGF-I receptor plays a critical role in myoblast proliferation and differentiation. Among known muscle factors, IGF-I is unique in its ability to stimulate both myoblast proliferation and terminal differentiation to postmitotic myotubes (Figure 2). Activation of MAPKs is required for developmental IGF-I receptor-dependent mitogenesis (Coolican *et al.* 1997). By contrast, PI3K signaling mediates three crucial

effects of IGF-I on muscle: fusion of myoblasts into myotubes, anabolic effects on protein and glucose uptake, and resistance to programmed cell death, or apoptosis (Kaliman *et al.* 1998).

During post-natal growth it was shown that the binding capacity of IGF-I receptor changes and the pattern of receptor abundance and sensitivity to nutritional stress differs between skeletal muscles and cell types within skeletal muscle (Oldham *et al.* 1996). In rat muscle, cell surface IGF-I receptor expression drops by 80% between infancy and young adulthood with a subsequent decrease of 10 to 60% during senescence (Dardevet *et al.* 1994, Willis *et al.* 1997).



Adapted from Singleton & Feldman 2001

Figure 2. Intracellular signaling pathways following cell surface type I IGF receptor (IGF-I receptor) by IGF-I. Access of IGF-I to IGF-I receptor is mediated by IGFBPs. IGF-I binds to the extracellular subunit of IGF-I receptor, inducing autophosphorylation of the transmembrane subunit. Downstream signaling diverges through activation of either the mitogen-activated protein kinase (MAPK) cascade or phosphatydilinositol3-kinase (PI3K). MAPKs mediate the proliferative effects of IGF-I, while the consequences of PI3K signaling (listed) are more diverse.

1.2.4 IGF-I regulation

IGF-I activity is regulated in a complex manner. Circulating IGF-I concentrations are very sensitive to GH (Coleman *et al.* 1994, Oldham *et al.* 1996) and nutritional status (Rivero *et al.* 1995, Wilson *et al.* 1995, Pfaffl *et al.* 1998b). GH and nutrition are also two important regulators of IGF-I mRNA (Florini & Ewton 1992, Coleman *et al.* 1994).

Liver may be the most GH-responsive tissue for IGF-I production and IGF-I gene expression, while most non-hepatic tissues do not show an increase in IGF-I message in response to GH (Duguay *et al.* 1994, Rivero *et al.* 1995, Oldham *et al.* 1996). Pell *et al.* (1993) found that muscle IGF-I mRNA in sheep was insensitive to GH treatment as well as nutritional status. Similarly, treatment of pigs with porcine GH increased IGF-I mRNA levels in liver and adipose tissue, but not in skeletal muscle, although there was a substantial increase in muscle growth (Oldham *et al.* 1996). In another species of farm animals, cattle, there was a similar lack of correlation between GH levels, growth, and muscle IGF-I mRNA levels (Duguay *et al.* 1994). Wilson *et al.* (1995) observed that GH administration increased muscle IGF-I expression but he also suggested that muscle IGF-I mRNA can be produced in a GH-independent manner. Thus it seems very clear that IGF-I gene expression can occur in muscle without stimulation by GH. In addition to GH and nutritional status, other factors like hormonal, tissue-specific and developmental factors, all modulate IGF-I gene expression (Pell *et al.* 1993).

Testosterone has been implicated in the increased levels of IGF-I responsible for growth rate and body composition differences among intact males, castrated males and females (Ford & Llindt 1989). Increased muscle growth due to testosterone is caused in part by increasing the level of circulating IGF-I independent of GH

concentrations (Arnold *et al.* 1996). Steers receiving steroid hormone implants had increased circulating IGF-I concentrations compared with nonimplanted steers and the longissimus and semimembranosus muscles of implanted steers showed an increase in IGF-I mRNA levels compared with the same muscle in the nonimplanted steers (Pampusch *et al.* 2003, White *et al.* 2003).

Circulating IGF-I and IGF-I binding protein concentrations are increased in association with increasing levels of energy or protein intake (Hannon *et al.* 1991). Fasting alters muscle metabolism, exhausts glycogen stores, converts muscle to fatty acid metabolism, and ultimately forces protein degradation to fuel gluconeogenesis. Studies showed that IGF-I mRNA levels in the liver fall to less than 40% of control in rats after 30 hours of fasting (Clemmons & Underwood 1991) and other studies reported that fasting caused a decrease in muscle IGF-I mRNA levels in pigs (Emler & Schalch 1987). Insulin-like growth factor I mRNA and serum IGF-I decrease during acute and chronic fasting and rebound with refeeding (Lewis *et al.* 1997). Chronic malnourishment also lowers the serum IGF-I in rats and humans (Beaune *et al.* 1997, Gautsch *et al.* 1998). In sheep, circulating levels of IGF-I in plasma are regulated by nutrition (Oldham *et al.* 1999); however, the expression of IGF-I in skeletal muscle is relatively insensitive to short-term fasting (Oldham *et al.* 1996).

The effect of testosterone on muscle IGF-I mRNA while holding diet, genotype and other factors constant was examined in this experiment.

1.3 Testosterone, androgen receptor and muscle growth.

Testosterone is a naturally occurring steroid that is synthesized primarily by the Leydig cells of the testis (95%). It is also synthesized by the adrenal cortex, and a small amount is produced by the ovaries. Testosterone can be converted by aromatase

to estradiol and by 5α-reductase to 5α-dihydrotestosterone. The actions of testosterone and its metabolites within target cells are transduced by the low-abundance intracellular androgen receptor. It has been generally accepted the existence of a single molecular species of androgen receptor that functions primarily as a transcription regulatory protein (Zhou *et al.* 1994), and in this context the diverse biologic effects of androgens are surprising. The biologic effects of androgens can be classified into two major categories, either androgenic or anabolic. Androgenic biological effects include stimulation of the male reproductive tract, external genitalia, and secondary male sexual characteristics. Anabolic biological effects are the stimulation of growth for many organs such as muscle, kidney, salivary glands, and liver.

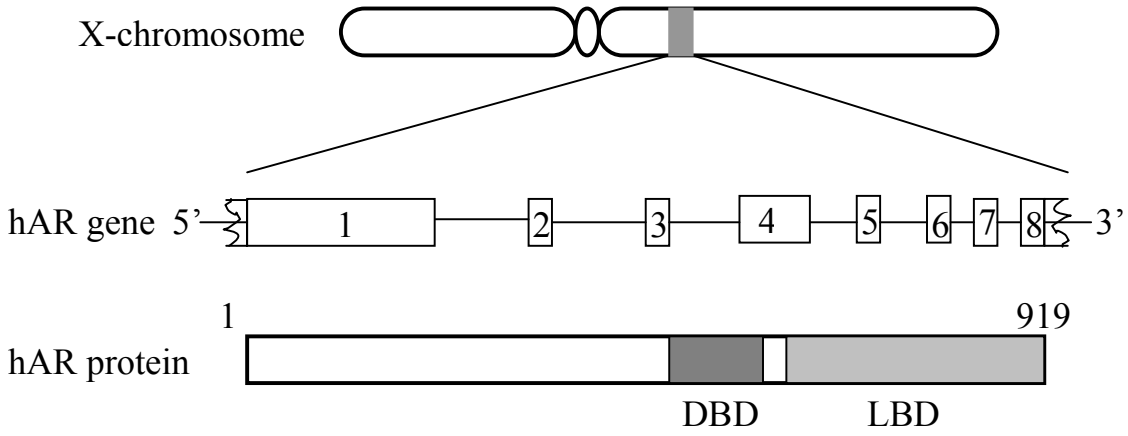
The androgen receptor is a member of the superfamily of nuclear transcription factors that mediate the action of steroid hormones. When activated by ligand binding, these transcription factors bind to specific DNA sequences on target genes, referred to as hormone response elements, and regulate the transcriptional activity of those genes. The receptor proteins are modular in nature and contain three common domains: a highly conserved central domain that is responsible for DNA binding, a variable N-terminal region that is involved in transcriptional activation, and a C-terminal domain that binds ligand. Specific regions within these domains that vary between receptors are involved in nuclear translocation, dimerization, phosphorylation, and interaction with accessory factors.

The human androgen receptor gene is located on the X chromosome and contains 8 exons, with a total length of 90 kb (Kuiper *et al.* 1989, Lubahn *et al.* 1989, Marcelli *et al.* 1990, Zhou *et al.* 1994) (Figure 3). The large first exon encodes the entire N-terminal domain. The small second and third exons separately encode the two DNA-binding zinc fingers that comprise the DNA-binding domain. The fourth exon encodes the hinge region that contains an important ligand-dependent nuclear

localization signal. The remaining four exons encode the hormone-binding domain (Zhou *et al.* 1994).

Androgen receptors are nuclear proteins that are activated by ligand binding (Marcelli *et al.* 1990). This initiates a complex cascade of events that include a conformation change, phosphorylation, dimerization, DNA binding of zinc fingers to androgen response elements, and interaction with accessory factors, other transcription factors, and RNA polymerase to alter the transcriptional activity of a specific gene (Poujol *et al.* 2000).

Unliganded androgen receptors are primarily localized to the cell nucleus, and androgens diffuse into the nucleus, where they bind to available receptors. Androgen binding to a receptor induces an allosteric conformational change of the protein that results in hormone-dependent, DNA-independent phosphorylation of the receptor at several sites and dissociation from associated factors (heat shock proteins). This dissociation allows the androgen receptor to dimerize, which is essential for binding to androgen response elements (Prins 2000). Due to specific androgen-induced



Adapted from Brinkmann 2001

Figure 3. Genomic organization of the human androgen receptor gene and functional domain structure of the androgen receptor protein. The androgen receptor gene has been mapped to the long arm of the X-chromosome. The androgen receptor protein is encoded by eight exons and consists of several distinct functional domains (DNA binding domain, ligand-binding domain).

conformational changes, the zinc fingers in the DNA-binding domain are available to associate with specific androgen response elements on regulated genes (Mangelsdorf *et al.* 1995).

In addition to their virilizing effects in the reproductive tracts of males, androgens have anabolic effects in some skeletal muscles. In adult animals, castration produces atrophy of the rat levator ani and bulbocavernosus muscles, frog flexor carpi radialis and flexor carpi centralis muscles, and several guinea pig muscles, such as the sternomastoid, spinotrapezius, and latissimus dorsi muscles, whereas the readministration of androgenic hormones to castrated animals results in normal growth of these muscles (Kochakian & Tillotson 1957, Thibert 1986, Rand & Breedlove 1992, Celotti & Negri 1992). Furthermore, Krieg *et al*. (1977) have also reported that

the number of ARs varies among skeletal muscles, which may explain regional differences in the physiological response to androgen administration.

Testosterone stimulates muscle growth by affecting the rate of protein synthesis, protein breakdown and the net gain or loss of muscle protein (Wong *et al.* 1993). When testosterone increases muscle protein synthesis, intramuscular mRNA concentrations of IGF-I are increased and concentrations of the inhibitory IGF binding protein 4 are decreased (Rooyackers & Nair 1997). The response to testosterone differs among muscle groups and this differential response may be explained by the variation of AR number among skeletal muscles (Urban 1999). Thus, sexual dimorphism can be explained partly by higher androgen sensitivities in muscles with pronounced growth under androgen stimulation (Sauerwein & Meyer 1989). A recent study in cattle showed that androgen receptor mRNA concentrations in muscles with different fiber type compositions and growth impetus is positively related to the individual growth patterns (Brandstetter *et al.* 2000).

1.4 Myostatin and the control of skeletal muscle mass

Myostatin, a member of the TGF-β superfamily also known as growth and differentiation factor-8 (GDF-8), is specifically expressed in muscle and is a key regulator of skeletal muscle development and growth. Although myostatin does not appear to be essential for either viability or fertility, its sequence is remarkably well conserved in animals, ranging from fish to birds and mammals, suggesting that the biological function of myostatin has been conserved throughout the animal kingdom (Brandstetter *et al.* 2000). The human myostatin gene comprises 3 exons and 2 introns and encodes for a 375-aa precursor protein (McPherron & Lee 1997) which contains a hydrophobic N terminus that acts as a secretory signal and a conserved domain that is important for proteolytic processing (McPherron *et al.* 1997, Thomas *et al.* 2000,

Sharma *et al.* 2001). Cleavage of the protein gives rise to an N-terminal latency associated peptide (LAP) noncovalently attached to the C-terminal mature signaling peptide, which varies in size from 12.5 to 30 kDa, depending on species and posttranslational modifications (Gonzalez-Cadavid *et al.* 1998, Thomas *et al.* 2000, Lee & McPherron 2001, Taylor *et al.* 2001). The mature signaling peptide binds to its receptor, suggested to be activin type II receptor, to elicit its biological functions (Lee & McPherron 2001).

Myostatin null mice generated by gene targeting showed a dramatic and widespread increase in skeletal muscle mass. Individual muscles in myostatin null mice weighed 2- to 3-fold more than those of wild-type mice, primarily due to an increase in the number of muscle fibers (hyperplasia) and an increase in fiber diameter (hypertrophy) (McPherron *et al.* 1997). In addition, myostatin knockout mice accumulate less fat than their wild type littermates (Lin *et al.* 2002, McPherron & Lee 2002), which may be a secondary consequence of increased muscle mass since a similar effect is seen in other genetic models of muscle hypertrophy (Sutrave *et al.* 1990, Musaro *et al.* 2001). Aside from having increased muscle and decreased fat, the myostatin knockout mice appear normal and healthy. Furthermore, the "double-muscle" phenotypes of three breeds of cattle (Belgian Blue, Piedmontese (Grobet *et al.* 1997) and Asturiana de los Valles (Dunner *et al.* 1997)) have been linked to nucleotide deletions, transitions, or transversions within the coding region of the myostatin gene. Belgian Blue and Piemontese double-muscled cattle have a 15 to 30% increase in skeletal muscle mass, in addition to higher birth weights and also lower fat and bone percentages compared to non-double muscled animals. Also, other breeds of cattle that are characterized by increased muscle mass (double muscling) have mutations in the myostatin coding sequence (Kambadur *et al.* 1997, Brandstetter *et al.* 2000). These mutations likely compromise the biological activity of the protein, which leads to

increased muscle via hyperplasia and hypertrophy. The inheritance of double muscling in Belgian Blue cattle has been identified as a monogenic autosomal segregation pattern (Hanset & Michaux 1985, Charlier *et al.* 1995). The muscular hypertrophy (mh) locus has been termed "partially recessive" because a single copy of the allele can have some effect, although the full double-muscled phenotype requires the cattle to be homozygous. Gene mapping (Charlier *et al.* 1995) of the Belgian Blue cattle localized the mh gene to the centromeric end of the bovine chromosome 2 linkage group. The myostatin gene has been mapped to the same interval as the mh locus by genetic linkage (Smith *et al.* 1997) indicating that the myostatin gene and the muscular hypertrophy gene are one and the same.

Initial studies from myostatin null mice and from double-muscled cattle breeds indicated that lack of myostatin activity during embryonic development causes growth of muscle tissue but there was no evidence that loss of myostatin activity during adult life has a similar effect. This question was addressed recently when Zimmers *et al.* (2002) showed that injection of high levels of myostatin into the thighs of adult mice produced, on average, 33% of total body weight loss. Part of this loss was due to a global decline in skeletal muscle mass and a near-total loss of adipose tissue. In contrast, inhibition of myostatin in adult mice for as little as 2 to 4 weeks leads to an increase in skeletal muscle size (Whittemore *et al.* 2003, Grobet *et al.* 2003). The increase in muscle mass, which varied from 13 to 30% was highly reproducible and occurred in both male and female mice of a variety of strains. The effect was observed in young adults as well as 24-week-old mice indicating that myostatin acts postnatally as a negative regulator of skeletal muscle growth.

