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EFFECTS OF TESTOSTERONE ON PROLIFERATION, DIFFERENTIATION AND ANDROGEN RECEPTOR CONTENT OF PORCINE SATELLITE CELLS *IN VITRO*

By

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ABSTRACT

EFFECTS OF TESTOSTERONE ON PROLIFERATION, DIFFERENTIATION AND ANDROGEN RECEPTOR CONTENT OF PORCINE SATELLITE CELLS *IN VITRO*

By

Matthew Eli Doumit

Androgens are known to increase skeletal muscle size and DNA content. While increased DNA content is essential for muscle growth, the mechanisms of androgen-induced DNA accretion in skeletal muscle are unclear. Immunochemical methods utilizing polyclonal androgen receptor (AR) antibodies (PG-21) were used to identify AR in clonal cultures of porcine muscle-derived fibroblasts, satellite cells and satellite cell-derived myotubes. Immunoblot analysis revealed an immunoreactive AR band of approximately 107 kDa in porcine epididymis, satellite cells, and myotubes, but not spleen. Immunocytochemical AR staining was confined to the nuclei of satellite cells, myotubes and fibroblasts. Exposure of satellite cell, myotube, and fibroblast cultures to 10^{-7} M testosterone increased immunoreactive AR. In satellite cells and myotubes, AR protein increased incrementally after 6, 12 and 24 h exposure to testosterone. Testosterone administration did not affect ($P>0.1$) satellite cell proliferation in serum-containing or serum-free media. Additionally, testosterone administered for 24 h did not alter subsequent responsiveness of cells to IGF-I, bFGF or PDGF. Satellite cell differentiation, as measured by cell fusion,

incidence of sarcomeric myosin-positive cells, and creatine kinase activity, was depressed ($P<0.01$) by testosterone. A 20-30% decrease in satellite cell differentiation was observed on days 2, 3 and 4 of treatment with 10^{-7} M testosterone. This effect was not reversible within 48 h after treatment withdrawal and replacement with control medium. These data indicate that satellite cells are direct targets for androgen action. *In vitro* testosterone administration increases immunoreactive AR protein and decreases differentiation of porcine satellite cells. A mechanism whereby androgens may increase the population of proliferative satellite cells *in vivo* by reducing cell differentiation is proposed.

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TABLE OF CONTENTS

	Page
LIST OF TABLES	ix
LIST OF FIGURES	x
INTRODUCTION	1
REVIEW OF THE LITERATURE	3
Intact Males versus Castrates in Livestock Production	3
Skeletal Muscle Growth	4
Androgen Effects on Skeletal Muscle	7
Circulating Androgens and Androgen Metabolism in Muscle	12
Androgen Receptors	14
Androgen Receptor Regulation	18
Evidence for Autologous Up-Regulation	20
Evidence for Autologous Down-Regulation	22
Cellular Responses to Androgens	24
CHAPTER ONE: IDENTIFICATION, LOCALIZATION AND REGULATION OF ANDROGEN RECEPTORS IN CULTURED PORCINE SATELLITE CELLS AND SATELLITE CELL-DERIVED MYOTUBES	
ABSTRACT	28
INTRODUCTION	30
EXPERIMENTAL PROCEDURES	32
Materials	32

Porcine Satellite Cell Isolation 32

Development of Satellite Cell and Fibroblast Clones 33

Satellite Cell Cultures 34

Western Immunoblots 35

Immunocytochemical Localization of the Androgen Receptor 38

RESULTS 39

Identification of Androgen and Estrogen Receptors 39

Regulation of Androgen Receptor Protein . . 46

DISCUSSION 58

CHAPTER TWO: EFFECTS OF TESTOSTERONE ON PROLIFERATION AND DIFFERENTIATION OF CLONALLY-DERIVED PORCINE SATELLITE CELLS

ABSTRACT 65

INTRODUCTION 67

EXPERIMENTAL PROCEDURES 70

Materials 70

Porcine Satellite Cell Isolation 70

Satellite Cell Cultures 71

Immunocytochemical Detection of Myosin Positive Cells 72

Assay of Creatine Phosphokinase 73

Statistical Analysis 73

RESULTS 75

Testosterone Effects on Satellite Cell Proliferation 75

Testosterone Effects on Satellite Cell Differentiation 78

DISCUSSION 87

SUMMARY AND IMPLICATIONS 94

REFERENCES 100

LIST OF TABLES

PAGE

TABLE 1.1 Porcine Satellite Cell Serum-Free Media . . 36

LIST OF FIGURES

PAGE

CHAPTER ONE

Fig. 1.1. Western blot analysis of satellite cells and myotubes using androgen receptor antiserum 40

Fig. 1.2. Western blot analysis of porcine spleen, satellite cells, and epididymis using PG-21 41

Fig. 1.3. Immunoblot analysis of porcine spleen, satellite cells, and epididymis using AR-antiserum 42

Fig. 1.4. Immunoblot of porcine estrogen receptors with ER-21. Porcine spleen, satellite cells, and epididymis using AR-antiserum . . 44

Fig. 1.5. Western blot analysis of satellite cells and myotubes maintained in SFM 45

Fig. 1.6. Western blot analysis of satellite cells and myotubes exposed to MEM+10% FBS 47

Fig. 1.7. Identification of non-specific 75 kDa band 48

Fig. 1.8. Immunocytochemical staining for androgen receptor in satellite cells and myotubes from primary culture 49

Fig. 1.9. Western blot analysis of myotubes exposed to testosterone 50

Fig. 1.10. Immunoblot analysis of testosterone-induced increases in myotube androgen receptor concentration 51

Fig. 1.11. Immunoblot analysis of testosterone -induced increases in satellite cell androgen receptor concentration 52

Fig. 1.12. Immunoblot analysis of myotubes exposed to estradiol-17β 54

Fig. 1.13. Immunocytochemical staining for androgen receptor in satellite cells exposed to testosterone 55

Fig. 1.14. Effect of testosterone on androgen receptor immunostaining of fibroblasts . . . 56

Fig. 1.15. Effect of dexamethasone on androgen receptor immunostaining of satellite cells . 57

CHAPTER TWO

Fig. 2.1. Effects of testosterone concentration on satellite cell proliferation in serum-containing media 76

Fig. 2.2. Effects of testosterone pre-treatment on satellite cell responsiveness to growth factors 77

Fig. 2.3. Effects of testosterone on satellite cell proliferation in serum-free medium 79

Fig. 2.4. Effects of testosterone pre-treatment on satellite cell proliferation in serum-free medium 80

Fig. 2.5. Effects of testosterone on myotube formation 81

Fig. 2.6. Effects of testosterone on appearance of myosin-positive cells 82

Fig. 2.7. Effects of testosterone on incidence of myosin-positive cells 83

Fig. 2.8. Effects of testosterone on creatine kinase activity 85

Fig. 2.9. Effects of testosterone on cell density of differentiating cultures 86

Fig. 2.10. Hypothetical model for testosterone-induced increases in DNA 96

INTRODUCTION

Skeletal muscle is the most abundant tissue in the animal body. The growth and function of skeletal muscle is critical to the survival of the animal, and as meat it provides society with a high quality, nutritious and palatable food. Better understanding the process of skeletal muscle growth should provide insight into ways of improving the rate and efficiency of meat animal production. Gonadally intact male livestock are more feed efficient and produce carcasses with less fat and more muscle than castrates and females at comparable weights (reviewed by Field, 1971; Seideman et al., 1982; Purchas, 1991). However, the direct effects of androgens on skeletal muscle are not well understood. Accretion of DNA, via satellite cell proliferation and differentiation, is considered to be a prerequisite for skeletal muscle growth (Allen et al., 1979). Gonadally intact males and testosterone-treated male castrates and females typically have more proliferating satellite cells, greater DNA per muscle and consequently larger muscles than untreated male castrates and females (Mulvaney et al., 1988; Joubert and Tobin, 1989; Thompson et al., 1989). However, androgens generally have no direct effects on proliferation of cultured skeletal muscle

cells (Gospodarowicz et al., 1976; Thompson et al., 1989). The apparent discrepancies between cells in culture and skeletal muscle *in vivo* have not been explained.