Male transgenic mice that overexpress myostatin protein selectively in the skeletal muscle had an 18 to 24% decrease of skeletal muscle mass and a decrease of muscle fiber size (Reisz-Porszasz *et al.* 2003). Female transgenic mice generated the

same way did not differ from wild-type controls in either body weight or skeletal muscle mass even though the expression of myostatin mRNA and protein was increased in the skeletal muscle and was not lower compared with male mice. However, it was shown that increased body and muscle mass in male compared with female mice is associated with decreased expression of processed myostatin (McMahon *et al.* 2003). Interestingly, this decreased expression of processed myostatin is due to regulation after translation and, presumably, after secretion, because there was no difference in mRNA or in the LAP form of myostatin. Although myostatin-null mice have two to three times more muscle mass than wild-type controls, males remain heavier than females (McPherron *et al.* 1997), implying that a reduction in myostatin does not regulate sexual dimorphism per se. Rather, it may enable anabolic factors like testosterone to enhance growth.

Experiments with muscle atrophy and mechanically-induced hypertrophy were conducted to address the possible myostatin function in hypertrophy of fibers of fully differentiated muscle. Muscle atrophy caused by either hindlimb unloading (Carlson *et al.* 1999, Wehling *et al.* 2000, Reardon *et al.* 2001) or by denervation (Sakuma *et al.* 2000) increased myostatin mRNA and protein level. These observations were supported by findings of Gonzales-Cadavid *et al.* (1998) who demonstrated that myostatin protein level increased in the muscle of HIV-infected patients undergoing weight loss. Myostatin protein level was also increased in mechanically-hypertrophied rat muscles.

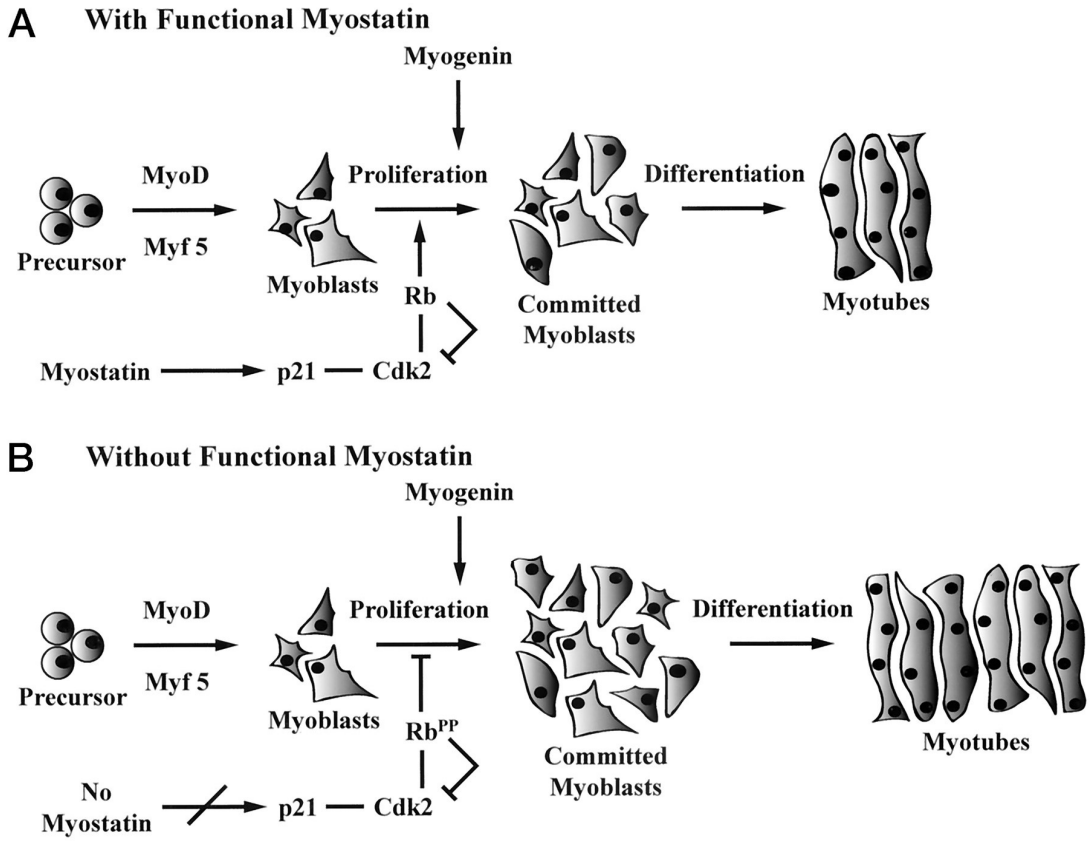
A series of reports have shown that myostatin mRNA and /or protein concentrations are increased in skeletal muscle in conditions associated with loss of muscle mass in postnatal life, such as sarcopenia of old age (Marcell *et al.* 2001, Schulte & Yarasheski 2001, Yarasheski *et al.* 2002) and disuse atrophy in men under prolonged bed rest (Zachwieja *et al.* 1999), after exposure to the microgravity

environment of a spacelift (Lalani *et al.* 2000), and in association with glucocorticoid-induced muscle loss (Lang *et al.* 2001, Ma *et al.* 2003). In contrast, recovery of muscle mass in the late stages of muscle regeneration is associated with a decrease of myostatin from the elevated levels detected immediately after injury (Kirk *et al.* 2000, Sakuma *et al.* 2000, Yamanouchi *et al.* 2000).

Myostatin was shown to inhibit differentiation of adipocytes in vitro (Kim *et al.* 2001). Furthermore, loss of myostatin prevents an age-related increase in adipose tissue mass and partially attenuates the obese and diabetic phenotypes of two mouse models of obesity and diabetes, agouti lethal yellow (A^y) and obese ($Lep^{ob/ob}$) (McPherron & Lee 2002).

Myostatin was shown to function by controlling the proliferation of muscle precursor cells (Thomas *et al.* 2000). Myostatin promotes an increase in *p21* expression, a cyclin-dependent kinase inhibitor, and a decrease in Cdk2 protein and activity thus resulting in an accumulation of hypophosphorylated Rb protein. This in turn, leads to the sequestration of the E2F transcription factors, a family of proteins that are essential for the G1/S progression, which leads to the arrest of myoblasts in the G1-phase of cell cycle (Thomas *et al.* 2000). Thus, myoblast number and, hence, fiber number, following differentiation, is regulated (limited), suggesting that the increased number of myofibers in cattle and mice with heavy muscling is a result of deregulated myoblast proliferation caused by the absence of functional myostatin (Figure 3). The inhibitory effect of myostatin on myoblast proliferation has been shown to be reversible, as myoblasts maintained their ability to proliferate after myostatin was removed. Recently it was demonstrated that myostatin also negatively regulates myoblast differentiation (Langley *et al.* 2002). The degree of differentiation inhibition is dependent on the concentration of myostatin and cultured myoblasts readily differentiate upon removal of myostatin from the media. The inhibition of

differentiation by myostatin was associated with decreased expression of the myogenic determining genes; *myoD*, *myf5*, *myogenin*, and *p21*.



Adapted from Thomas et al. 2000

Figure 4. A model for the role of myostatin in muscle growth.
A, during embryonic myogenesis, Myf-5 and MyoD specify cells to adopt the myoblast fate. Myoblasts then migrate and proliferate. In response to myostatin signaling, p21 is up-regulated, inhibiting cyclin-E·Cdk2 activity, which causes Rb inactivation and G_1 arrest. Thus, myoblast number and, hence, fiber number, following differentiation, is regulated (limited).
B, in the absence of functional myostatin, the signal for p21 up-regulation is lost and Rb remains in a hyperphosphorylated form, resulting in deregulated (increased) myoblast proliferation, which leads to increased fiber number.

CHAPTER II

MATERIALS AND METHODS

2.1 Animals

East Friesian x Dorset sired ram lambs born from Dorset ewes were used in a 2 x 4 factorial experiment. Twenty sets of twins were assigned randomly to four age groups corresponding to 77, 105, 133 and 161 days of age. The ages were chosen to start just before puberty (77 days of age) followed by 3 more ages at 4 week intervals. Within two days after birth, one of the twins was randomly selected and castrated using a rubber band. Four sets of twins were assigned for each of the first two age groups and 6 sets for each of the last 2 age groups to ensure sufficient sets of twins were available for sampling at older ages. During the time of the experiment, one twin from each of two sets assigned to the 161-day sampling age were lost, therefore only 4 sets of twins were available for this age group. Lambs were fed the standard Cornell high energy barley based diet with 15% soy hulls added for digestible fiber, 14% soybean meal for protein and limestone, sheep salt, ammonium chloride and vitamin premix to satisfy the vitamin and mineral requirements. All animals were slaughtered in the Department of Animal Science abattoir within a 3-day range surrounding the assigned slaughter age. The animals were weighed before slaughter and the carcass weight was recorded after removal of the head, feat, skin and internal organs. Semitendinosus (ST) and splenius (SP) muscles were completely removed, trimmed of visible fat and weighed. Within 15 min after exsanguination, samples from both muscles were snap-frozen in liquid nitrogen, and stored at -70°C until they were subsequently assayed.

2.2 RNA isolation

Total RNA was isolated using the guanidine thiocyanate, acid phenol:chloroform procedure of Chomczynski and Sacchi (ToTALLY RNA™ Total RNA Isolation Kit, Ambion). Using a hand-held Tissue Tearor, about 140 mg muscle tissue were homogenized on ice in guanidinium-based lysis solution. Samples were extracted sequentially with Phenol:Chloroform:IAA (phenol, chloroform and isoamyl alcohol at a ratio of 25:24:1, pH 6.6) and Acid-Phenol:Chloroform (phenol and chloroform at a 5:1 ratio, pH 4.7). The RNA was then precipitated with isopropanol and the RNA pellet was resuspended in 60 to 70 μL of 1 mM sodium citrate, pH 6.4 +/- 0.2 (The RNA Storage Solution, Ambion). RNA integrity was verified by inspection of the 28S and 18S rRNA bands using denaturing agarose gel electrophoresis and ethidium bromide stain.

The concentration and purity of RNA in the final preparations was determined by diluting an aliquot of the preparation (1:50) in TE (10 mM Tris-HCl pH 8, 1 mM EDTA) and reading the absorbance in a spectrophotometer at 260 nm and 280 nm. The ratio of A260 to A280 values was a measure of RNA purity, and it should fall in the range of 1.8 to 2.1.

2.3 RNase Protection Assay

2.3.1 Preparation of the AR, myostatin, GAPDH labeled antisense and unlabeled sense riboprobes. Full-length cDNA was generated from 500 ng of total ovine muscle RNA using a gene specific primer (Appendix Table 8) in the reverse transcription (Display THERMO-RT Kit). Total RNA, 1xRT buffer, 0.5 mM dNTPs (each of dATP, dGTP, dCTP, dTTP), 1 μM reverse primer, and display Terminator Mix were incubated for 40 min at 42°C followed by 10 min at 65°C. Specific regions

were amplified from 1 μL of cDNA in 10 μL PCR amplification reactions (Appendix Table 8) using an Idaho Technologies Rapid Cycler. Each 10 μL reaction contained Idaho Technologies buffer (50 mM Tris, pH 8.3; 0.25 mM crystalline BSA; 2 mM or 4 mM $MgCl_2$; and sucrose), 0.4 U *Taq* DNA polymerase (Gibco BRL), 0.2 mM dNTPs (Promega Corporation) and 0.5 μM each primer. The 453 bp myostatin PCR product containing parts of exons 1 (63 bp) and 3 (16 bp) and all of exon 2 (374 bp) and the 556 bp GAPDH PCR product containing parts of the exons 6 (74 bp) and 8 (70 bp) and all of exon 7 (412 bp) were cloned into pCR™ II using Invitrogen's TA cloning kit. The 910 bp oAR PCR product spanning 90 bp of exon 2, all of exons 3 (118 bp), 4 (297 bp), 5 (136 bp), 6 (130 bp) and 139 bp of exon 7 was cloned into pCR ™2.1.

The plasmids were subsequently transfected into INVαF' competent cells (Stratagene, CA). Positive transformants were selected on the basis of ampicillin resistance and a selection of these were grown in LB broth containing ampicillin (50 μg/μL). The ovine cDNA structures for AR, myostatin, GAPDH and the orientations in the plasmid were confirmed by sequencing. The myostatin and GAPDH PCR products were found to be in reverse sequence in the vector. The plasmids were named pCR(MSTN) and pCR(GAPDH) to designate cDNA templates for ovine myostatin and GAPDH respectively, and pCR(ARs) and pCR(ARas) to designate cDNA templates for ovine AR sense and AR antisense (because the pCR ™2.1 had only the T7 promoter, two plasmids were required; one for each orientation), respectively (Appendix Figures 17, 18, 19). Large scale plasmid DNA purification was carried out using a Qiagen Plasmid Kit.

The vectors pCR(MSTN), pCR(ARs) and pCR(ARas) were linearized by BamHI, BamHI and HincII digestion, respectively, and the cloned ovine cDNAs were transcribed with T7 RNA polymerase at 37°C for 1 hour using the reagents supplied with the Ambion's MaxiScript Kit to produce sense myostatin, sense AR and antisense

AR, respectively. The vectors pCR(MSTN) and pCR(GAPDH) were linearized by DraI and EcoO109I digestion, respectively, and the cloned ovine cDNAs were transcribed with SP6 RNA polymerase (Ambion) at 37°C for 1 hour to produce antisense myostatin and antisense GAPDH. At the end of the reaction, 1 μL RNase-free DNase I (10 U) was added and the reaction was incubated for 15 min at 37°C. Subsequently, all sense and antisense RNA transcripts were gel purified and the concentration was calculated from the OD_{260} after gel elution. The antisense RNA transcripts were labeled with Psoralen-Biotin using the BrightStar™ Psoralen-Biotin Nonisotopic Labeling Kit (Ambion). Due to the abundance of GAPDH, the probe concentration was adjusted to generate a signal in the same range as the myostatin and AR signal. This adjustment was accomplished by mixing biotin-labeled and unlabeled GAPDH probe at a ratio of 1:25.

2.3.2 RNase protection assay. Forty micrograms of SP and ST muscle total RNA were coprecipitated with 1 ng of labeled myostatin, AR and GAPDH riboprobes and hybridization was performed at 42°C overnight using the protocol and the reagents supplied in the RPA III™ Kit (Ambion) as described in the standard procedure. The next day, the mixture was treated with an RNase cocktail (0.04 U of RNase A and 1.5 U of RNase T1) to degrade single-stranded RNA. The protected fragments were precipitated, the pellets were washed once with 75% ethanol, dried, resuspended in 10 μL of gel-loading buffer, denatured at 95°C for 5 min, loaded onto a 6% polyacrylamide/8M urea denaturing gel and run at 200V for approximate one hour. The RNA was then electrophoretically transferred to a positively-charged nylon membrane (Bright Star-Plus, Ambion) by using a mini-gel blotting apparatus (BioRad) and 1xTBE as transfer buffer. The transfer was carried on for 1 hour at 100 mA, and the membrane was UV irradiated to crosslink the RNA to the nylon. The Ambion BrightStar BioDetection Kit was used for chemiluminescent detection of the

protected fragments. At the end of the procedure, the membrane was exposed to X-ray film (Kodak) overnight at room temperature and for shorter periods (between 30 min and 6 hours) the following day.