Studies comparing many aspects of *in vivo* skeletal muscle growth among gonadally intact male pigs, androgen-treated male castrates and male castrates have been instigated by R.A. Merkel and co-workers. Accordingly, procedures for isolation and culture conditions which optimize proliferation and differentiation of primary porcine satellite cell cultures have been described (Doumit and Merkel, 1992). Furthermore, clonal cultures of highly myogenic porcine satellite cells have been established. Serum-free media which promote proliferation and differentiation of porcine satellite cells have been formulated and used to examine the effects of several growth factors on proliferation of clonally-derived porcine satellite cells (Cook et al., 1993; Doumit et al., 1993). The following studies utilized this culture system to 1) identify, localize, and study regulation of androgen receptors in satellite cells and myotubes, and, 2) examine the effects of testosterone on satellite cell proliferation and differentiation. The information derived from these studies, as well as the questions provoked, will hopefully improve our understanding of androgen action on skeletal muscle growth.

REVIEW OF THE LITERATURE

Intact Males versus Castrates in Livestock Production

Castration of the male is a traditional practice in meat animal production in the United States. Castrated livestock are easier to manage, and are generally thought to produce carcasses with more desirable qualitative properties than gonadally intact males. Nevertheless, numerous studies have shown that gonadally intact males grow more rapidly, utilize feed more efficiently and produce trimmer, more muscular carcasses than castrates (Field, 1971; Seideman et al., 1982; Purchas, 1991). Thus, if the negative aspects of producing gonadally intact males could be overcome, considerable economic advantages could be realized by their use for meat production.

Several studies have shown that boars have 20-30% less fat at market weight than barrows (Prescott and Lamming, 1967; Mulvaney, 1984; Knudson et al., 1985). Boars also utilize feed more efficiently and produce carcasses with 3-20% greater muscle mass than barrows (Prescott and Lamming, 1967; Field, 1971; Hansson et al., 1975; Mulvaney, 1984; Knudson et al., 1985). Variability in comparisons of muscle mass between boars and barrows likely results from differences in breed,

weight at slaughter and the level of dietary protein fed.

Despite obvious advantages of utilizing boars for meat production, consumer acceptance of boar meat is hindered by an objectionable odor in the heated fat of meat from some boars (Malmfors and Lundstrom, 1983). This unpleasant odor is due to the pheromonal steroid, androstenone and related compounds, which accumulate in adipose tissue after being synthesized in the testes (Brooks and Pearson, 1986; Purchas, 1991). The unpleasant odor may be synergistically strengthened by skatole, a metabolite of tryptophan breakdown by intestinal microorganisms (Brooks and Pearson, 1986). The incidence of boars with strong odor was 1% in a survey by Walstra (1974), and 28% in the study of Williams et al. (1963). Only a low proportion of the human population can detect the odor (Walstra 1974). Although practical methods for preventing boar odor have not yet been adopted, it is not considered a problem in countries in which hogs are slaughtered at relatively light weights (Purchas, 1991).

Skeletal Muscle Growth

In the first stages of skeletal muscle development, embryonic myoblasts arise from mesodermal cells located in pairs of somites which flank the developing notochord and neural tube. This process, termed determination, results in a population of proliferative cells that, along with their descendants, are committed to the myogenic lineage (reviewed by Stockdale, 1992; Ivarie, 1993). Distinct classes of

proliferative myoblasts are present during embryonic, fetal, and adult skeletal muscle development, although the precise origins of these lineages are not clear. Ultimately, these myoblasts withdraw from the cell cycle, activate muscle-specific genes and fuse to form multinucleated myotubes. Activation of muscle-specific gene expression, accompanied by permanent withdrawal from the cell cycle, is referred to as terminal differentiation. Both determination and differentiation appear to be controlled by a family of basic helix-loop-helix proteins that transactivate many muscle-specific promoters (Emerson, 1990; Stockdale, 1992; Ivarie, 1993).

Myotubes synthesize and accumulate muscle proteins. During maturation of myotubes into myofibers, centrally located nuclei migrate to the periphery of muscle cells as myofibrils assemble and form a centrally located contractile apparatus. Muscle growth is achieved primarily by increased fiber diameter and length, and this is accomplished primarily by an increase in myofibril content (Goldspink, 1991). Myofibrils increase in number by longitudinal splitting (Goldspink, 1969; Goldspink, 1971), and in length by addition of sarcomeres to the ends of myofibrils and minor increases in sarcomere length (Williams and Goldspink, 1971; Stromer et al., 1974).

Studies of myogenesis *in vitro* (Stockdale and Holtzer, 1961; Okazaki and Holtzer, 1966) and *in situ* (Shafiq et al.,

1968; Moss and Leblond, 1971) demonstrated that nuclei contained within the sarcolemma of myotubes and myofibers do not synthesize DNA. Nevertheless, skeletal muscle DNA accretion parallels myofiber hypertrophy (Enesco and Puddy, 1964; Moss, 1968; Cardasis and Cooper, 1975), suggesting that a population of proliferative cells must reside within skeletal muscle tissue. Satellite cells were first described by Mauro (1961) as being mononucleated cells wedged between the sarcolemma and basement membrane of muscle fibers. In this initial report, Mauro (1961) suggested that satellite cells were the source of nuclei added to myofibers during regeneration. Satellite cells are now widely accepted as the source of nuclei contributed to normally growing (Moss and Leblond, 1971; Allen et al., 1979), newly formed (Appell et al., 1988; Kennedy et al., 1988), and damaged or diseased myofibers (Allbrook, 1981; Carlson and Faulkner, 1983).

In chickens ranging from 0 to 266 days of age, increased cross-sectional area of muscle fibers is proportional to increased DNA content in pectoral and gastrocnemius muscles, and over 95% of the DNA in these muscles accumulates postnatally (Moss, 1968). Powell and Aberle (1975) reported that total DNA in the biceps femoris muscle of heavy and light muscled pigs increased from 1 to 210 days of age. In this study, heavy muscled pigs had more DNA per muscle than lighter muscled pigs, yet protein to DNA ratios were similar. Similarly, DiMarco et al. (1987) suggested that, in cattle

weighing over 350 kg, muscle growth was influenced more by DNA accretion than by changes in protein to DNA ratio. These findings are consistent with the concept that myonuclei dominate a finite volume of cytoplasm (Cheek et al., 1971). Total DNA content of physically separable muscle was greater in muscular than obese pigs, and was the measurement most highly related to total muscle content (Harbison et al., 1976). Additionally, Purchas et al. (1985) showed that satellite cell proliferative activity was greater in a lean strain of mice than in an obese strain. Hindlimb muscles of the lean strain weighed nearly 100% more than those of the obese strain at 8 weeks of age. Therefore, DNA accretion via satellite cell proliferation and differentiation appears to be a critical regulatory component of postnatal skeletal muscle growth.

Androgen Effects on Skeletal Muscle

Early studies on the mechanism of androgen-induced skeletal muscle growth indicated that testosterone stimulated protein synthesis in rat skeletal muscle (Florini and Breuer, 1966) and this effect was additive with growth hormone-stimulated protein synthesis. Testosterone and growth hormone both stimulated RNA polymerase activity, yet only the androgen enhanced the priming efficiency of DNA (Breuer and Florini, 1966). These studies indicated that testosterone and growth hormone stimulated RNA synthesis in muscle by different mechanisms. Florini (1970) also demonstrated that under

conditions in which testosterone stimulated both RNA and protein synthesis by 60%, no differences in the population of proteins synthesized in skeletal muscle could be detected.

Castration of pigs within 1 week of birth decreased protein deposition, increased fat deposition, and decreased serum IGF-I concentration relative to gonadally intact male pigs at 60 and 90 kg body weight (Taylor et al., 1992). Castration at birth did not affect muscle weight, *in vitro* protein synthesis or *in vivo* fractional synthesis rates of skeletal muscle of neonatal pigs at 1, 2 or 4 weeks of age (Skjaerlund et al., 1994). However, postpubertal boars have greater muscle protein synthesis and accretion rates than castrates (Mulvaney, 1984).

Testosterone administration to intact male rabbits improved feed efficiency, stimulated weight gain, increased serum insulin, and increased skeletal muscle RNA content and myofibrillar protein synthesis (Grigsby et al., 1976). Martinez et al. (1984) found that daily administration of testosterone (1 mg/kg body weight) for 7 days stimulated growth rates of female rats, but had no significant effect on carcass composition. Feed intake was not influenced by treatment, but feed conversion efficiency was increased by testosterone. In their study, testosterone stimulated fractional synthesis rate (FSR) and calculated fractional breakdown rate (FBR) but to a lesser extent than FSR, thus resulting in greater protein gain in the gastrocnemius muscle.

In contrast, Sinnett-Smith et al., (1983) reported that administration of trenbolone acetate (TBA), a testosterone analogue, decreased muscle FSR and decreased FBR to a greater extent than FSR in ewe lambs. The apparent discrepancy between the mode of action of TBA and testosterone has not been explained. The methyl derivative of trenbolone (17-alpha-methyl-trienolone, or R1881) has been shown to bind androgen receptors of skeletal muscle cytosol (Snochowski, 1980; 1981a; 1981b).

Treatment of young female rats with TBA increased total weight gain, weight gain per gram of feed consumed, muscle DNA content and semimembranosus muscle weight (Thompson et al., 1989). Snochowski et al. (1981a) observed that boars had a greater skeletal muscle DNA concentration than gilts and castrates (0.58 $\pm$ 0.11 *vs* 0.46 $\pm$ 0.07 or 0.43 $\pm$ 0.06 mg/g tissue, respectively). Additionally, gonadally intact male pigs had semitendinosus muscle DNA contents that were 39% and 73% greater than male pigs castrated at 15 kg and 74 kg, respectively (Mulvaney and Merkel, unpublished data). In the same study, semitendinosus muscle weights of boars were 28% and 65% greater (5 weeks after castration) than pigs castrated at 15 kg and 74 kg, respectively. Live weight did not differ between boars and barrows compared in this study (Mulvaney and Merkel, unpublished data).

Castration of male pigs within 24 h after birth reduced total myofiber nuclei and the incidence of proliferating

satellite cells per millimeter of myofiber length within 3 weeks of age (Mulvaney et al., 1988). Castrates implanted with testosterone propionate had greater satellite cell proliferative activity than that of control pigs. Likewise, injection of adult female rats with testosterone (25 mg/100 g body weight) stimulated satellite cell proliferation in the androgen-sensitive levator ani muscle and subsequently increased myonuclei by 80% within 30 days (Joubert and Tobin, 1989). In this study, an increase in myofiber diameter was observed, with no change in myofiber number.

The apparent *in vivo* effects of androgens have been difficult to demonstrate in cultured skeletal muscle cells. Neither testosterone nor growth hormone stimulated α-actin accumulation in rat satellite cell-derived myotubes (Allen et al., 1983). Likewise, Roeder et al. (1986) reported that testosterone did not alter protein synthesis or degradation rates in either L6 rat myoblast or myotube cultures. Powers and Florini (1975) reported a 25% increase in DNA labeling index of primary rat myoblasts and L6 myoblasts exposed to 10^{-8} M testosterone. In this study, testosterone shortened the G_1 phase of the cell cycle by 8.5 h, but estradiol, pregnanediol, dihydrotestosterone and androstenedione had no effect on myoblast proliferation. However, androgens have been shown to have no direct effect on DNA synthesis of cultured bovine myoblasts (Gospodarowicz et al., 1976) or proliferation of rat satellite cells (Thompson et al., 1989). Trenbolone did not

affect myotube formation or the responsiveness of primary rat satellite cell cultures to IGF-I or FGF (Thompson et al., 1989). However, satellite cells isolated from muscles of TBA-treated rats were more responsive to the mitogenic effects of IGF-I and FGF than satellite cells from control rats, and serum collected from TBA-treated rats was more mitogenic than serum from control rats (Thompson et al., 1989). These authors concluded that the primary cellular response to TBA may be an increased sensitivity to growth factors that stimulate satellite cell proliferation, rather than a direct mitogenic effect.

Steroid hormones also have priming effects on the biological actions of IGF-I in chondrocyte proliferation and metabolism. Testosterone, estradiol and progesterone had no effect on chondrocyte DNA synthesis, although pre-exposure of cells to these steroids for 24 h increased subsequent responsiveness to a maximal dose of IGF-I (Itagane et al., 1991). In contrast, no difference in proliferation or differentiation was observed between satellite cells isolated from tom and hen turkeys and grown in serum-containing medium (Doumit et al., 1990). However, when a turkey satellite cell clone was exposed to serum collected from tom and hen turkeys at 3, 9, and 15 weeks of age, an age-related decline in mitogenic activity was detected in serum from hen but not tom turkeys (Doumit et al., 1990). These results indicate that gender-related differences in skeletal muscle DNA accretion

and growth are more likely to be controlled by extracellular signals than inherent differences in the responsiveness of satellite cells.

Circulating Androgens and Androgen Metabolism in Muscle

Testosterone is primarily produced in Leydig cells of the testes, but is also produced in the adrenal glands. Adrenal androgen may be important for determining the onset of puberty (Wiedeman, 1982). Concentrations of serum testosterone in male pigs were found to be elevated in three phases of life: from 40 to 60 days of gestation (Colenbrander et al., 1978), in the second and third week after birth, and in the postpubertal period beyond 18 weeks of age (Colenbrander et al., 1978; Ford, 1983; Schwarzenberger et al., 1993). Elevated neonatal testosterone has been shown to have little anabolic effect on skeletal muscle growth of pigs (Skjaerlund et al., 1994). Testosterone elicits complex biological responses in a variety of tissues, acting directly, through conversion to 5α-dihydrotestosterone (DHT) or aromatization to estradiol (Mooradian et al., 1987). DHT binds more tightly to the AR than testosterone, but the B_{max} of binding of the two androgens is identical (Grino et al., 1990). Furthermore, DHT was 10 times more potent than testosterone (half-maximal stimulation of 0.018 nM *vs* 0.2 nM, respectively) in activating a reporter gene linked to the mouse mammary tumor virus (MMTV-CAT) promoter that contains an androgen response element, but the maximal activity achieved was the same for the two

androgens (Deslypere et al., 1992).