2.3.3 Quantitative analysis of myostatin and AR mRNAs. On each sample gel, known amounts (ranging from 52 to 0.25 pg) of in vitro synthesized myostatin and AR sense RNA were hybridized with an excess of labeled antisense probe (1 ng) to construct standard curves. The values were plotted as the log of the amount of sense RNA vs. the log of the intensity of the protected band in the standard curve. The absolute amount of the protected RNA in the muscle sample RNA was determined by comparing the intensity of the band to the standard curve. In some cases, the band density in the sample was outside the range of the standard curve. Extrapolating was possible because these points were close to the range of the standard curve and also, the points in the standard curve fitted the linear equation precisely (r^2 ranging from 0.956 to 0.999). The amount determined using the standard curve was then corrected for size differences and for RNA loading. The protected fragments in the experimental samples differed in size from the protected fragments in the standard curve; therefore the difference in the amount of biotin had to be corrected. The following equation was used to correct for size difference:

$$\text{Amount} = \text{amount} \times \frac{\text{protected fragment in the standard curve (bp)}}{\text{protected fragment in the experimental samples (bp)}}$$

To correct for variations in RNA loading, the amount corrected for size differences was multiplied by the ratio between the highest GAPDH amount from all gels (considered to correspond to the 40 μg RNA loaded into the gel) and the GAPDH amount from the respective lane.

2.4 RT-PCR

2.4.1 Construction of a heterologous competitor. The relationship between the initial amount A of target mRNA present in the tissue and the amount Y_n of DNA produced after n PCR cycles can be expressed as $Y_n = A \cdot (1+E)^n$, where E is the amplification efficiency of one reaction cycle (Chelly *et al.* 1988). Small variations in reaction efficiency, therefore, translate into large differences in the amount of RT-PCR product generated after n cycles. These limitations in quantitative analyses can be overcome by parallel co-amplification of the native mRNA together with known amounts of an internal standard cRNA in a competitive PCR. The amplification efficiency should affect both templates similarly. Several designs have been used in quantitative RT-PCR to obtain an internal standard cRNA that has identical amplification efficiency to native mRNA template and is easily distinguishable from it. One common practice is the use of a PCR MIMIC, a heterologous internal standard that "mimics" the primer binding and amplification characteristics of the target (Gaudette & Crain 1991, Siebert & Larrick 1993), obtained by the addition of primers specific to gene products such as ubiquitously expressed "housekeeping" genes, like GAPDH (Murphy *et al.* 1990, Gaudette & Crain 1991).

To quantify ovine muscle IGF-I mRNA abundance by RT-PCR, a heterologous competitor (MIMIC) was constructed based on the ovine GAPDH sequence obtained by RT-PCR using 20 nt primers homologous to ovine GAPDH sequences to which an additional 20 nt were appended on the 5' ends. The additional 20 nucleotides corresponded to the IGF-I forward and reverse primers. These composite primers (Appendix Table 8) amplified a 325 bp product, including 20 bp for IGF-I at both ends.

Reverse transcription was used to produce the MIMIC DNA. Using the Display THERMO-RT Kit, 500 μg of total ovine muscle RNA was amplified with an IGFGAPDH reverse primer in the reverse transcription. One μL of cDNA from the reverse transcription reaction was used in PCR amplification reaction using the Idaho Technologies Rapid Cycler and the two composite primers (IGFGAPDH forward primer and IGFGAPDH reverse primer). The 325 bp GAPDH MIMIC PCR product was cloned into pCR™ II using Invitrogen's TA cloning kit. The plasmid was subsequently transfected into INVαF' competent cells (Stratagene, CA). Positive transformants were selected on the basis of ampicillin resistance and, from all positive colonies, 10 were grown in LB broth containing ampicillin (50 μg/μL). The orientation in the plasmid was checked by digestion with AluI restriction enzyme which has 28 restriction sites, one in the plasmid. Large scale plasmid DNA purification was carried out using the Qiagen Plasmid Kit. The vector was linearized by EcoRV digestion, and the cloned ovine cDNA was transcribed with SP6 RNA polymerase at 37°C for 1 hour using the reagents supplied with the Ambion's MaxiScript kit to create a 422 bp template containing the 325 bp MIMIC and 97 bp of the plasmid. At the end of the reaction, 1 μL RNase-free DNase I (10 U) was added and the reaction was incubated for 15 min at 37°C. The concentration of RNA MIMIC was determined using spectrophotometry.

2.4.2 Establishment and validation of quantitative RT-PCR. To ensure a parallel start in all individual reactions and to increase specificity, yield and precision of the PCR, Zombie-Taq (Clontech) DNA polymerase was used. In the Zombie-Taq DNA polymerase, the polymerase activity is inhibited by the Taq antibody until the first denaturation step inactivates the antibody. To quantify the native IGF-I mRNA in different tissues, a preliminary estimation of the IGF-I cRNA start-molecule concentration range to be used for individual tissues was required. Initially, a broad

range of dilutions was titrated to obtain a rough estimate of the amount of RNA present. This was performed by 6 titration steps ranging from 2500 to 0.025 cRNA start-attomoles of MIMIC in a 1:10 dilution scheme added to 600 ng total tissue RNA. A finer titration was then performed over a narrower range of MIMIC (24.1 to 0.01 attomoles 1:3 dilution scheme). The final dilution series had MIMIC amounts above and below the equivalence point for target IGF-I in tissue RNA.

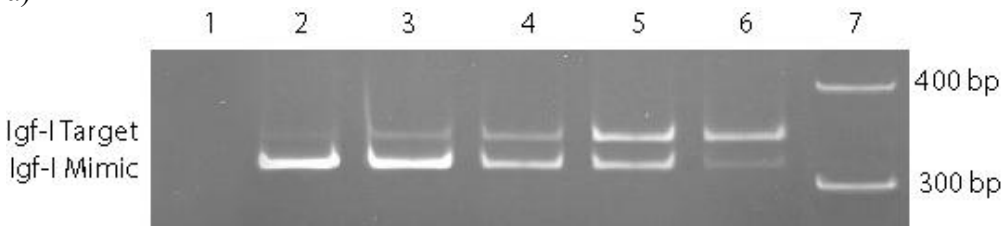
2.4.3 Competitive RT-PCR. For each of the 2 muscle tissues, five RT reactions were performed using 600 ng of total RNA and fixed concentrations of the MIMIC IGF-I cRNA. Because the amount of IGF-I mRNA in muscle decreased with age, two different dilution series of the MIMIC IGF-I cRNA were used in order to keep the equivalence point close to the middle (9, 3, 1, 0.33, 0.11attomoles for the first 18 animals and 3, 1, 0.33, 0.11, 0.04 attomoles for the remaining 18 animals). First-strand cDNAs of target and MIMIC were generated using the Omniscript^{TM} RT Kit (Qiagen) in an Amplitron (Barnstead/Thermolyne, Dubuque, IA) thermocycler. The RNA, 1xRT buffer, 0.5 mM of each dNTP, 1 μM reverse primer, and 2U Omniscript Reverse Transcriptase were incubated for 60 min at 37°C. At the end of the reaction, the Omniscript Reverse Transcriptase was inactivated by heating the reaction mixture to 93°C for 5 min followed by rapid cooling on ice. The PCR was performed in a Rapid Cycler (Idaho Technologies) with 1 μL cDNA; Idaho Technologies buffer (50 mM Tris, pH 8.3; 0.25 mM crystalline BSA; 2 mM $MgCl_2$; and sucrose), 0.4 U *Taq* DNA polymerase (Gibco BRL), 0.2 mM dNTPs (Promega Corporation) and 0.5 μM each primer. The PCR used 30 cycles (0 sec at 94°C, 0 sec at 60°C, 15 sec at 75°C) followed by an additional 30 sec at 75°C for complete amplification of all PCR products. In each experiment, a negative control, with water replacing template, was included.

2.4.4 Quantification of PCR products. The ten μL PCR product was electrophoresed on a 5% polyacrylamide gel, which was then stained in a solution of 0.1 μg/mL ethidium bromide (Figure 5a). The gel was scanned using a gel documentation and analysis system (Alpha Innotech Corp.) under UV-light. The area of optical densities of the target and the MIMIC peaks were quantified using the Alpha Innotech software (Appendix Figure 16). Because the size of the MIMIC cDNA was smaller than the size of the target cDNA, the decrease in the incorporation of ethidium bromide had to be corrected. The optical density associated with the 325 bp competitor (MIMIC) product was corrected by a multiplication factor corresponding to the bp ratio of both fragments as follows:

$$\text{Corrected (band density)} = \text{band density} \times \frac{\text{native bp length}}{\text{competitor (MIMIC) bp length}}$$

A $\log_{10}$ transformation was used and the regression of $\log_{10}$ of ratio of the corrected MIMIC band densities to the target band densities (Y variable) against $\log_{10}$ of concentration of the MIMIC (X variable) was performed. As shown in Figure 5b, the association was linear. When the ratio of MIMIC to target was 1 (bands densities equal) the amount of the target and the MIMIC was equal. To determine the amount of target IGF-I in the sample, the solution to the equation with Y set to 0 was transformed from $\log_{10}$ to attomoles (10^X).

a)



b)

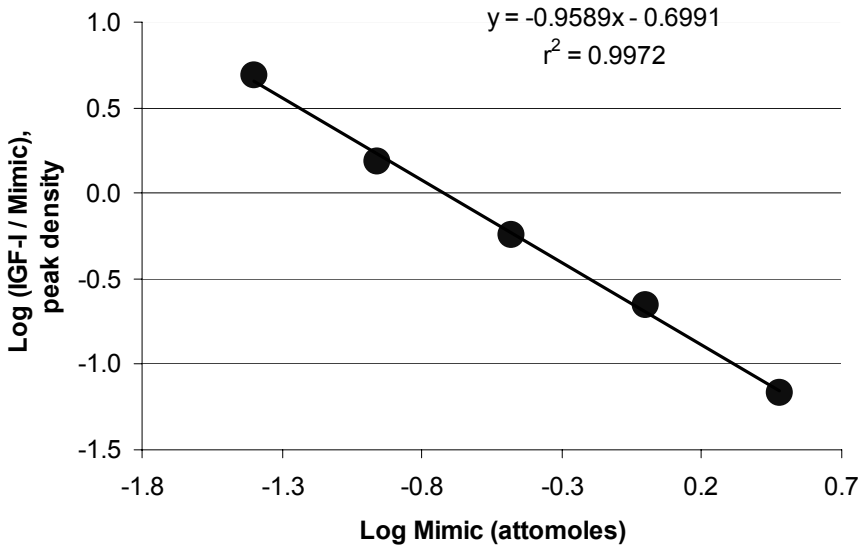


Figure 5. Competitive RT-PCR.
a) Total RNA (600 ng) was coamplified with a dilution series of IGF-I MIMIC cRNA (9, 3, 1, 0.33, 0.11 attomoles for the first 18 animals and 3, 1, 0.33, 0.11, 0.04 attomoles for the rest 16 animals [lanes 2-6]). Lane 1 was a negative control with water replacing template. Lane 8 represented the 100 bp DNA ladder (Life Technology, MD).
b) Following gel electrophoresis and staining with ethidium bromide, the gel was scanned and the density of each peak was integrated. After correcting for size differences, the $\log_{10}$ of the ratio of IGF-I product peak density to IGF-I mimic peak density was plotted against the $\log_{10}$ of the amount of IGF-I Mimic cRNA added. The quantity of IGF-I mRNA was determined by setting Y = 0 and transforming the solution for X from $\log_{10}$ to attomoles (10^X).

2.5 Statistical analyses.

Carcass weight.

After an initial fit of the model (1) on carcass weight, the plot of the absolute residuals against the fitted values for carcass weight detected heterogeneity of residual variance. A natural log transformation was effective in correcting this problem. The log of carcass weight was analyzed using the following fixed effects statistical model:

(1) $$Y_{ijkl} = \mu + A_i + S_{ij} + P_{ki} + e_{ijkl}$$

where

Y_{ijkl} = log carcass weight from the l^{th} animal, within the k^{th} pair, of the j^{th} sex, in the i^{th} age class,

μ = overall mean,

$A_i = i^{th}$ age class (i = 77d, 105d, 133d, 161d),

$S_{ij} = j^{th}$ sex within i^{th} the age class (j = ram, wether),

$P_{ki} = k^{th}$ pair within i^{th} the age class (k = 1, 2,…18),

e_{ijkl} = error associated with $ijkl^{th}$ observation, assumed to be normally distributed N (0, σ^2_e).

The fixed effects model had 35 degrees of freedom: the age class effect with 3; the sex within age effect with 4; the pair within age effect with 14 and the error term with 14 degrees of freedom. The sex within age effect was evaluated using four one degree of freedom orthogonal contrasts that compared the log carcass weight between rams and wethers for each of the four age classes.

SP and ST muscle weights.

The difference between the two muscle weights within each individual was considered to evaluate the effect of sex on the growth of the two muscles over time.

Because ST was much larger than SP, a natural log transformation was used to transform the two muscle weights to comparable scales. A new variable was created as the difference between the log SP and log ST muscle weights within each individual, DMW = (lnSP - lnST). The difference between the two muscle weights was analyzed using the same fixed effects statistical model (1).

The sex effect was evaluated using four one degree of freedom orthogonal contrasts that compared the difference in log SP and log ST muscle weight between rams and wethers for each of the four age classes.

Gene expression.

The main objective was to compare the gene expression in two specific muscles. Because the two muscles were sampled from each individual, the gene expression in each muscle represented paired observations with respect to the individual. A new variable was created as the difference between mRNA concentration (attomoles/μg total RNA) in the two muscles for each gene studied, D = (SP - ST).

The difference in gene expression in the SP and ST muscles was analyzed using the same fixed effects statistical model (1).

The sex effect was evaluated using four one degree of freedom orthogonal contrasts that compared the difference in gene expression in the SP and ST muscles between rams and wethers for each of the four age classes.

To test if there was a difference between sexes in gene expression between the two muscles, a hypothesis test was performed. The null hypothesis was H_o: d = 0 and the alternative hypothesis was H_a: d > 0 for IGF-I and AR, and H_a: d < 0 for myostatin.

The assumptions of normality and equal variance were checked with a normal probability plot and a plot of the absolute values of residuals against the predicted values. All tests were performed for an α level of 0.05.

CHAPTER III
RESULTS AND DISCUSSION

Eighteen sets of twins born during the March 2002 lambing season between March 15th and April 2nd were used in this study. All the animals were slaughtered throughout the summer of 2002 from June 4th to September 9th. For a general description of the data, the subclass means and standard deviations for all variables in this experiment are presented. Additional information on individual animals is presented in Appendix Table 9.

Table 1. Carcass, semitendinosus (ST) and splenius (SP) muscle weights.

Sex	Number of animals	Age group (days)	Carcass weight (kg)	ST muscle (g)	SP muscle (g)
Rams	4	77	6.58 ± 0.96	34.50 ± 10.14	3.37 ± 0.69
Wethers	4	77	9.018 ± 2.47	51.50 ± 13.85	4.99 ± 1.50
Rams	4	105	13.558 ± 3.15	75.02 ± 12.43	8.42 ± 2.38
Wethers	4	105	14.178 ± 1.49	83.97 ± 5.23	7.12 ± 1.00
Rams	6	133	19.208 ± 4.04	96.25 ± 15.89	13.20 ± 3.29
Wethers	6	133	17.358 ± 3.07	89.82 ± 15.48	9.08 ± 1.12
Rams	4	161	17.978 ± 2.75	99.32 ± 16.84	14.27 ± 5.61
Wethers	4	161	18.26 ± 0.86	96.97 ± 14.39	9.75 ± 3.36

Table 2. IGF-I, AR and myostatin mRNA concentrations (attomoles per μg RNA).