Snochowski et al. (1981b) observed that methyltrienolone, testosterone and dexamethasone were not metabolized by human skeletal muscle cytosol during a 22 h incubation at 0-4°C, while 43 and 80% of DHT was metabolized when exposed to cytosol obtained from two different human subjects. Krieg et al. (1974) found that over 70% of the testosterone recovered from striated muscle was not metabolized, while approximately half of the DHT administered to rats was recovered as 5α-androstane-3α,17β-diol (3α-diol) within 30 min. In that study, more than 65% of the androgen recovered from seminal vesicles and prostate was DHT. In rat skeletal muscle, Snochowski et al. (1980) demonstrated that 3α-diol accounts for 86% of the total metabolites of DHT, while 3β-diol accounts for the remaining 14%. In cultured L6 rat myoblasts, the major metabolite of testosterone was also found to be 3α-diol (Inoue et al., 1990). These authors suggested that L6 myoblasts, similar to skeletal muscle tissue, rapidly convert DHT to 3α-diol.

Development of skeletal muscle occurs normally in human patients with 5α-reductase deficiency (Imperato-McGinley et al., 1974). In the aforementioned study by Powers and Florini (1975), testosterone shortened the cell cycle of myoblasts, yet other steroids were ineffective. The actions of the male hormones testosterone and DHT, while diverse, are believed to be mediated by the same intracellular receptor protein during

both embryonic development and in postnatal life (Tilley et al., 1990). The available evidence suggests that if androgens exert direct effects on skeletal muscle, these effects are likely mediated by testosterone rather than its metabolites, DHT or estradiol.

The anabolic effects of androgens may result from their effects on other hormones (Tucker and Merkel, 1987). For example, elevated circulating androgens in the neonate and adult appear to be important for normal growth hormone secretion in rats (Jansson and Frohman, 1987). Intact male sheep and androgen-treated wethers also have higher growth hormone (GH) concentrations than control wethers. Additionally, testosterone administration to prepubertal hypopituitary boys did not directly affect plasma IGF-I or augment the effect of GH to raise plasma IGF-I (Craft and Underwood, 1984). This suggests that the androgen-stimulated increase in circulating GH is most likely responsible for elevated IGF-I observed in the gonadally intact male. These studies demonstrate that the anabolic effects of androgens may be due, at least in part, to their influence on other hormones.

Androgen Receptors

In order to be directly regulated by androgens, it is generally assumed that a cell must possess specific intracellular androgen receptors. The androgen receptor is a member of the steroid/thyroid/retinoic acid family of

receptors (O'Malley, 1990). Hormone binding is the primary event in androgen receptor-mediated gene activation. The molecular mechanism of transactivation by these receptors involves structural modifications of the receptor upon ligand binding. As with other steroid receptors, the androgen receptor is thought to activate gene transcription through the following series of events: hormone binding, receptor activation, dimerization, and interaction of the ligand-receptor complex with specific cis-acting regulatory elements such that expression of a target gene is modified (Beato, 1989). All members of this receptor family have a similar functional domain structure which includes: a variable N-terminal region which is involved in modulation of gene expression, a short well conserved DNA-binding domain characterized by two zinc finger motifs, and a partially conserved C-terminal ligand binding domain which is important for receptor dimerization and transactivation (Beato, 1989; O'Malley, 1990). The complementary DNA and amino acid sequences of the human and rat androgen receptors have recently been identified (Chang et al., 1988b; Lubahn et al., 1988; Trapman et al., 1988).

Specific receptors for androgens have been identified in a variety of tissues, among which is skeletal muscle. Traditionally, the detection of androgen receptors has been performed by biochemical binding assay (Liao, 1975). Cytosolic androgen receptor binding in human (Snochowski et

al., 1981b), rat (Michel and Baulieu, 1980), mouse (Dahlberg et al., 1981) bovine (Sauerwein and Meyer, 1989), fetal pig (Heyns and Pape, 1991) and market weight pig (Snochowski et al., 1981a) skeletal muscle has been reported. The ligand specificity of the androgen receptor in muscle is similar to that in the prostate (Saartok et al., 1984). Methyltrienolone has the highest relative binding affinity, followed by 19-nortestosterone and testosterone. Although DHT binds the androgen receptor in prostate with high affinity, the apparent low affinity of DHT for the androgen receptor in skeletal muscle is probably due to rapid metabolism of DHT in muscle (Saartok et al., 1984).

Studies of androgen binding to cytosolic skeletal muscle receptors have been performed under a variety of physiological conditions. Females and/or castrates have consistently been shown to have greater cytosolic androgen receptor concentrations (2 to 5-fold) than gonadally intact males (Dahlberg et al., 1981; Sauerwein and Meyer, 1989; Snochowski et al., 1981a; Rance and Max, 1984). In fact, Snochowski et al. (1981a) were unable to detect androgen receptors in cytosol from muscle of boars. Administration of androgens to castrates has been shown to return cytosolic androgen receptor concentrations to that of gonadally intact males (Rance and Max, 1984).

The cytosolic androgen receptor content of skeletal muscle appears to increase transiently within 72 h after

physical exercise (Tchaikovsky et al., 1986). During denervation atrophy the ratio of cytosolic glucocorticoid receptor to androgen receptor of the levator ani muscles increased within 3 days and this was maintained for at least 4 weeks (Hughes and Krieg, 1986). Denervation caused 40% and 17% increases in cytosolic androgen receptor binding in skeletal muscles of male and female rats, respectively (Bernard and Max, 1986). In this study, however, total homogenate androgen receptor binding in skeletal muscles did not change, suggesting altered intracellular distribution of androgen receptors in response to denervation.

The observed increase in cytosolic androgen receptors in denervated muscle and that of females or castrated males is believed to result from a greater number of unoccupied receptors which are cytosolic or loosely bound to skeletal muscle nuclei (Snochowski et al., 1981a; Rance and Max, 1984). Similarly, steroid binding assays indicated that unoccupied estrogen receptors were cytosolic, yet immunocytochemical studies demonstrated that estrogen receptors are located in the nuclei of cells, even in the absence of ligand (King and Greene, 1984; Welshons et al., 1984). It has been proposed that estrogen receptors recovered in the cytosolic fraction of a homogenate represent receptors that are loosely associated with nuclei and binding of estradiol to its receptor leads to tighter association (King and Greene, 1984). Furthermore, cytosolic estrogen receptors determined with biochemical

binding methods may be artifacts due to release of loosely bound nuclear receptors during cell disruption (Welshons et al., 1984). This may also be true for binding assays which detect cytosolic androgen receptors.

Until recently, specific antibodies against androgen receptors were not available due to difficulties in purification of androgen receptors. However, the cloning of androgen receptor cDNA has led to the production of peptides and fusion proteins containing specific androgen receptor sequences, which have been used to develop anti-androgen receptor antibodies (Chang et al., 1989). Recent immunological studies have shown that androgen receptors appear to be confined to the nuclei of cells in skeletal muscle, cardiac muscle and various other tissues (Takeda et al., 1990; Sar et al., 1990; Kimura et al., 1993). Based on this information, it seems probable that observed differences in cytosolic androgen receptor numbers in skeletal muscle, measured by ligand binding, represent only differences in receptor affinity for DNA. Therefore, assays of ligand binding to skeletal muscle cytosol are not valid for comparison of androgen receptor number. Still, the presence of androgen receptors indicates that androgens potentially have direct effects on skeletal muscle.

Androgen Receptor Regulation

The levels of androgen receptor protein and mRNA are thought to reflect the degree of androgen responsiveness of a

tissue. Androgen-receptor concentration in prostatic carcinomas is related to the androgen sensitivity of various tumors (Voight et al., 1975). Additionally, Quarmby et al. (1990a) demonstrated that, in lines of Dunning rat prostatic adenocarcinoma cells, loss of androgen binding and responsiveness is accompanied by decreased androgen receptor mRNA and protein. Positive androgen receptor immunostaining in different cell types of the adult rat prostate also corresponds with androgen sensitivity of these cell types (Prins et al., 1991).

A low level of 10 kilobase (kb) androgen receptor mRNA was detected in extracts of rat skeletal muscle (Chang et al., 1988; Tan et al., 1988; Shan et al., 1990). Chang et al. (1988) reported that the level of AR mRNA per unit of poly $(A)^+$ RNA in thigh muscle was less than 10% of that found in seminal vesicle. More recently, Shan et al. (1990) observed a prominent 10 kb band in poly(A) RNA isolated from rat ventral prostate, seminal vesicle, epididymis, kidney, and skeletal muscle, and another androgen receptor mRNA species of approximately 8 kb in kidney and skeletal muscle. The significance of the 8 kb band is unknown at this time. Unlike skeletal muscle, spleen lacks androgen receptor mRNA (Tan et al., 1988, Shan et al., 1990) and stains negatively for androgen receptor (Takeda et al., 1990; Prins et al., 1991). Therefore, spleen is commonly used as a negative control in androgen receptor studies.

Regulation of androgen receptor protein or mRNA levels in skeletal muscle has not been reported, although Takeda et al., (1990) stated that nuclear androgen receptor staining in rat skeletal muscle was "slightly weaker" in female than in male rats. Regulation of androgen receptors in other tissues has been studied more extensively and is discussed below.

Evidence for Autologous Up-Regulation

Studies using several cell lines have shown that androgen-stimulated cell proliferation is associated with an augmentation of androgen receptors (Syms et al., 1983a; Syms et al., 1983b). Testosterone up-regulates the androgen receptor in isolated adipose precurser cells (De Pergola et al., 1990). Exposure of hamster ductus deferens smooth muscle (DDT_1MF-2) cells to androgens increases cell proliferation (Norris et al., 1974) and also induces a doubling in androgen receptor concentration over a 6 h period (Syms et al., 1983a). Addition of glucocorticoid overcomes the mitogenic action of androgens and the elevation in androgen-receptor levels (Syms et al., 1983a; Syms et al., 1983b), and also inhibits production of *c-sis* proto-oncogene mRNA transcripts which encode PDGFs (Norris et al., 1984). Exogenous PDGF prevents the glucocorticoid-induced arrest of DDT_1MF-2 cells in the G_1 phase of the cell cycle, but has no effect on the action of glucocorticoid on androgen-receptor augmentation (Syms et al., 1984).

Syms et al. (1985) determined rates of androgen synthesis

and degradation in DDT_1MF-2 cells using isotopically dense amino acids. In the absence or presence of 1 nM [^{3}H]R1881 (methyltrienolone), half-life of the androgen receptor was 3.1 h and 6.6 h, and the rate constant for receptor synthesis was 1.35 and 2.23 fmol/μg DNA per h, respectively. Thus, androgen receptor concentration was increased through stabilizing existing receptors and by increasing *de novo* receptor synthesis in DDT_1MF-2 cells. This androgen receptor augmentation was specific for androgens and androgen receptor synthesis was completely inhibited by cycloheximide. Autologous up-regulation of androgen receptors has also been demonstrated in fibroblasts cultured from human genital skin (Kaufman et al., 1983; Grino et al., 1990), and this effect is suppressible by cycloheximide. In these cells, DHT had a higher affinity for the receptor than testosterone, and 2 nM DHT was more effective in thermostabilizing and promoting up-regulation of the receptor than 2 nM testosterone (Grino et al., 1990). However, differences observed between these ligands were overcome by increasing the testosterone concentration to 20 nM.

Up-regulation of androgen receptor protein and mRNA by androgens has been observed in cultured rat penile smooth muscle cells (Gonzalez-Cadavid et al., 1993). It is possible that ligand-bound steroid receptors stabilize mRNA (Nielson and Shapiro, 1990). However, Gonzalez-Cadavid et al. (1993) demonstrated that androgen receptor up-regulation occurs, at

least in part, at the transcriptional level. In their studies, incubation with R1881 for 24 h increased androgen receptor binding and this increase was prevented by addition of cycloheximide or actinomycin D, an inhibitor of transcription. Furthermore, DHT increased androgen receptor mRNA in confluent androgen-deprived smooth muscle cells (Gonzalez-Cadavid et al., 1993). In contrast, *in vivo* androgen receptor binding (Takane et al., 1990) and expression of androgen receptor mRNA in penile smooth muscle cells declines at puberty (Takane et al., 1991).

Evidence for Autologous Down-Regulation

Androgens have been shown to elicit autologous down-regulation of AR mRNA concentration in some target tissues (Chang et al., 1988a; Tan et al., 1988; Quarmby et al., 1990c). Castration increased the steady state levels of 10 kb androgen receptor mRNA in rat ventral prostate, and this effect was reversed by administration of 2 mg testosterone propionate (Tan et al., 1988). Androgen withdrawal or castration caused a decrease in the amount of 10 kb androgen receptor mRNA both in the LnCAP human prostate cancer cell line and *in vivo* in the rat kidney, brain, epididymis and coagulating gland, respectively, and this effect was also reversed by testosterone administration (Quarmby et al., 1990c). In their study, autologous down-regulation of androgen receptor mRNA was not observed in the kidneys of androgen insensitive testicular feminized (*Tfm*) rats,

indicating that down-regulation requires a functional androgen receptor.

Receptor down-regulation is mediated by a decreased rate of transcription and/or half-life of receptor mRNA. Androgen withdrawal increased steady state levels of androgen receptor mRNA and androgen receptor protein *in vivo* in rat ventral prostate and seminal vesicles and in cultured human hepatoma (HepG2) cells (Shan et al., 1990). In each case these effects were reversed by androgen replacement which consisted of testosterone-releasing implants (400 μg/day), or culture medium containing 10^{-7} M testosterone for HepG2 cells. Autologous receptor down-regulation has also been shown for the glucocorticoid (Dong et al., 1988) and progesterone (Alexander et al., 1989) receptors, and is thought to be analogous to receptor-mediated inhibition of target gene transcription.

Androgen receptor concentration is usually highest in androgen-responsive tissues with high endogenous androgen content, such as prostate, epididymis, and testes (Voight et al., 1975; Quarmby et al., 1990a; Shan et al. 1990; Prins et al., 1991). Thus, the physiological importance of androgen-induced down-regulation of androgen receptor mRNA and protein is difficult to establish. Collectively, there exists no consensus in the literature on the topic of androgen-receptor regulation, and it appears likely that tissue differences and possibly species differences in androgen receptor regulation

exist.