Sex	Age group (days)	Attomoles / μg RNA		
		IGF-I mRNA SP-ST	AR mRNA SP-ST	MSTN mRNA SP-ST
Rams	77	0.11 ± 0.11	0.21 ± 0.22	-0.36 ± 0.29
Wethers	77	0.25 ± 0.10	0.30 ± 0.16	-0.12 ± 0.46
Rams	105	-0.05 ± 0.07	0.74 ± 0.38	-0.16 ± 0.29
Wethers	105	-0.05 ± 0.09	0.23 ± 0.26	-0.18 ± 0.21
Rams	133	0.16 ± 0.06	0.90 ± 0.31	-0.27 ± 0.47
Wethers	133	-0.05 ± 0.06	0.05 ± 0.21	-0.24 ± 0.50
Rams	161	0.16 ± 0.04	0.95 ± 0.19	0.04 ± 0.13
Wethers	161	-0.02 ± 0.02	0.04 ± 0.19	-0.27 ± 0.28

3.1 Carcass weight

A plot of carcass weight data against age for the two sexes is presented in Figure 6.

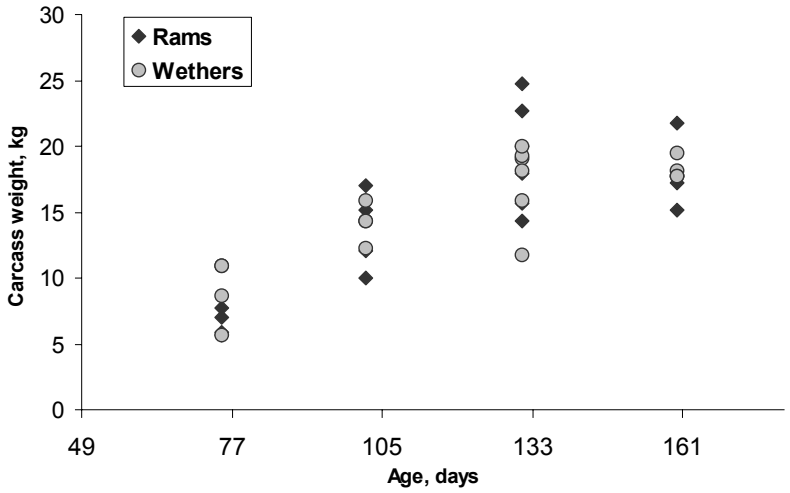


Figure 6. Carcass weight for rams and wethers across ages.

Wethers were heavier than rams at 77 days of age and the difference approached significance ($p = 0.07$) but no sex-related differences were found for the other age groups (Table 3 and Appendix Table 10). When evaluating carcass weight, the small sample size for each age group and the fact that the rams and the wethers in this experiment are pairs of twins need to be considered. Within each twin pair one individual is likely to be heavier than the other one especially at younger ages. Therefore the difference in carcass weight between the two sexes found in the first age group could be attributed to chance alone. Even though no statistically significant difference in carcass weight between rams and wethers after puberty was found, carcasses from wethers at older ages appeared to be fatter, in line with the report that when animals are allowed *ad libitum* access to a high-energy diet, anabolic effects of hormones are sometimes observed only in body composition differences because control animals consume enough food to gain significant amounts of fat (Rosemberg *et al.* 1989).

Table 3. Contrasts evaluating sex within age effect on log carcass weight.

Model: Lg(Carcass Weight) = Age + Sex(Age) + Pair(Age)					
Contrast	*DF*	*SS*	*MS*	*F Value*	*p value*
Sex within 77 days	1	0.17	0.17	3.75	0.07
Sex within 105 days	1	<0.01	<0.01	0.17	0.69
Sex within 133 days	1	0.02	0.02	0.64	0.44
Sex within 161 days	1	<0.01	<0.01	0.02	0.88

3.2 Semitendinosus and splenius muscle weights

The raw data for SP and ST muscles classified by sex and age and by sex and carcass weight are presented in Figure 7. There was a difference in the SP muscle weight between rams and wethers at later ages in this experiment while the ST muscle weight was similar between the two sexes across all ages (Figure 7a). The same difference for the SP muscle weight was associated with heavier carcass weights (Figure 7b).

The genetic similarity between twins was accounted for by the pair effect.

The statistical significance of the sex effect on the difference between the two muscles was evaluated using orthogonal contrasts (Table 4 and Appendix Table 11). No significant difference was found between rams and wethers for the difference in the weight of the two muscles at 77 days of age but the sex effect was significant at 105, 131 and 161 days of age ($p = 0.05$, $p = 0.04$ and $p = 0.02$). The heavier weight of the SP muscle in rams relative to wethers for the same age supports the hypothesis that the presence of testosterone is an important factor in sexual dimorphism of SP muscle.

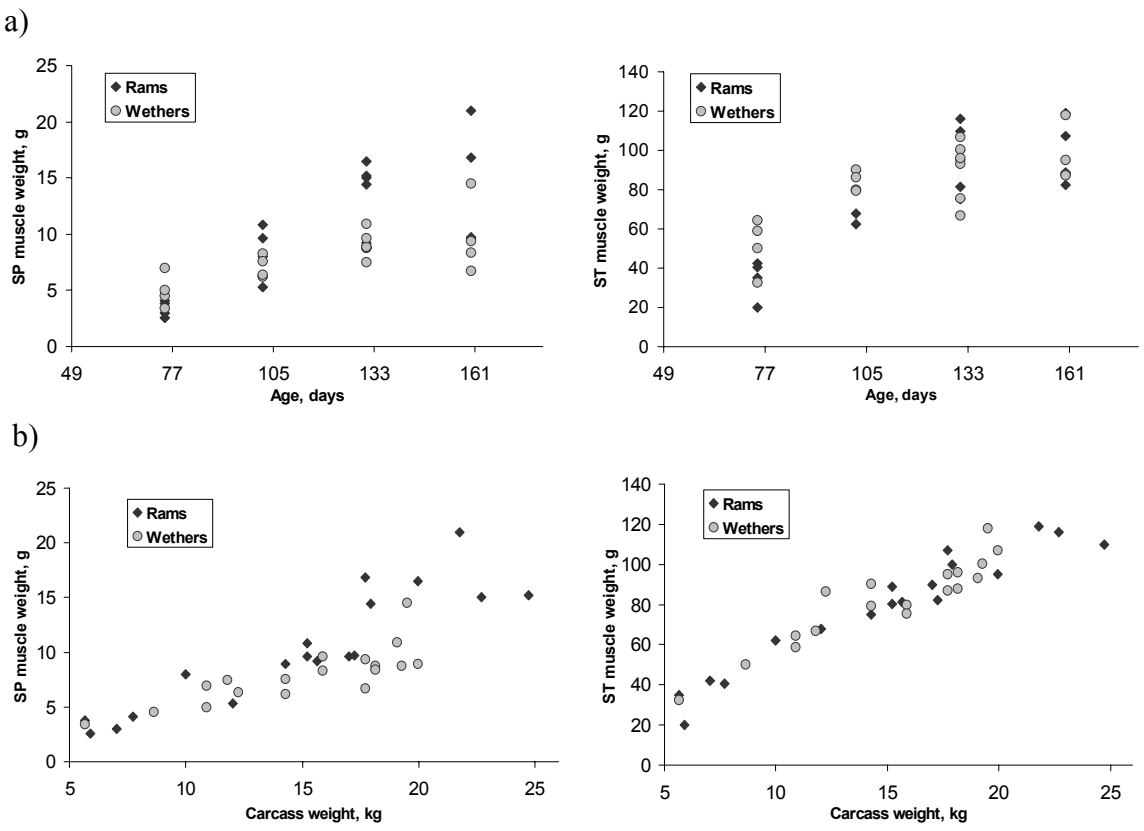


Figure 7. a) Splenius and semitendinosus muscle weights for rams and wethers versus age. b) Splenius and semitendinosus muscle weights for rams and wethers versus carcass weight.

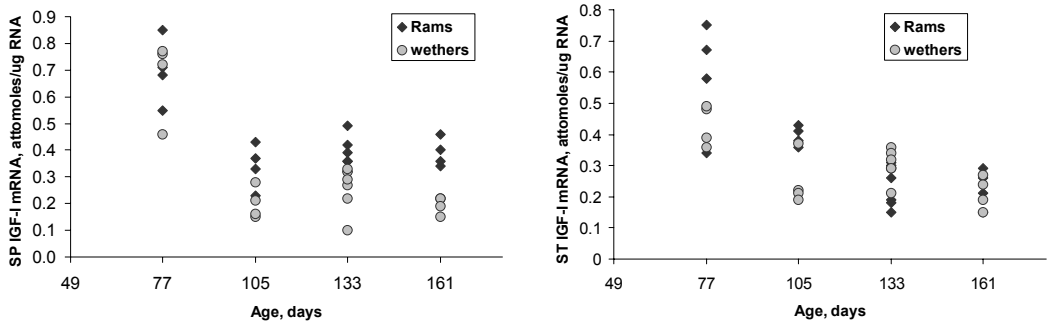
Table 4. Contrasts evaluating sex within age effect on the difference between the log of the two muscle weights.

Model: Lg(SP) – Lg(ST) = Age + Sex(Age) + Pair(Age)					
Contrast	*DF*	*SS*	*MS*	*F Value*	*p value*
Sex within 77 days	1	<0.01	<0.01	0.08	0.78
Sex within 105 days	1	0.14	0.14	4.25	0.06
Sex within 133 days	1	0.23	0.23	7.26	0.02
Sex within 161 days	1	0.23	0.23	7.26	0.02

3.1 IGF-I mRNA expression

IGF-I mRNA expression was determined using a competitive RT-PCR (Appendix Figures 11, 12, 13, 14). The raw data for IGF-I mRNA expression in SP and ST muscles classified by sex and age are presented in Figure 8.

a)



b)

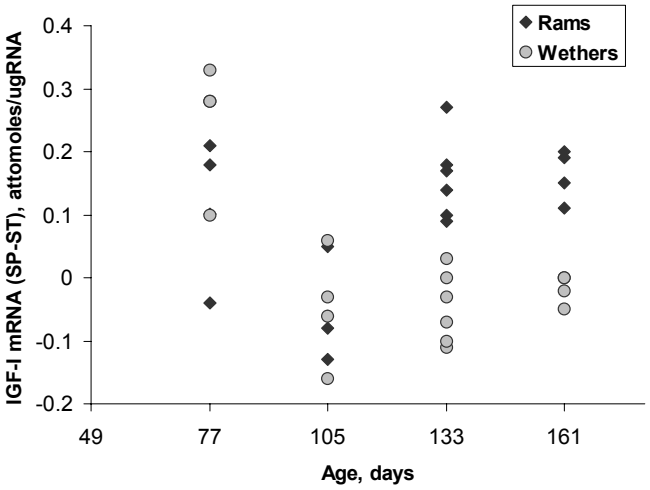


Figure 8. a) IGF-I mRNA level in splenius and semitendinosus muscles in rams and wethers at different ages.
b) IGF-I difference between SP and ST muscles in rams and wethers at different ages.

The IGF-I mRNA concentration was higher at 77 days and lower at later ages in both muscles, but for SP it was higher in rams while for ST the two sexes had similar concentrations at these later ages (Figure 8a). The differences in IGF-I mRNA expression between SP and ST muscles for rams and wethers at different ages show a similar trend (Figure 8b). These observations suggest that IGF-I expression is decreasing with increasing maturity of muscle tissue, a relation which is supported by reports that skeletal muscle IGF-I mRNA expression in postnatal animals decreases with increasing age (Dickson *et al.* 1991, Oldham *et al.* 1996).

The statistical significance of the sex effect on the difference in IGF-I gene expression between the two muscles was evaluated using orthogonal contrasts (Table 5 and Appendix Table 12). The difference in IGF-I mRNA expression between SP and ST was not greater in rams than in wethers at 77 or 105 days of age but it was greater at 133 and 161 days of age ($p = 0.001$ and $p = 0.014$). These results support the hypothesis that the increase in splenius muscle weight in rams relative to wethers is associated with an increase in locally produced IGF-I in SP muscle in response to testosterone. It is not surprising that no difference was found between rams and wethers at 105 days of age which is the age when puberty is thought to occur in sheep. It is possible that some of the

Table 5. Contrasts evaluating sex within age effect on the difference in IGF-I gene expression in the SP and ST muscles.

Model: $IGF_{SP} - IGF_{ST}$ = Age + Sex(Age) + Pair(Age)					
Contrast	*DF*	*SS*	*MS*	*F Value*	*p value*
Sex within 77 days	1	0.04	0.04	5.52	0.97
Sex within 105 days	1	<0.01	<0.01	0.02	0.90
Sex within 133 days	1	0.13	0.13	19.11	<0.01
Sex within 161 days	1	0.06	0.06	9.82	<0.01

rams had not reached puberty at this point in time or that higher levels of testosterone might be necessary for an effect on IGF-I gene expression.

These results are consistent with a study of IGF-I gene expression in cattle (Pfaffl *et al.* 1998a). Insulin-like growth factor I mRNA expression was measured in two muscles selected because of their overproportional (m. splenius) and underproportional (m. gastrocnemius) growth response to testicular steroids. In bulls, higher IGF-I mRNA concentration was found in the m. splenius compared to m. gastrocnemius. Thus, local differences in IGF-I expression is one of the mediators of the differential growth of these muscles in intact males.

3.2 AR mRNA expression

AR gene expression in the two muscles showing differential growth patterns was measured by a ribonuclease protection assay using GAPDH to normalize AR data for differences in RNA loading (Appendix Figure 15). The raw data for the difference in AR mRNA between SP and ST muscles classified by sex and age are presented in Figure 9.

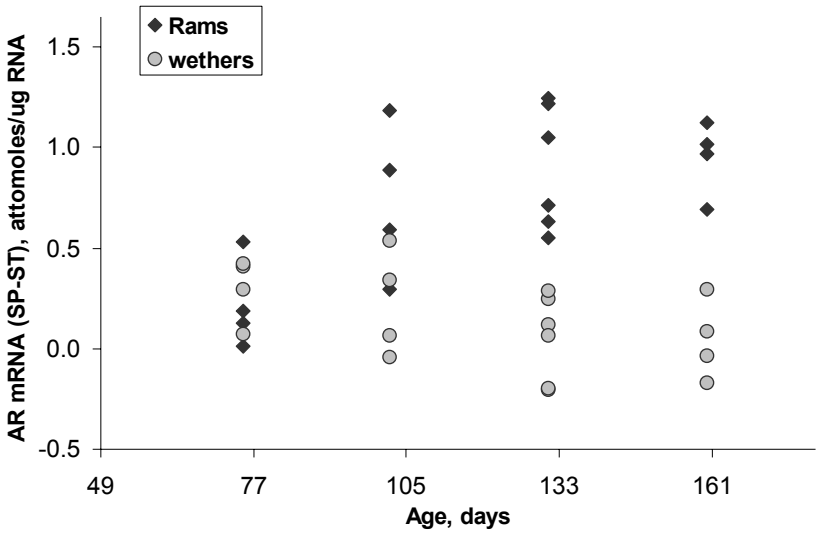


Figure 9. AR mRNA difference between SP and ST muscles in rams and wethers at different ages.

There was similar AR mRNA expression in SP and ST muscles in the two sexes at 77 days (Figure 9). At later ages, the AR mRNA expression was higher in SP relative to ST muscle in rams, while in wethers the difference between SP and ST AR mRNA was about 0 suggesting that the expression level of this gene remained similar in the two muscles for wethers.

The statistical significance of the sex effect on the difference in AR mRNA expression between the two muscles was evaluated using orthogonal contrasts (Table 6 and Appendix Table 12). The difference in AR mRNA expression between SP and ST was not greater in rams than in wethers at 77 days of age but it was significantly greater at 105, 133 and 161 days of age ($p = 0.002$, $p < 0.0001$ and $p < 0.0001$).

Table 6. Contrasts evaluating sex within age effect on the difference in AR gene expression in the SP and ST muscles.