Cellular Responses to Androgens

It has been postulated that the immediate response to androgen binding is the modulation of a few regulatory genes whose products amplify the androgen effect via regulation of a multitude of structural genes by transcription and/or post-transcriptional mechanisms (reviewed by Berger and Watson, 1989). A small number of androgens-regulated genes have been characterized (Berger and Watson, 1989). Among the best studied are the C3 component of prostatic steroid binding protein (Tan et al., 1992; Claessens et al., 1989) and prostate-specific antigen genes (Reigman et al., 1991). The response elements from these genes AGTACGtgaTGTTCT and AGAACAgcaAGTGCT, respectively, confer androgen regulation to a reporter gene. These elements are similar to the derived consensus glucocorticoid response element (GRE) sequence (GGTACAnnnTGTTCT), which can function as a response element for all steroid classes except estrogens (Beato, 1989).

Androgen receptors in the nuclear fraction from LNCaP cells (human prostate cancer cell line) have been shown to be phosphorylated in the presence of synthetic androgen, R1881, while only a small amount of phosphorylated androgen receptor was detected in the absence of ligand (van Laar et al., 1990). LNCaP cells have been shown to respond to androgens by increasing the secretion of a 42-kDa protein (Berns et al., 1986). A 2-fold increase in EGF receptors was also observed

24 h after androgen administration to LNCaP cells, suggesting that androgens may affect growth of these cells by autocrine or paracrine mechanisms (Mulder et al., 1989).

Because androgen receptor response depends on sequences that can also function as GRE's, expression of androgen-responsive genes may involve accessory factors that favor interaction of androgen receptor over glucocorticoid receptor. One mechanism by which specific hormone responses may be mediated in cells containing more than one type of receptor is interaction with other regulatory proteins. For example, strong stimulation of transcription from the MMTV-CAT promoter was observed when c-*jun* was co-expressed with the AR, but not progesterone or glucocorticoid receptors (Shemshedini et al., 1991). However, when c-*fos* was co-expressed with c-*jun*, transcriptional activation was inhibited by the ligand bound androgen receptor.

Adler et al. (1992) demonstrated that androgen induction requires both a consensus response element and auxiliary elements also present within a 120 base-pair DNA fragment within the hormone response element within the androgen activated enhancer of the sex-limited protein (*Slp*) gene. Glucocorticoid receptors can bind, but cannot activate expression of this gene. The *Slp* hormone-dependent enhancer is specifically regulated by androgens and not glucocorticoids or progestins. This stringent hormonal control requires both the androgen receptor binding site and adjacent sequences.

In addition to their stabilizing effect on the androgen receptor, androgens have also been shown to prolong the half-life of other proteins. Testosterone propionate administration to male mice has been shown to increase the half-life of ornithine decarboxylase and S-adenosylmethionine decarboxylase, two enzymes involved in polyamine metabolism, which normally have rapid turnover rates (Seely et al., 1982).

While the anabolic effects of androgens are well documented, the mechanisms of androgen action on skeletal muscle are unclear. The objectives of this study were to: 1) determine if cultured porcine satellite cells and satellite cell-derived myotubes possess androgen receptors, 2) demonstrate androgen receptor autoregulation by testosterone, and 3) examine the effects of testosterone on proliferation and differentiation of porcine satellite cells.

CHAPTER 1

IDENTIFICATION, LOCALIZATION AND REGULATION OF ANDROGEN RECEPTORS IN CULTURED PORCINE SATELLITE CELLS AND SATELLITE CELL-DERIVED MYOTUBES

ABSTRACT

Immunochemical methods were used to verify the presence of androgen receptors (AR) in porcine skeletal muscle satellite cells and myotubes. The effect of testosterone on AR content of clonally derived porcine satellite cells and myotubes was examined. Satellite cells were isolated from the semimembranosus muscles of 6- to 8-week-old pigs and myogenic clones were established using glass cloning rings. Cells were grown on gelatin coated 16-mm diameter wells (immunocytochemistry) or 35-mm diameter wells (immunoblotting) in Minimum Essential Medium (MEM) containing 10% fetal bovine serum. Myotube formation was induced by exposure to serum-free medium containing 10^{-6} M insulin. Polyclonal rabbit antibodies raised against amino acids 1-21 of the rat AR (PG-21) were used to detect the porcine AR. Western immunoblot analysis of porcine spleen, epididymis, satellite cells and myotubes revealed an immunoreactive AR protein of approximately 107 kDa in all tissues except spleen. Exposure of satellite cell or myotube cultures to serum-free medium containing 10^{-7} M testosterone for 6, 12, or 24 h induced a time-dependent increase in immunoreactive AR protein relative to control cultures. Androgen receptor immunostaining of

paraformaldehyde-fixed satellite cells and myotubes revealed positive nuclear staining, which was stronger in cells exposed to testosterone. Treatment of cells with 10^{-7} M estradiol-17β or dexamethasone had no apparent effect on AR content. Nuclear AR were also detected immunocytochemically in primary satellite cell and myotube cultures, and clonal cultures of fibroblasts. These results indicate that skeletal muscle satellite cells, myotubes, and fibroblasts possess AR which appear to be confined to nuclei in the absence or presence of ligand. Testosterone up-regulates AR in cultured porcine satellite cells and myotubes, while estradiol and dexamethasone have no apparent effect on AR regulation.

INTRODUCTION

The anabolic effects of androgens on skeletal muscle are well documented. In livestock species, intact males grow more rapidly, utilize feed more efficiently, and produce trimmer, more muscular carcasses than castrates (reviewed by Seideman et al., 1982; Purchas, 1993). *In vivo*, testosterone administration has been shown to stimulate myofibrillar protein synthesis (Florini and Breuer, 1966; Grigsby et al., 1976; Martinez et al., 1984), and enhance satellite cell mitotic activity (Mulvaney et al., 1988; Joubert and Tobin, 1989). Skeletal muscle DNA content is greater in boars compared to barrows or gilts (Snochowski et al., 1981) and is also increased in androgen treated female rats compared to untreated females (Thompson et al., 1989). Still, direct effects of androgens on cultured skeletal muscle cells have been difficult to demonstrate. Powers and Florini (1975) reported a 25% increase in DNA labeling index of myoblasts exposed to testosterone. However, androgens have generally been shown to have no direct effect on proliferation (Gospodarowicz et al., 1976; Thompson et al., 1989) or protein accretion (Allen et al., 1983; Roeder et al., 1986) in skeletal muscle cell cultures.

To be directly regulated by androgens, it is generally assumed that a cell must contain specific androgen receptors (AR). Cytosolic AR binding in skeletal muscle tissue has been reported (Michel and Baulieu, 1980; Dahlberg et al., 1981; Snochowski et al., 1981). More recently, immunohistochemical studies revealed AR in cardiac muscle nuclei (Takeda et al., 1990; Kimura et al., 1993) and in "nearly every nucleus" of skeletal muscle (Takeda et al., 1990).

The apparent discrepancy between *in vivo* and *in vitro* skeletal muscle responsiveness to androgen has not been explained. In the present study, an immunochemical approach was used to determine if satellite cells and satellite cell-derived myotubes possess AR. Additionally, cellular location and hormonal regulation of AR were investigated.

EXPERIMENTAL PROCEDURES

Materials-Minimum Essential Medium (MEM), antibiotic-antimycotic and gentamicin were purchased from GIBCO BRL (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Hazleton (Lenexa, KS). Tissue culture dishes (10-cm diameter) and plates (24- and 6-well) were obtained from Corning Glass Works (Corning, NY). Pronase, MCDB-110 medium, bovine serum albumin (BSA; RIA grade, A-7888), dexamethasone (D-8893), bovine insulin (I-1882), bovine transferrin (T-8027), water-soluble linoleic acid (L-5900), porcine skin gelatin, goat serum, non-immune rabbit serum, rabbit IgG (I-5006), biotinylated anti-rabbit IgG (B-7389), Extravidin-peroxidase (E-2886) and 3,3′-Diaminobenzidine (DAB; D-8160) were obtained from Sigma Chemical Co. (St. Louis, MO). Nitrocellulose was purchased from Schleicher and Schuell (Keene, NH), and Immobilon-P was obtained from Millipore (Bedford, MA).

Porcine Satellite Cell Isolation-Satellite cells were isolated from the semimembranosus muscles of 6 to 8-week-old pigs as described by Doumit and Merkel (1992). This is a modification of the procedure outlined by Bischoff (1974) for rodent skeletal muscle. Briefly, semimembranosus muscles were

excised, trimmed of visible connective tissue, sectioned and ground in an aseptically prepared meat grinder. Ground muscle was incubated for 40 min at 37°C in a solution of 0.8 mg/ml Pronase dissolved in phosphate buffered saline (PBS) (2 parts muscle:3 parts Pronase solution v/v). Following enzymatic digestion, cells were separated from tissue fragments by repeated centrifugation at 300 x *g* for 5 min, followed by sequential filtration through 500 and 53 μm mesh nylon cloth. Isolated cells were frozen or cultured under conditions previously described (Doumit and Merkel, 1992). An aliquot of the cell suspension from each isolation was plated to determine the yield of viable cells.

Development of Satellite Cell and Fibroblast Clones-In all culture experiments, plating medium consisted of MEM containing 10% FBS, 0.5% antibiotic-antimycotic and 0.1% gentamicin. Cells were seeded into gelatin-coated culture wells or dishes and maintained in a humidified CO_2 incubator containing 95% air and 5% CO_2 at 37°C. Gelatin coating (0.1%) was performed using the method of Richler and Yaffe (1970). Porcine satellite cells were cloned as outlined by Doumit et al. (1993), using a cloning ring technique adapted from those described for other myogenic cells (Rutz and Hauschka, 1982; Minshall et al., 1990; Quinn et al., 1990). Primary satellite cells were plated at 100 or 200 cells per 10 cm diameter culture dish and incubated for 7 to 10 days in MEM containing 10% FBS. Colonies were then exposed to MEM containing 2% FBS

or horse serum for 24 h or until myotube formation was initiated. Myogenic colonies, which typically contained fewer than 5% myotube nuclei at this stage, were isolated using 1.0 cm diameter sterile glass cloning rings dipped in silicone grease. These colonies were immediately trypsinized (trypsin-EDTA diluted 1:10 in PBS) and transferred into cell culture wells containing MEM and 10% FBS. Cultures were expanded and subsequently transferred (3-5 passages) into increasingly larger cell culture flasks, then frozen and stored in liquid nitrogen for later use. We have partially characterized four clonally-derived porcine satellite cell cultures. These clones yield 5° cultures that typically contain 40 to 60% myotube nuclei after 4 days in serum-free differentiation medium. The clones with the highest apparent myogenic capacity, determined by the extent of myotube formation, were used in this study. Clonal cultures of fibroblast-like cells were isolated from primary colonies which did not exhibit myotube formation. These clones were prepared as described for satellite cell clones. No myotube formation, creatine kinase induction, or myosin positive cells (determined as described in Experimental Procedures of Chapter 2) were observed in these cultures under conditions which induce differentiation of porcine satellite cells.

Satellite cell cultures-Unless otherwise specified, clonally-derived satellite cells (fourth passage) were suspended in MEM containing 10% FBS (5000 cells/ml medium).

For immunocytochemistry, cells were seeded on gelatin coated 24-well plates (5000 cells/16 mm-diameter well). For Western blots, cells were plated on gelatin coated 6-well plates (2 x 10^4 cells/35 mm-diameter well). Fresh growth medium (MEM+10%FBS) was supplied at 48-h intervals. When cells reached confluence (approximately day 5), myotube formation was induced by daily exposure to serum-free medium (0.3 ml/16 mm-diameter well or 2 ml/35 mm-diameter well). This medium was originally formulated to maintain viability and support proliferation of cultured porcine satellite cells (Merkel et al., 1993), and has been modified to promote differentiation of porcine satellite cells. The components of the original serum-free medium and the differentiation-promoting medium are listed in Table 1.1.

Western Immunoblots-At times specified in figure legends, cells were solubilized by addition of hot (95°C) electrophoresis sample buffer (62 mM Tris-HCL, pH 6.8, 2% SDS, and 10% glycerol; 100 µl/35-mm diameter well). Cells were scraped and contents of three wells per treatment were pooled. Porcine spleen (obtained at slaughter from a mature pig) and epididymal tissues (obtained at castration of 10-day-old boars) were immediately frozen in liquid nitrogen, then pulverized under liquid nitrogen in a mortar and pestle. Powdered tissues were solubilized directly in 9 volumes of boiling 1X sample buffer, boiled 5 min and clarified by centrifugation at 2000 x *g* for 10 min. Protein concentration

TABLE 1.1

Porcine Satellite Cell Serum-Free Media

COMPONENT	FINAL CONCENTRATION	
	Growth	Differentiation
MEM:MCDB-110 Medium	4:1	4:1
Basic Fibroblast Growth Factor	10.0 ng/ml *	-
Bovine Serum Albumin	0.5 mg/ml	0.5 mg/ml
Dexamethasone	10^{-7} M	10^{-10} M
Insulin	10^{-6} M	10^{-6} M
Linoleic Acid	0.5 µg/ml	0.5 µg/ml
Platelet-Derived Growth Factor-BB	5.0 ng/ml *	-
Transferrin	100.0 µg/ml	100.0 µg/ml

* Concentration used was previously determined to stimulate maximal proliferation of porcine satellite cells (Doumit et al., 1993).

was determined by the bicinchoninic acid method of Smith et al. (1985). Immediately prior to electrophoresis, 2-mercaptoethanol was added to 5% v/v and samples were boiled for 5 min. Proteins were separated by electrophoresis in 8% polyacrylamide separating gels with 4% stacking gels as described previously (Laemmli, 1970). Resolved proteins were transferred to nitrocellulose or Immobilon-P at 4°C for 5 h at 0.8 amp in a buffer containing 25 mM Tris, 193 mM glycine, and 15% methanol. Lanes containing molecular weight standards were stained with Coomassie blue. To prevent non-specific

antibody binding, blots were blocked with 10% Carnation non-fat dry milk, 0.3% Triton X-100 in PBS, pH 7.4 for 2 h. Antibody incubations were carried out in blocking buffer at room temperature and blots were washed three times with blocking buffer after each incubation. Blots were incubated for 2 h with 1 μg/ml PG-21, an antibody raised against a peptide (AR_{21}) containing the first 21 amino acids of the rat and human AR (Prins et al., 1991). PG-21 was generously provided by Dr. Geoffrey L. Greene of the Ben May Institute, University of Chicago, Chicago, IL. Normal rabbit IgG (1 μg/ml) served as the negative control. Alternatively, blots were incubated with a 1:5000 dilution of polyclonal (rabbit) antiserum raised against the N-terminal domain of the human AR (Affinity Bioreagents, Neshanic Station, NJ), or non-immune rabbit serum. Blots were then exposed to biotinylated goat anti-rabbit IgG (2 μg/ml) for 1 h, followed by 2.5 μg/ml Extravidin-peroxidase in PBS containing 10% blocking solution for 1 h. Blots were washed three times with PBS and immunoreactive bands were visualized by treatment with DAB (10 mg in 20 ml PBS) in the presence of 0.003% H_2O_2 (20 μl of 3% H_2O_2). In some studies, estrogen receptors were detected using polyclonal antibodies (ER-21) under identical conditions to those described for PG-21. ER-21 was raised against amino acids 1-21 of the human estrogen receptor and was kindly provided by Dr. Geoffrey Greene.