Model: $AR_{SP} - AR_{ST}$ = Age + Sex(Age) + Pair(Age)					
Contrast	*DF*	*SS*	*MS*	*F Value*	*p value*
Sex within 77 days	1	0.01	0.01	0.45	0.51
Sex within 105 days	1	0.52	0.52	17.44	<0.01
Sex within 133 days	1	2.15	2.15	71.39	<0.01
Sex within 161 days	1	1.65	1.65	54.98	<0.01

The increased responsiveness of neck muscles to testosterone could be correlated with the AR level as changes in the number of receptors are a point of modulation of sensitivity to hormones. Divergent densities of AR in individual muscles have been reported previously in cattle (Sauerwein & Meyer 1989) and data on the developmental regulation of AR mRNA expression in three bovine muscles that differed in muscle fiber composition, metabolic activity and growth pattern showed a relationship between AR

mRNA concentration and differential growth (Krieg *et al.* 1977). The higher AR mRNA concentrations in SP muscle in rams shown in this experiment are in agreement with those previous studies and help to explain the pronounced muscle growth in the neck of maturing rams.

3.3 Myostatin mRNA expression

The ribonuclease protection assay indicated the presence of myostatin mRNA in both skeletal muscles (Appendix Figure 15). The raw data for the difference in myostatin mRNA between SP and ST muscles classified by sex and age are presented in Figure 10.

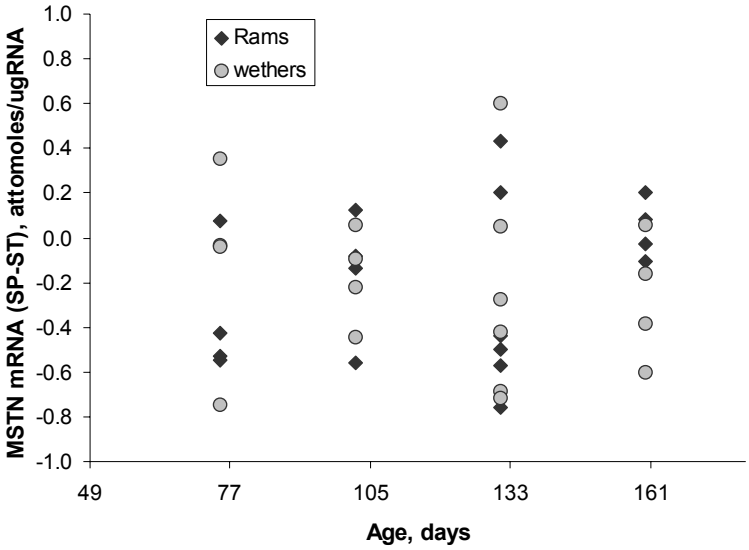


Figure 10. Myostatin mRNA difference between SP and ST muscles in rams and wethers at different ages.

There was no difference between the SP and ST myostatin mRNA expression in rams and wethers (Figure 10).

The statistical significance of the sex effect on the difference in myostatin mRNA expression between the two muscles was evaluated using orthogonal contrasts (Table 7 and Appendix Table 12). No significant sex effect was found in the expression of myostatin gene in the two muscles.

Table 7. Contrasts evaluating sex within age effect on the difference in myostatin gene expression in the SP and ST muscles.

Model: $MSTN_{SP} - MSTN_{ST}$ = Age + Sex(Age) + Pair(Age)					
Contrast	*DF*	*SS*	*MS*	*F Value*	*p value*
Sex within 77 days	1	0.12	0.12	1.32	0.27
Sex within 105 days	1	<0.01	<0.01	0.01	0.94
Sex within 133 days	1	<0.01	<0.01	0.03	0.86
Sex within 161 days	1	0.19	0.19	2.12	0.17

Lower myostatin gene expression in the SP muscle in rams was expected to explain the increased muscle mass. The possible role of myostatin in the sexual dimorphic muscle growth of SP muscle can not be ruled out even if there were no differences in myostatin mRNA between rams and wethers. Others have reported that, while myostatin mRNA concentration does not differ between sexes there are different levels of processed myostatin expressed in male and female mice associated with increased body and muscle mass (McMahon *et al.* 2003).

CHAPTER IV

CONCLUSIONS

This experiment is consistent with the hypothesis that the increased splenius muscle mass of the neck associated with sexual maturity of rams is mediated by an increase in IGF-I and AR gene expression. However, the regulation of the other components of the IGF-I system should be studied to be able to judge the relevance of the IGF system for regulation of differential muscle growth. Also, the AR role in the muscle growth regulation should be investigated further as other genes that are regulated by this transcription factor could be associated with muscle growth regulation.

There was no difference in the myostatin gene expression associated with the sexually dimorphic muscle growth. It would be of considerable interest to determine if there is a difference in the expression of processed myostatin in the sexually dimorphic muscles in rams and wethers.

APPENDIX

Note: Table 1, 2, 3, 4, 5, 6 and 7 are presented on pages 37, 38, 40, 42, 44, 46 and 48.

Table 8. Primer pairs and the PCR conditions.

Amplified fragment	Primer name	Primer sequence	PCR conditions
910 bp of the androgen receptor (AR) gene	AR Forward Reverse	5'-GCC TGA TCT GTG GAG ATG AA-3' 5'-AGC TTG GTG AGC TGG TAG AA-3'	30" 94°C 30 cycles: 0"94°C, 0"55°C, 20"72°C 1'72°C; 5'25°C 2 mM $MgCl_2$
453 bp of the myostatin (MSTN) gene	MSTN Forward Reverse	5'-GCT CCT TGG AAG ACG ATG AC-3' 5'-CTT CTA AAA AAG GAT TCA GT-3'	30" 94°C 30 cycles: 0"94°C, 0"62°C, 20"72°C 1'72°C; 5'25°C 4 mM $MgCl_2$
556 bp of the GAPDH gene	GAPDH Forward Reverse	5'- C TGC ACC ACC AAC TGC TTA G-3' 5'- T TAC TCC TTG GAG GCC ATG T-3'	30" 94°C 30 cycles: 0"94°C, 0"55°C, 20"72°C 1'72°C; 5'25°C 2 mM $MgCl_2$
358 bp of the insulin-like growth factor I (IGF-I) gene	IGF-I Forward Reverse	5'-CGC ATC TCT TCT ATC TGG CC-3' 5'-TTG TTT CCT GCA CTC CCT CT-3'	30" 94°C 30 cycles: 0"94°C, 0"55°C, 20"72°C) 1'72°C; 5'25°C 4 mM $MgCl_2$
325 bp of the MIMIC IGF-I	IGFGAPDH Forward Reverse	5'-CGC ATC TCT TCT ATC TGG CCC TGC ACC ACC AAC TGC TTA G-3' 5'-TTG TTT CCT GCA CTC CCT CTT TAC TCC TTG GAG GCC ATG T-3'	30" 94°C 30 cycles: 0"94°C, 0"58°C, 20"72°C) 1'72°C; 5'25°C 2 mM $MgCl_2$

Table 9. Animal number, pair and animal id, sex and age class for experimental animals.

Animal Number	Pair ID	Animal ID	Sex	Age class (days)
1	4	P2097	Wether	77
2	4	P2098	Ram	77
3	5	P2099	Ram	77
4	5	P2100	Wether	77
5	12	P2117	Ram	77
6	12	P2118	Wether	77
7	14	P2121	Wether	77
8	14	P2122	Ram	77
9	1	P2091	Wether	105
10	1	P2092	Ram	105
11	6	P2101	Wether	105
12	6	P2102	Ram	105
13	16	P2125	Wether	105
14	16	P2126	Ram	105
15	18	P2129	Ram	105
16	18	P2130	Wether	105
17	2	P2093	Ram	133
18	2	P2094	Wether	133
19	3	P2095	Wether	133
20	3	P2096	Ram	133
21	8	P2105	Wether	133
22	8	P2106	Ram	133
23	10	P2109	Ram	133
24	10	P2110	Wether	133
25	15	P2123	Wether	133
26	15	P2124	Ram	133
27	17	P2127	Ram	133
28	17	P2128	Wether	133
29	9	P2107	Wether	161
30	9	P2108	Ram	161
31	13	P2119	Ram	161
32	13	P2120	Wether	161
33	19	P2131	Wether	161
34	19	P2132	Ram	161
35	20	P2135	Wether	161
36	20	P2136	Ram	161

Table 10. The output of analysis of variance performed with the model: $Y_{ijkl} = \mu + Ai + S_{ij} + P_{ki} + e_{ijkl}$ for log carcass weight (kg). For a description of the model, see Materials and Methods.

Source	*DF*	*Sum of Squares*	*Mean Square*	*F Value*	*Pr > F*
Model	21	4.93740178	0.23511437	5.22	0.0013
Error	14	0.63006986	0.04500499		
Corrected Total	35	5.56747164			

R-Square	*Coeff Var*	*Root MSE*	*lgcarcasswt Mean*
0.886830	8.049044	0.212144	2.635640

Source	*DF*	*Type III SS*	*Mean Square*	*F Value*	*Pr > F*
days	3	4.31363943	1.43787981	31.95	<.0001
sex(days)	4	0.20617721	0.05154430	1.15	0.3757
pair(days)	14	0.41758513	0.02982751	0.66	0.7744

Contrast	*DF*	*Contrast SS*	*Mean Square*	*F Value*	*Pr > F*
sex within time1	1	0.16864803	0.16864803	3.75	0.0734
sex within time2	1	0.00763146	0.00763146	0.17	0.6867
sex within time3	1	0.02880940	0.02880940	0.64	0.4370
sex within time4	1	0.00108831	0.00108831	0.02	0.8786

Table 11. The output of analysis of variance performed with the model: $DMW_{ijkl} = \mu + Ai + S_{ij} + P_{ki} + e_{ijkl}$ for the difference between the log of the two muscle weights (g). For a description of the model, see Materials and Methods.

Source	*DF*	*Sum of Squares*	*Mean Square*	*F Value*	*Pr > F*
Model	21	1.58126808	0.07529848	2.33	0.0542
Error	14	0.45324248	0.03237446		
Corrected Total	35	2.03451056			

R-Square	*Coeff Var*	*Root MSE*	*lgspl_st Mean*
0.777223	-8.068059	0.179929	-2.230141

Table 11 (Continued).

Source	*DF*	*Type III SS*	*Mean Square*	*F Value*	*Pr > F*
days	3	0.29076233	0.09692078	2.99	0.0667
sex(days)	4	0.61014616	0.15253654	4.71	0.0128
pair(days)	14	0.68035959	0.04859711	1.50	0.2284

Contrast	*DF*	*Contrast SS*	*Mean Square*	*F Value*	*Pr > F*
sex within time1	1	0.00256680	0.00256680	0.08	0.7824
sex within time2	1	0.13761179	0.13761179	4.25	0.0583
sex within time3	1	0.23498642	0.23498642	7.26	0.0175
sex within time4	1	0.23498115	0.23498115	7.26	0.0175

Table 12. The output of analysis of variance performed with the model: $D_{ijkl} = \mu + A_i + S_{ij} + P_{ki} + e_{ijkl}$ for the difference in gene expression in the SP and ST muscles (attomoles per µg RNA). For a description of the model, see Materials and Methods.

IGF-Isplenius - IGF-Isemitendinosus

Source	*DF*	*Sum of Squares*	*Mean Square*	*F Value*	*Pr > F*
Model	21	0.50443750	0.02402083	3.64	0.0081
Error	14	0.09236250	0.00659732		
Corrected Total	35	0.59680000			

R-Square	*Coeff Var*	*Root MSE*	*Isp_st Mean*
0.845237	128.2483	0.081224	0.063333

Source	*DF*	*Type III SS*	*Mean Square*	*F Value*	*Pr > F*
days	3	0.21527083	0.07175694	10.88	0.0006
sex(days)	4	0.22743750	0.05685938	8.62	0.0010
pair(days)	14	0.06172917	0.00440923	0.67	0.7698

Contrast	*DF*	*Contrast SS*	*Mean Square*	*F Value*	*Pr > F*
sex within time1	1	0.03645000	0.03645000	5.52	0.9666
sex within time2	1	0.00011250	0.00011250	0.02	0.8980
sex within time3	1	0.12607500	0.12607500	19.11	0.0006
sex within time4	1	0.06480000	0.06480000	9.82	0.0073

Table 12 (Continued).

ARsplenius - ARsemitendinosus

Source	*DF*	*Sum of Squares*	*Mean Square*	*F Value*	*Pr > F*
Model	21	6.04474722	0.28784511	9.56	<.0001
Error	14	0.42174167	0.03012440		
Corrected Total	35	6.46648889			

R-Square	*Coeff Var*	*Root MSE*	*Asp_st Mean*
0.934780	39.95075	0.173564	0.434444

Source	*DF*	*Type III SS*	*Mean Square*	*F Value*	*Pr > F*
days	3	0.32839722	0.10946574	3.63	0.0397
sex(days)	4	4.34565833	1.08641458	36.06	<.0001
pair(days)	14	1.37069167	0.09790655	3.25	0.0175

Contrast	*DF*	*Contrast SS*	*Mean Square*	*F Value*	*Pr > F*
sex within time1	1	0.01361250	0.01361250	0.45	0.5124
sex within time2	1	0.52531250	0.52531250	17.44	0.0009
sex within time3	1	2.15053333	2.15053333	71.39	<.0001
sex within time4	1	1.65620000	1.65620000	54.98	<.0001

MSTNsplenius - MSTNsemitendinosus

Source	*DF*	*Sum of Squares*	*Mean Square*	*F Value*	*Pr > F*
Model	21	3.12129722	0.14863320	1.67	0.1643
Error	14	1.24862500	0.08918750		
Corrected Total	35	4.36992222			

R-Square	*Coeff Var*	*Root MSE*	*Msp_st Mean*
0.714268	-147.2759	0.298643	-0.202778

Source	*DF*	*Type III SS*	*Mean Square*	*F Value*	*Pr > F*
days	3	0.11588056	0.03862685	0.43	0.7326
sex(days)	4	0.30987500	0.07746875	0.87	0.5068
pair(days)	14	2.69554167	0.19253869	2.16	0.0811

Contrast	*DF*	*Contrast SS*	*Mean Square*	*F Value*	*Pr > F*
sex within time1	1	0.11761250	0.11761250	1.32	0.2701
sex within time2	1	0.00045000	0.00045000	0.01	0.9444
sex within time3	1	0.00270000	0.00270000	0.03	0.8644
sex within time4	1	0.18911250	0.18911250	2.12	0.1674

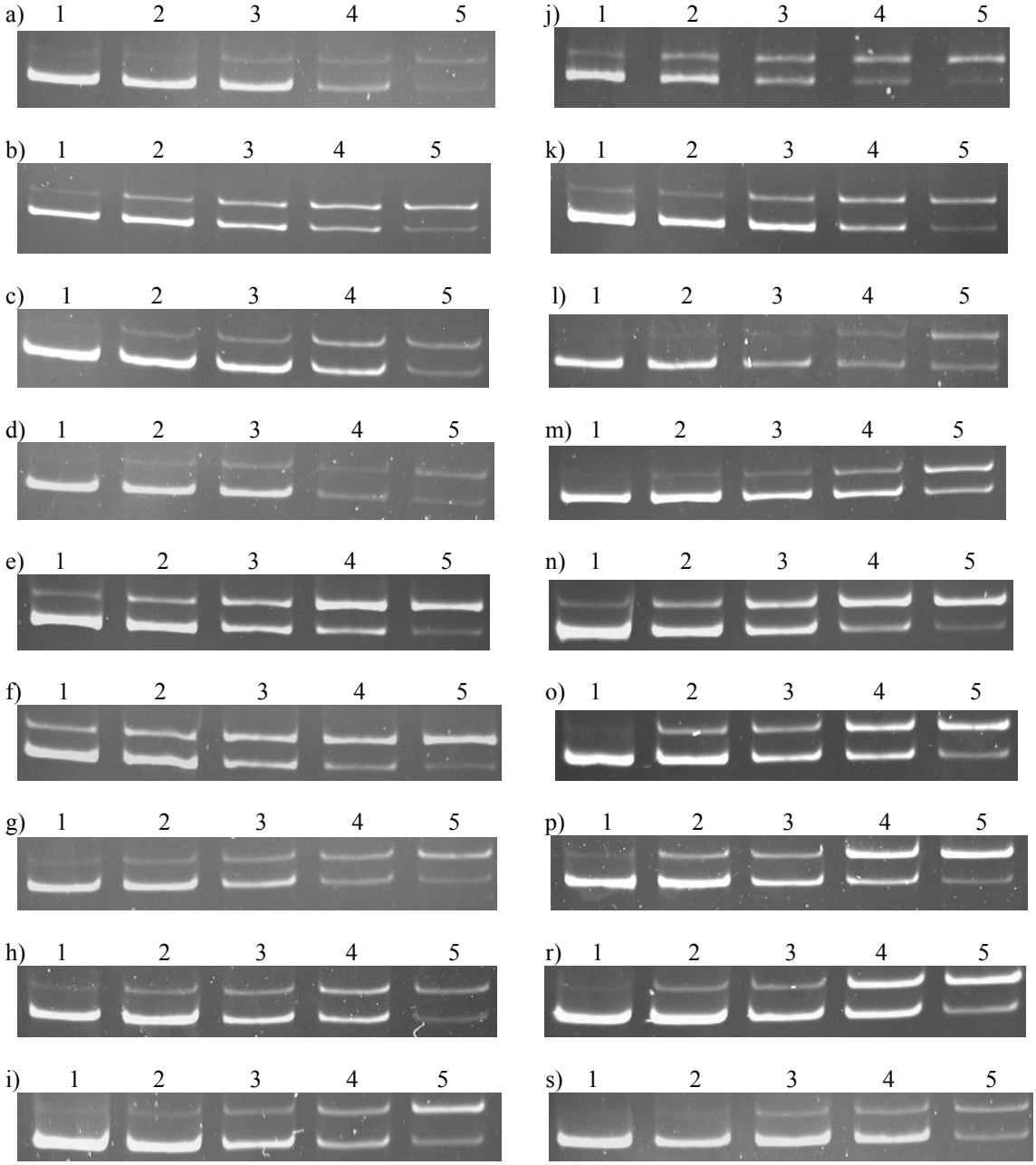


Figure 11. Quantitative RT-PCR analyses for insulin-like growth factor I (IGF-I) mRNA abundance in ovine splenius muscle. Gel electrophoresis of PCR products for wild-type and mimic (358 and 325 bp, respectively) DNA templates. Lane 1 to 5: native mRNA spiked with decreasing mimic cRNA concentrations (9, 3, 1, 0.33, 0.11 attomoles). The letters *a* to *s* represent animals 1 to 18.

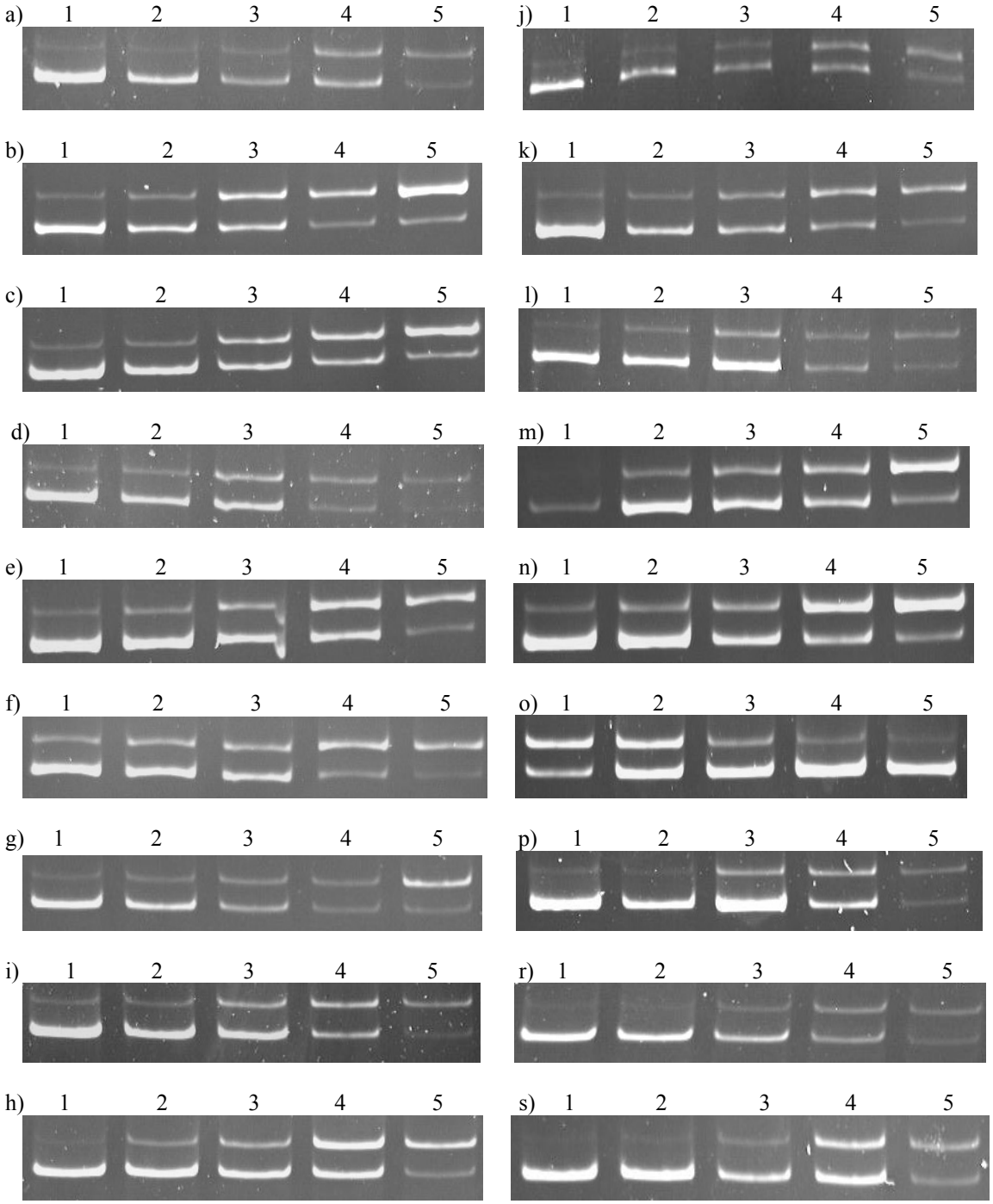


Figure 12. Quantitative RT-PCR analyses for insulin-like growth factor I (IGF-I) mRNA abundance in ovine semitendinosus muscle. Gel electrophoresis of PCR products for wild-type and mimic (358 and 325 bp, respectively) DNA templates. Lane 1 to 5: native mRNA spiked with decreasing mimic cRNA concentrations (9, 3, 1, 0.33, 0.11 attomoles). The letters *a* to *s* represent animals 1 to 18.

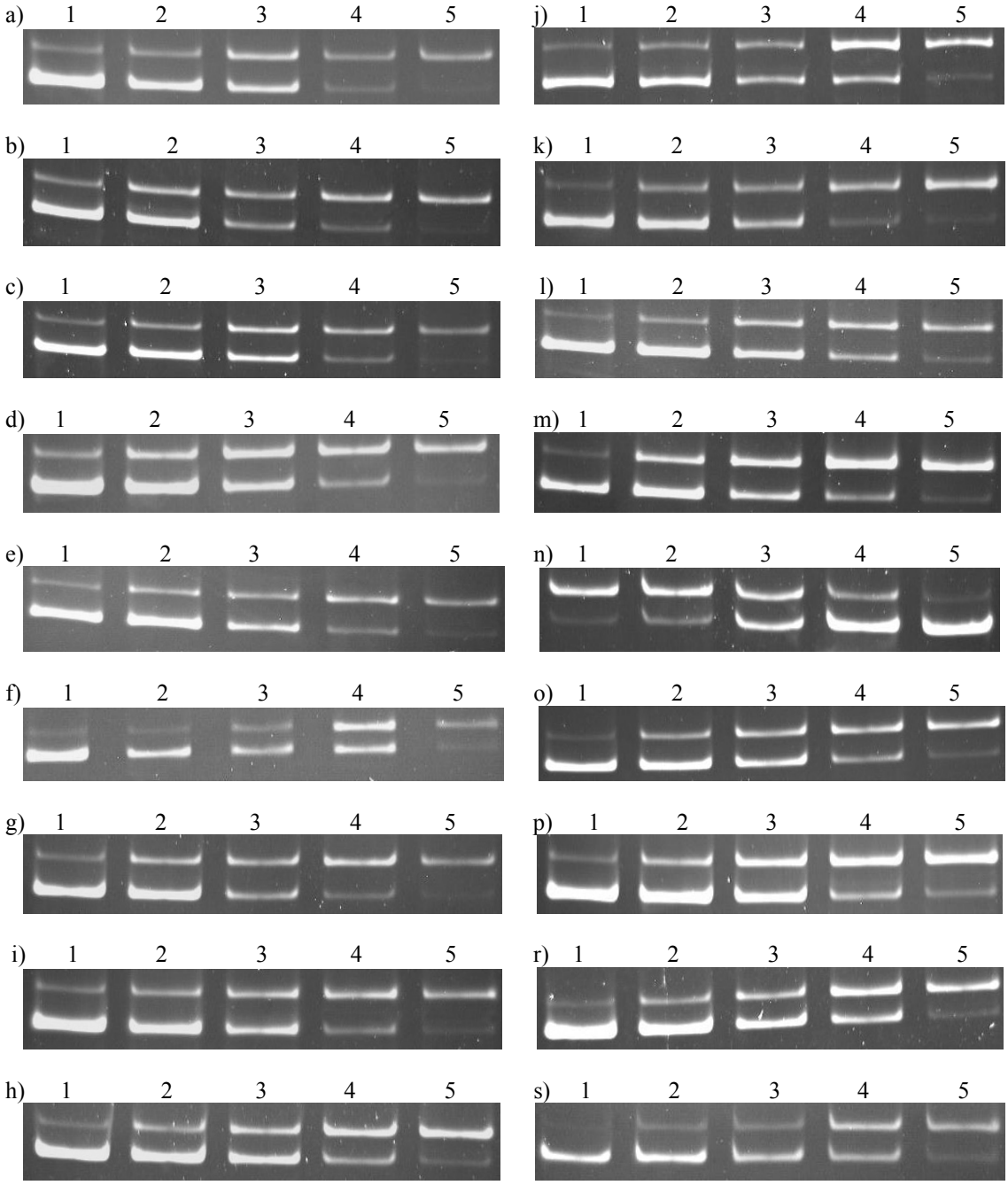


Figure 13. Quantitative RT-PCR analyses for insulin-like growth factor I (IGF-I) mRNA abundance in ovine splenius muscle. Gel electrophoresis of PCR products for wild-type and mimic (358 and 325 bp, respectively) DNA templates. Lane 1 to 5: native mRNA spiked with decreasing mimic cRNA concentrations (3, 1, 0.33, 0.11, 0.04 attomoles). The letters *a* to *s* represent animals 19 to 36.

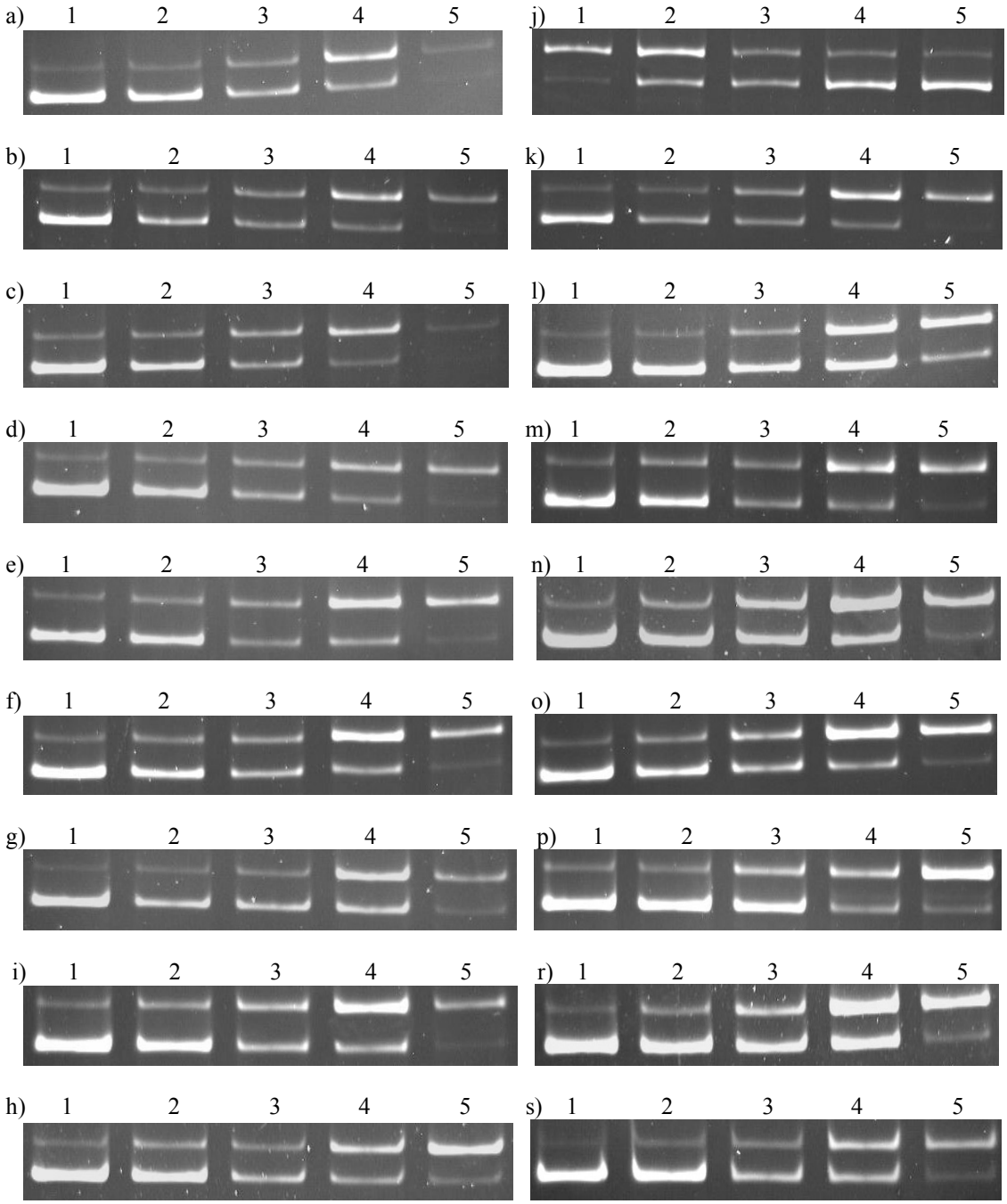
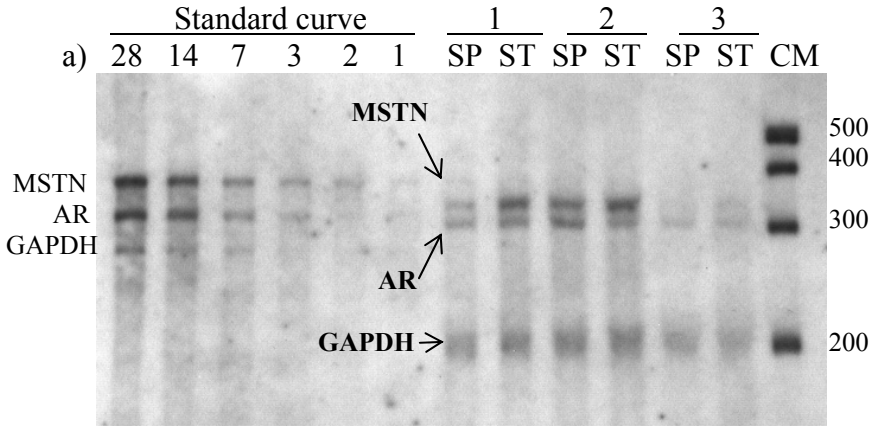
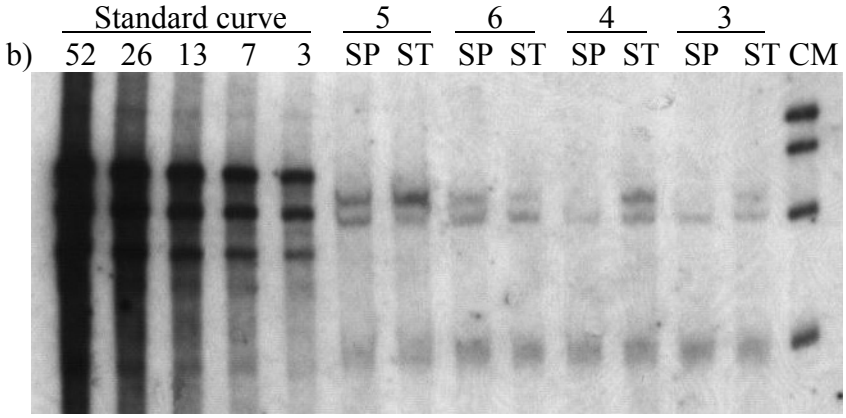


Figure 14. Quantitative RT-PCR analyses for insulin-like growth factor I (IGF-I) mRNA abundance in ovine semitendinosus muscle. Gel electrophoresis of PCR products for wild-type and mimic (358 and 325 bp, respectively) DNA templates. Lane 1 to 5: native mRNA spiked with decreasing mimic cRNA concentrations (3, 1, 0.33, 0.11, 0.04 attomoles). The letters *a* to *s* represent animals 19 to 36.

Figure 15. Quantitative analyses of myostatin (MSTN) and androgen receptor (AR) mRNA in ovine tissues using GAPDH as an internal control. The standard curve was constructed by hybridizing 1 ng of MSTN, AR and GAPDH antisense probe to known amounts, ranging from 52 to 0.25 pg of sense MSTN, AR and GAPDH cRNA, respectively. Lane SP and ST represents the hybridization of 1 ng MSTN, AR and GAPDH antisense probe with 40 ug semitendinosus and splenius muscle RNA. Lane CM represents the RNA molecular weight marker (Century Marker, Ambion). The numbers 1 to 36 represent the animals. The exposure time varied between 30 minutes and 6 hours.


Standard curve
1
2
3
a) 28 14 7 3 2 1 SP ST SP ST SP ST CM
MSTN
AR
GAPDH
MSTN
AR
GAPDH
500
400
300
200


Standard curve
5
6
4
3
b) 52 26 13 7 3 SP ST SP ST SP ST SP ST CM

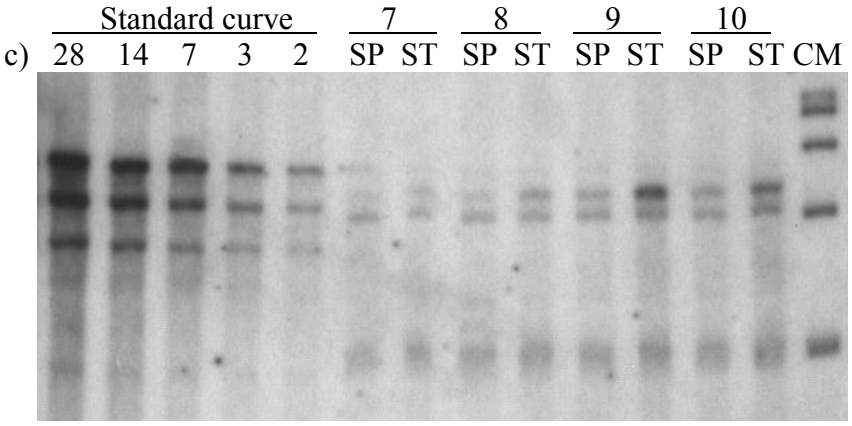
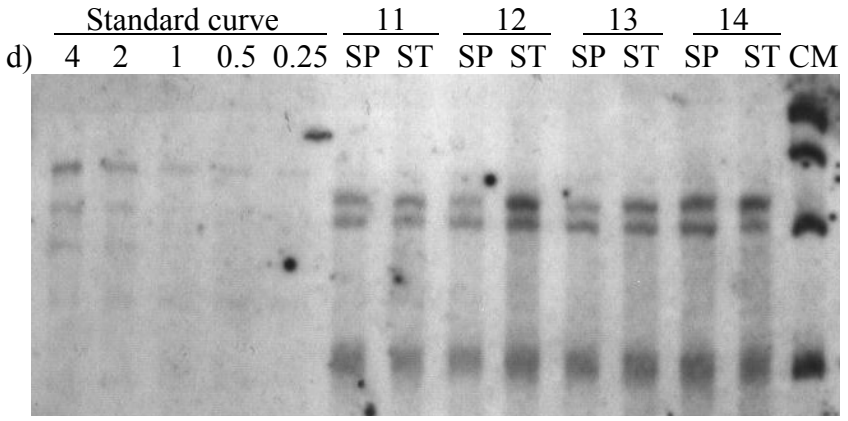
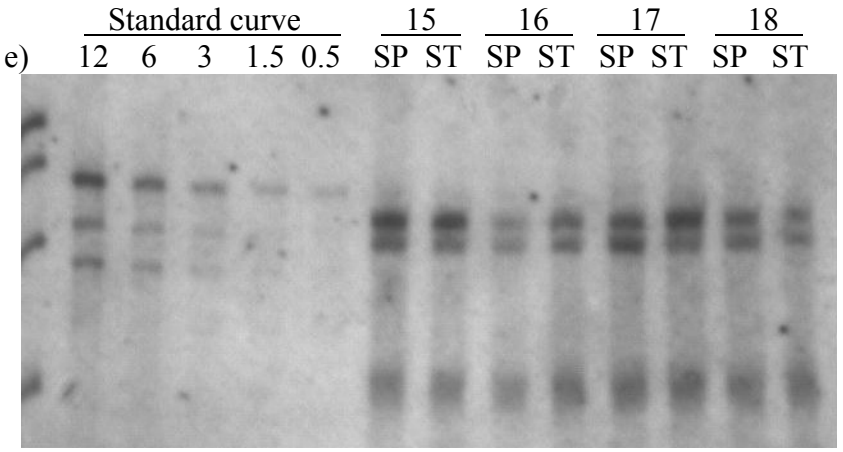

Standard curve
7
8
9
10
c) 28 14 7 3 2 SP ST SP ST SP ST SP ST CM

Figure 15 (Continued)





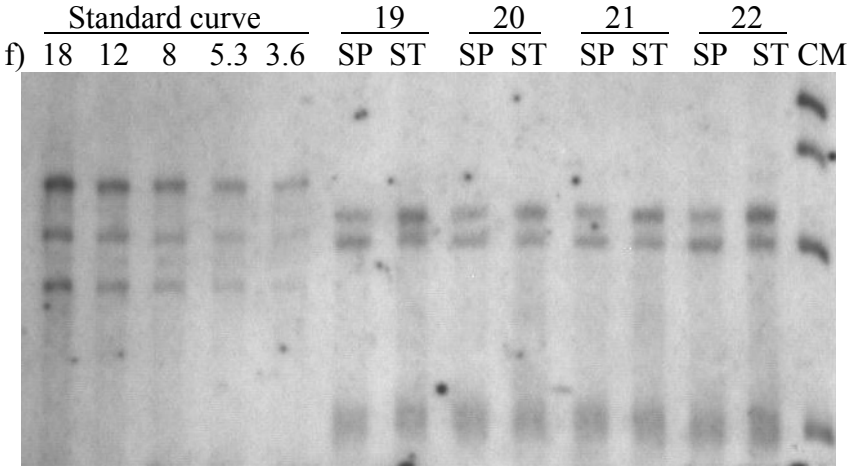
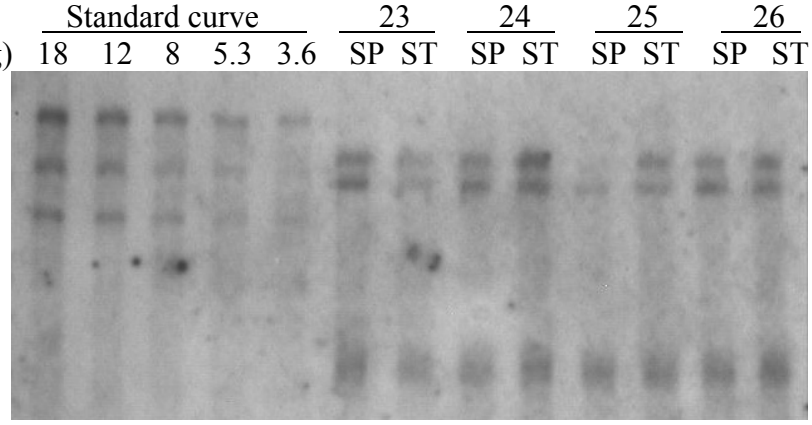
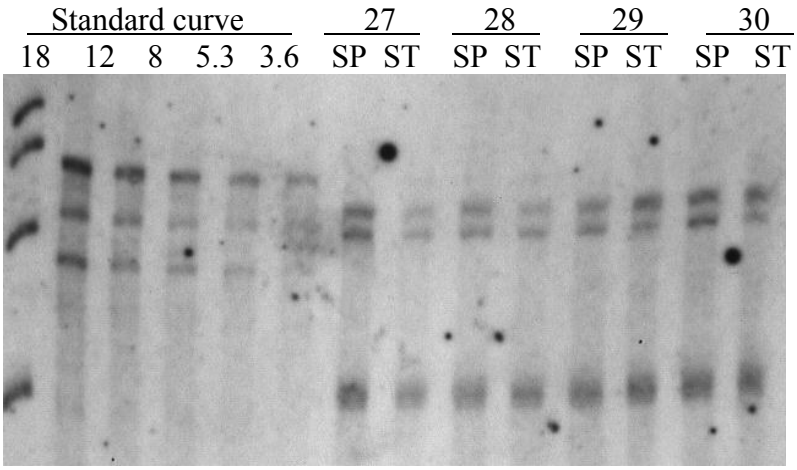


Figure 15 (Continued)

g)



h)



i)

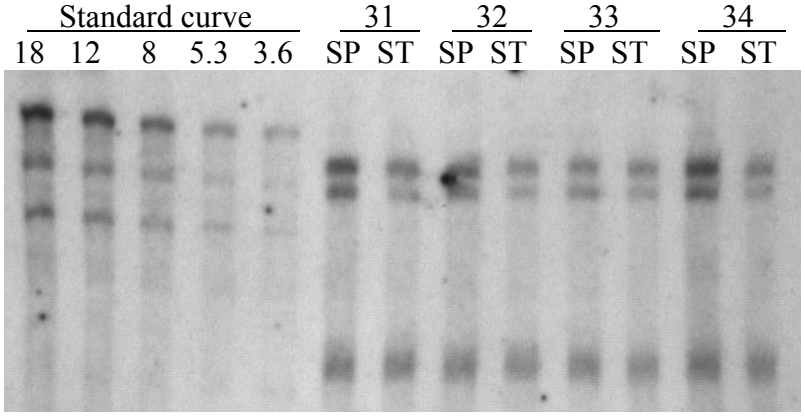


Figure 15 (Continued)

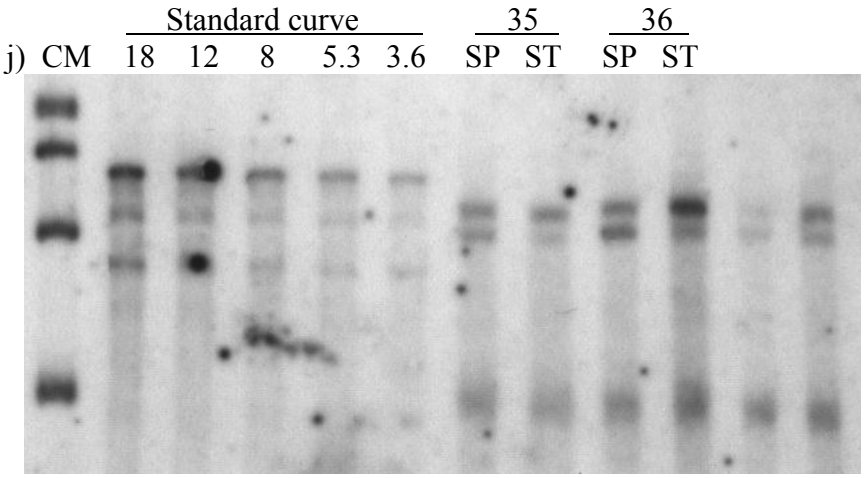
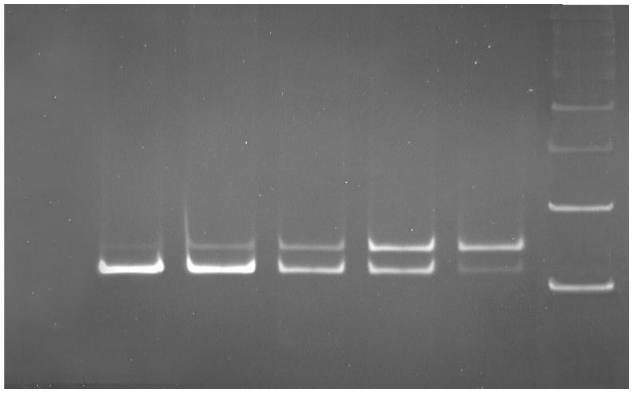
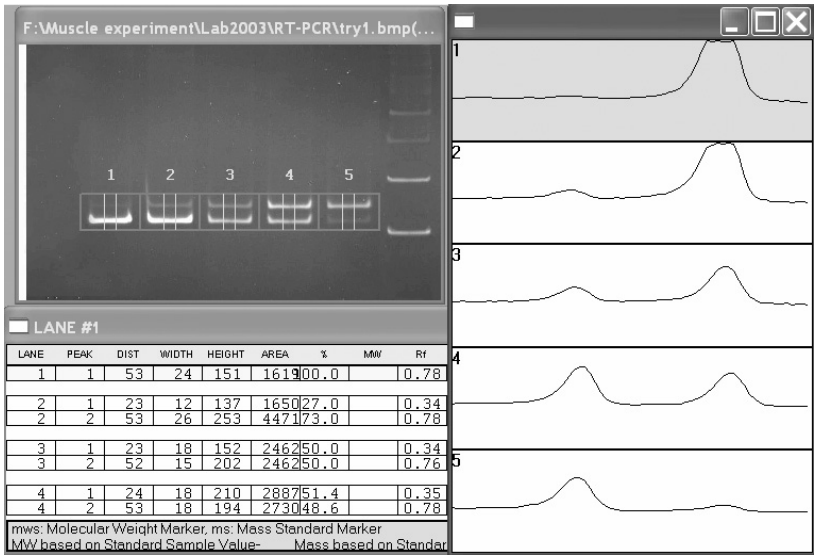


Figure 16. Quantification of optical densities of the insulin-like growth factor I (IGF-I) target and the MIMIC peaks using the Alpha Innotech software.
a) Using the picture of the gel, the number of lanes is specified and the placement of the template is adjusted so that it coincides with the positions of the lanes on the image. Also, the Scan Width (the area in which the pixel density actually is measured) is specified.
b) Using the Scan command, the density within the Scan Width of each lane is measured. The image is redisplayed in a smaller window in the upper left of the screen and the graphs of each lane are displayed on the right. The peaks on the graph correspond to the bands in a lane.
c) One way to quantify the band in a lane is to use the Auto Peak command, which automatically identifies the peaks, based on the parameters defined. The information associated with the peaks is displayed in the data table below the image. The value that we used was "Area" which represents the integrated density of each peak (in total pixel value).

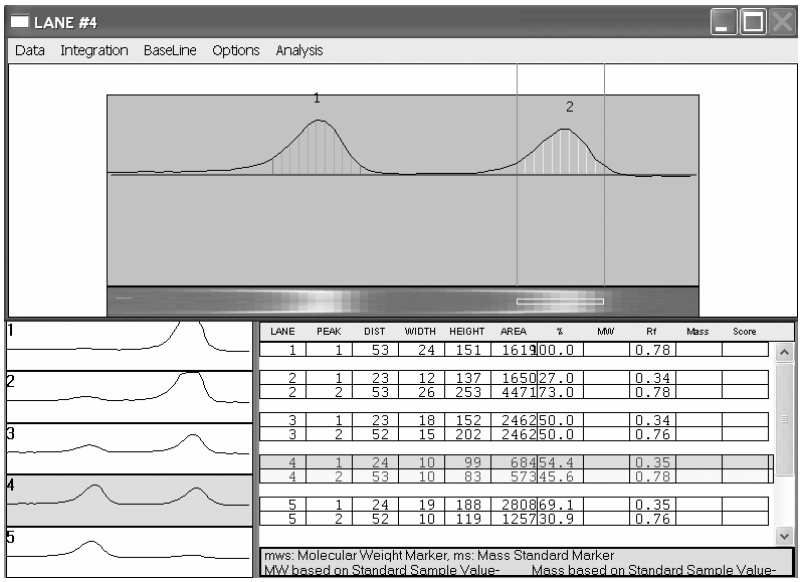
a)



b)


F:\Muscle experiment\Lab2003\RT-PCR\try1.bmp(...
1
2
3
4
5
LANE #1
LANE PEAK DIST WIDTH HEIGHT AREA % MW Rf
1 1 53 24 151 1619 100.0 0.78
2 1 23 12 137 1650 27.0 0.34
2 2 53 26 253 4471 73.0 0.78
3 1 23 18 152 2462 50.0 0.34
3 2 52 15 202 2462 50.0 0.76
4 1 24 18 210 2887 51.4 0.35
4 2 53 18 194 2730 48.6 0.78
mws: Molecular Weight Marker, ms: Mass Standard Marker
MW based on Standard Sample Value- Mass based on Standar

c)


LANE #4
Data Integration BaseLine Options Analysis
1
2
LANE PEAK DIST WIDTH HEIGHT AREA % MW Rf Mass Score
1 1 53 24 151 1619 100.0 0.78
2 1 23 12 137 1650 27.0 0.34
2 2 53 26 253 4471 73.0 0.78
3 1 23 18 152 2462 50.0 0.34
3 2 52 15 202 2462 50.0 0.76
4 1 24 10 99 684 54.4 0.35
4 2 53 10 83 573 45.6 0.78
5 1 24 19 188 2808 69.1 0.35
5 2 52 10 119 1257 30.9 0.76
mws: Molecular Weight Marker, ms: Mass Standard Marker
MW based on Standard Sample Value- Mass based on Standard Sample Value-

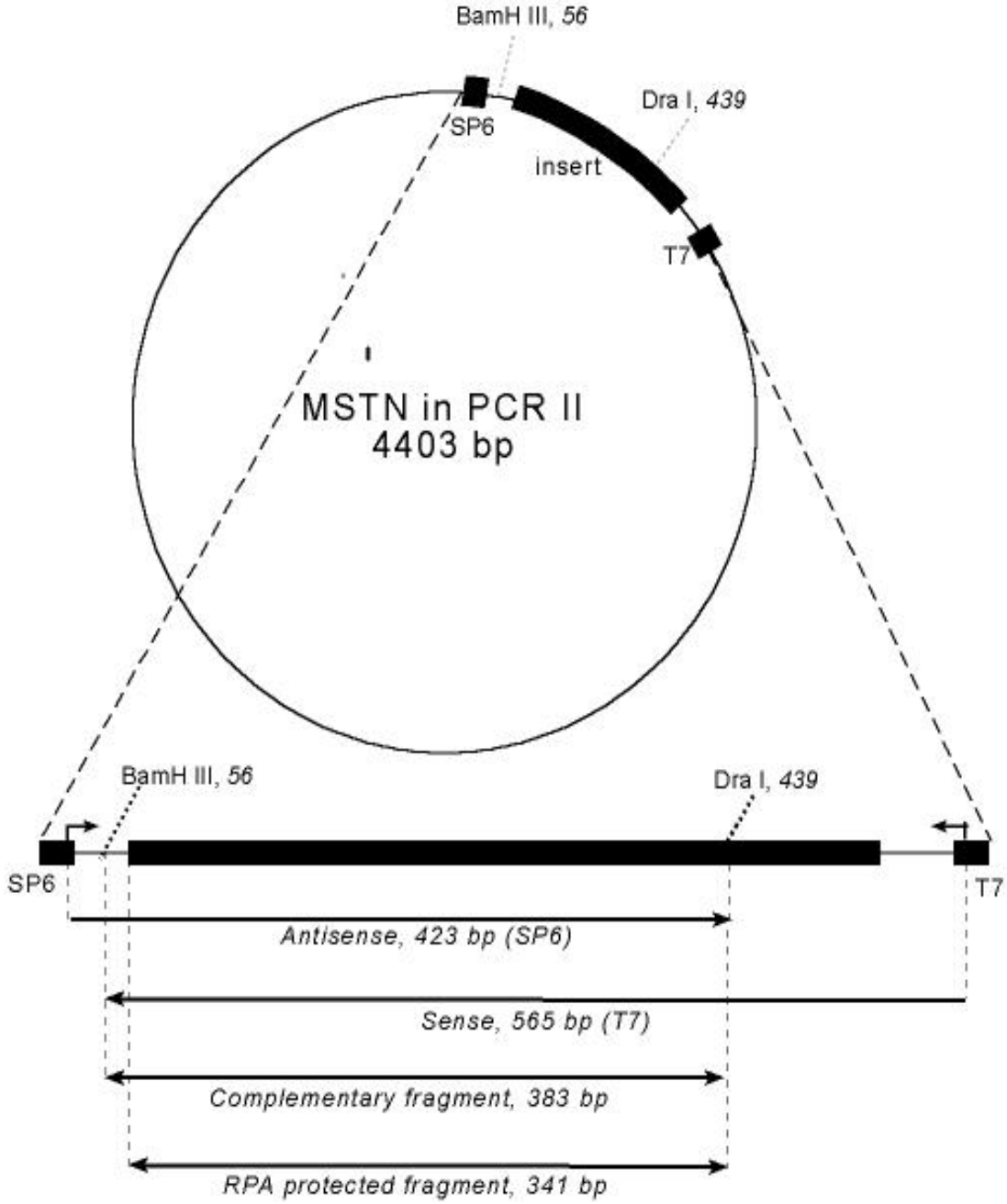


Figure 17. pCR (MSTN)
The plasmid was composed of a 3.95 kb pCR II vector (Invitrogen, CA) and a 453 bp PCR product insert (myostatin) which was inserted in inverse orientation into the vector.

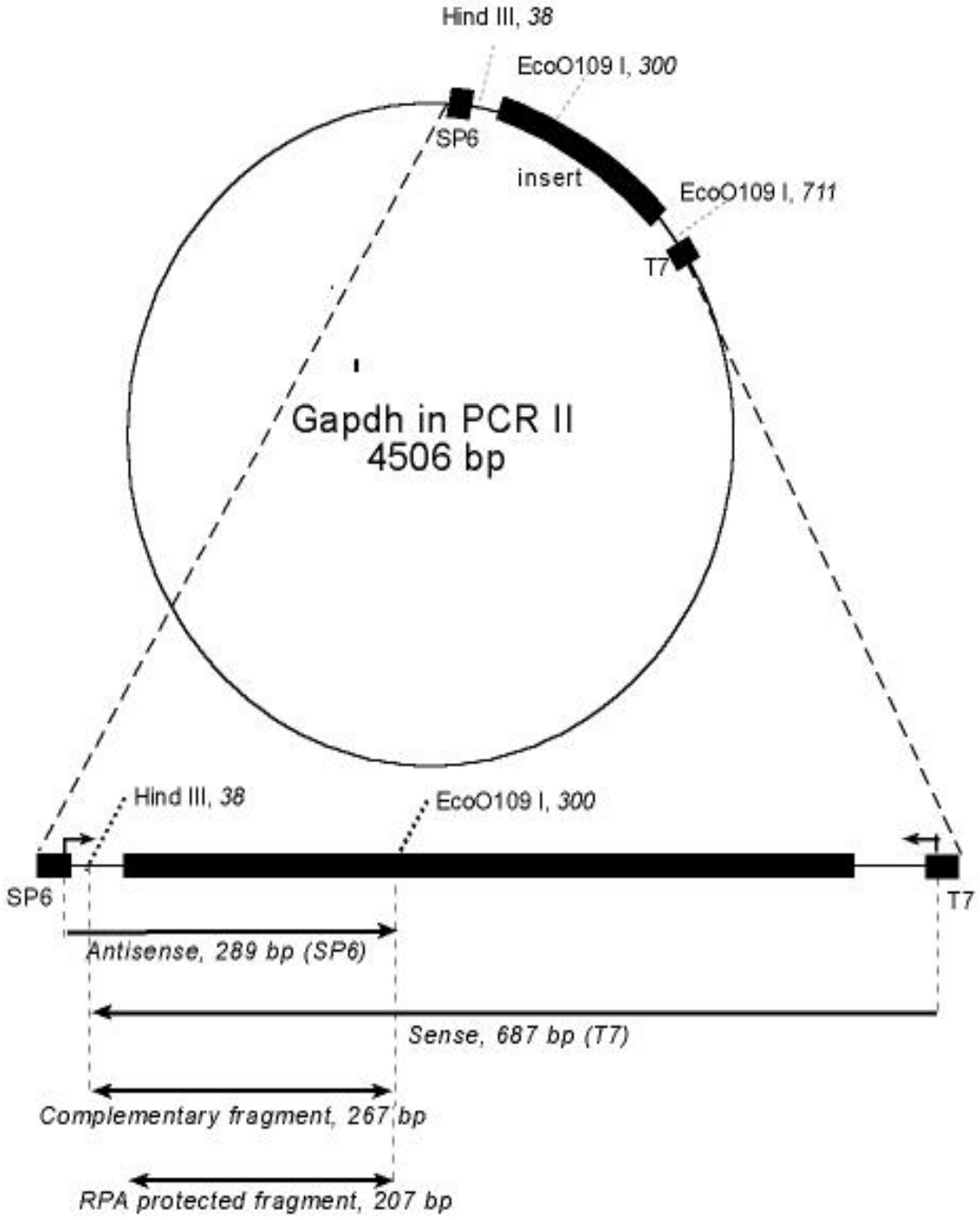


Figure 18. pCR (GAPDH)
The plasmid was composed of a 3.95 kb pCR II vector (Invitrogen, CA) and a 556 bp PCR product insert (GAPDH) which was inserted in inverse orientation into the vector.

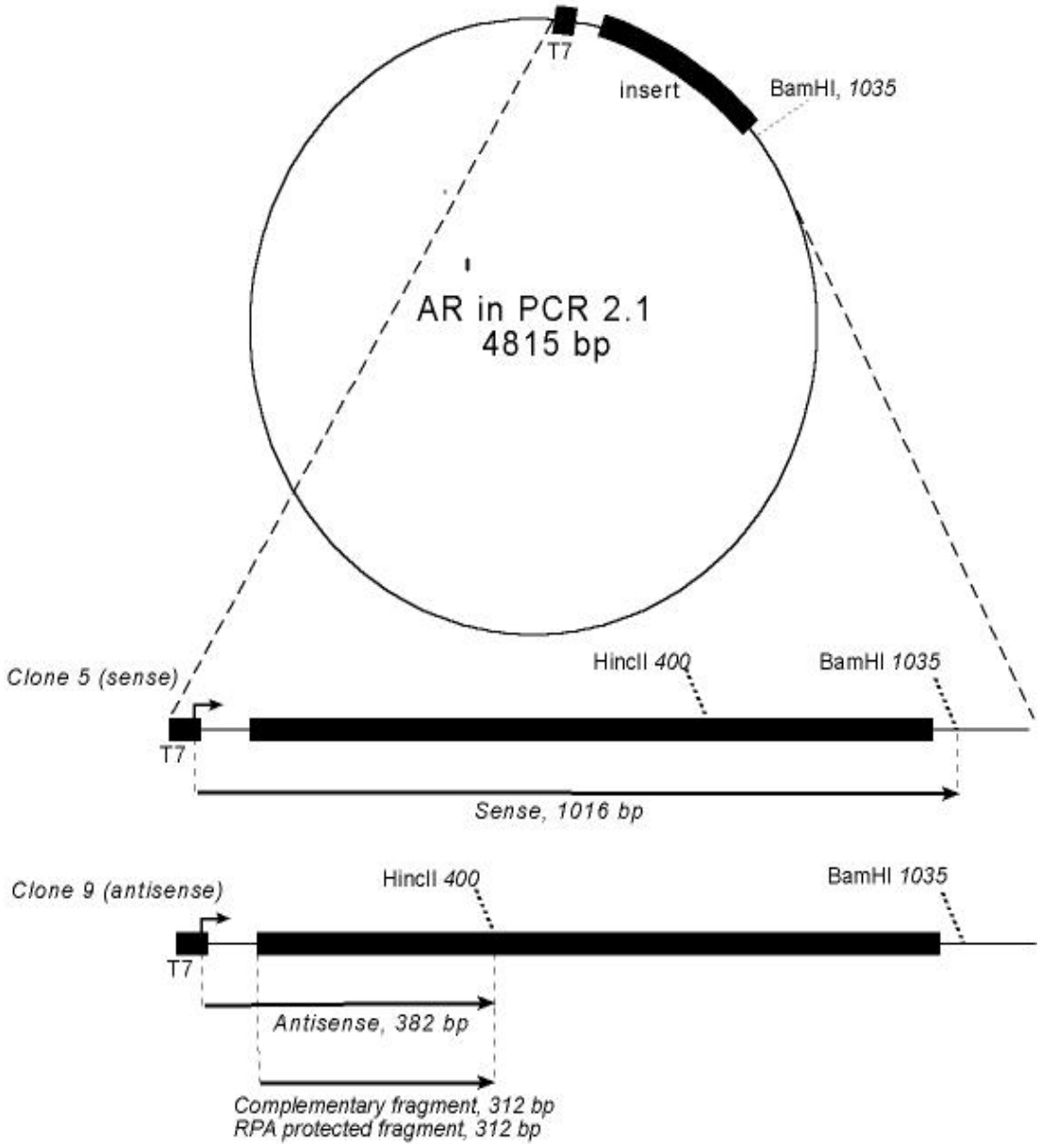


Figure 19. pCR(ARs) and pCR(ARas)
The plasmids were composed of a 3.9 kb pCR 2.1 vector (Invitrogen, CA) and a 910 bp PCR product insert (androgen receptor). Clone 5 contained the insert in direct orientation into the vector and clone 9 contained the insert in inverse orientation into the vector.

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