Immunocytochemical Localization of the Androgen Receptor- At specified times, cells were washed three times in PBS and fixed for 10 min in PBS containing 2% paraformaldehyde and 10% sucrose (pH 7.2), then permeabilized in 0.3% Triton X-100 in PBS for 30 min. Non-specific binding was blocked with PBS containing 1% goat serum for 30 min, and cells were incubated with 1 μg/ml PG-21 in blocking reagent overnight at 4° C. Normal rabbit IgG (1 μg/ml), and/or PG-21 pre-incubated for 1 h with a 10-fold molar excess of AR_{21} (kindly provided by Dr. Geoffrey Greene) served as negative control for each assay. Cells were washed three times in PBS and exposed to biotinylated goat anti-rabbit IgG (1:200) for 1 h. Following three washes in PBS, cells were incubated with Extravidin-peroxidase (1:100) in PBS for 40 min. Sites with enzyme activity (indicating antibody binding) produced a brown reaction product when exposed to DAB (1 mg/ml PBS) containing 0.006% H_2O_2 (20 μl of 3% H_2O_2). Photomicrographs were taken through a Zeiss inverted microscope equipped with a Nikon 35-mm camera (Morgan Instruments, Inc., Cincinnati, OH), using Ektar 100 or Tmax 100 film (Eastman Kodak, Rochester, NY).

RESULTS

Identification of Androgen and Estrogen Receptors-Two polyclonal AR antibodies were used to analyze extracts of porcine satellite cells, satellite cell-derived myotubes, epididymis and spleen for AR. In preliminary Western immunoblots, polyclonal (rabbit) AR antiserum interacted specifically with a protein of ~64 kDa in both satellite cell and myotube cultures (Fig. 1.1). Exposure of cultures to 10^{-7} M testosterone had no apparent effect on this protein (Fig. 1.1). Affinity purified anti-AR antibody, PG-21, was used for immunoblotting of porcine spleen, cultured satellite cells, and epididymis. Extracts from satellite cell and epididymis revealed a major immunoreactive band of ~107 kDa (Fig. 1.2). The mass of this protein is consistent with the known molecular mass of human, rat and bovine AR (Mulder et al., 1989; Prins et al., 1991). Androgen receptor immunostaining was more intense in epididymis than satellite cells, and was absent in spleen, which is generally considered an AR-negative tissue. Immunoblotting with anti-AR antiserum produced a 64 kDa band in spleen, satellite cells and epididymis (Fig. 1.3, lanes 1-3). This band was not observed in blots incubated with normal rabbit serum (Fig. 1.3, lanes 4-6) or PG-21 (Fig.

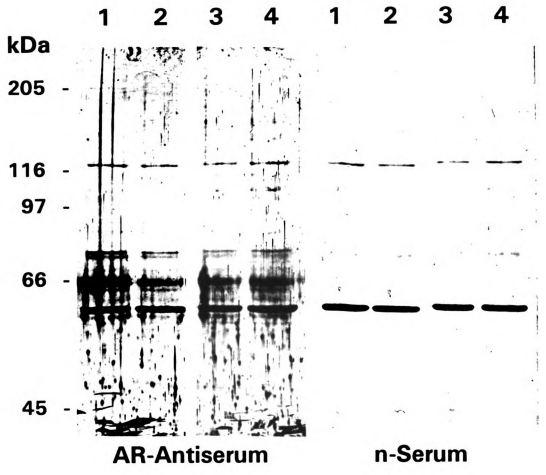

Fig. 1.1. Western blot analysis of satellite cells and myotubes using AR antiserum. Extracts were from confluent satellite cells exposed to MEM+2% FBS (lane 1), or MEM+2% FBS containing 10^{-7} M testosterone for 24 h (lane 2), cultures containing approximately 50% myotube nuclei (lane 3) or myotube cultures exposed to testosterone for 24 h (lane 4). Proteins present in cell extracts (50 μl/lane) were separated and transferred to nitrocellulose. Strips were incubated with polyclonal AR antiserum (left panel) or normal rabbit serum (right panel). In this preliminary experiment, antibody binding was visualized using reagents from a universal anti-rabbit immunostaining kit (Sigma Diagnostics). Positions of molecular weight markers are shown on the left. AR antiserum produced specific immunoreactive bands at ~64 kDa in all lanes. Bands observed in the presence of normal rabbit serum are non-specific.

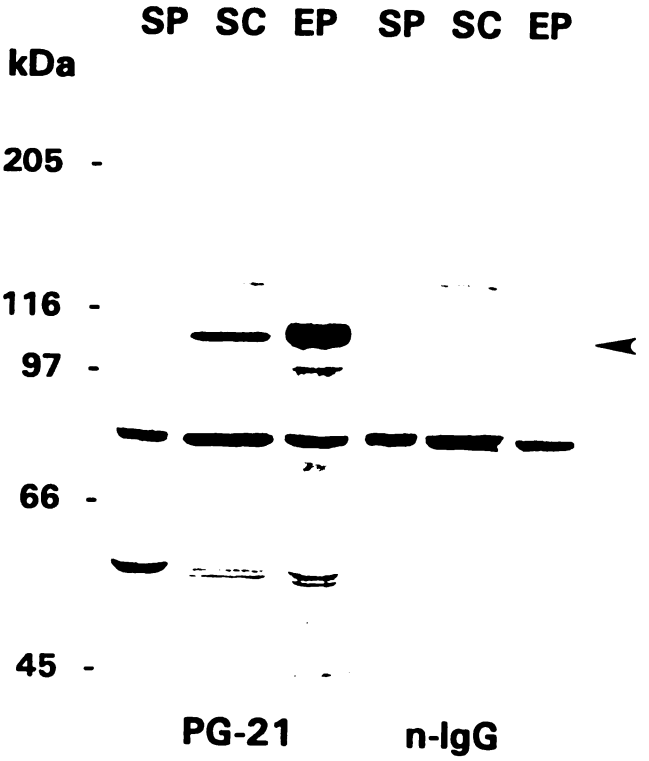

Fig. 1.2. Western blot analysis of porcine spleen (SP), satellite cells (SC), and epididymis (EP) using PG-21. Satellite cells were grown to confluence in MEM+10% FBS, then harvested. Extracts containing 250 μg protein were loaded in each lane. Nitrocellulose strips were incubated with affinity purified PG-21 (left panel), or control rabbit IgG (right panel). Positions of molecular weight markers are shown on the left. Incubation with PG-21 revealed a major immunoreactive band at ~107 kDa in porcine satellite cells and epididymis (indicated by arrow). The mass of this protein is consistent with the full-length AR protein detected in rat and human tissues. Bands observed in spleen and in right panel are non-specific.

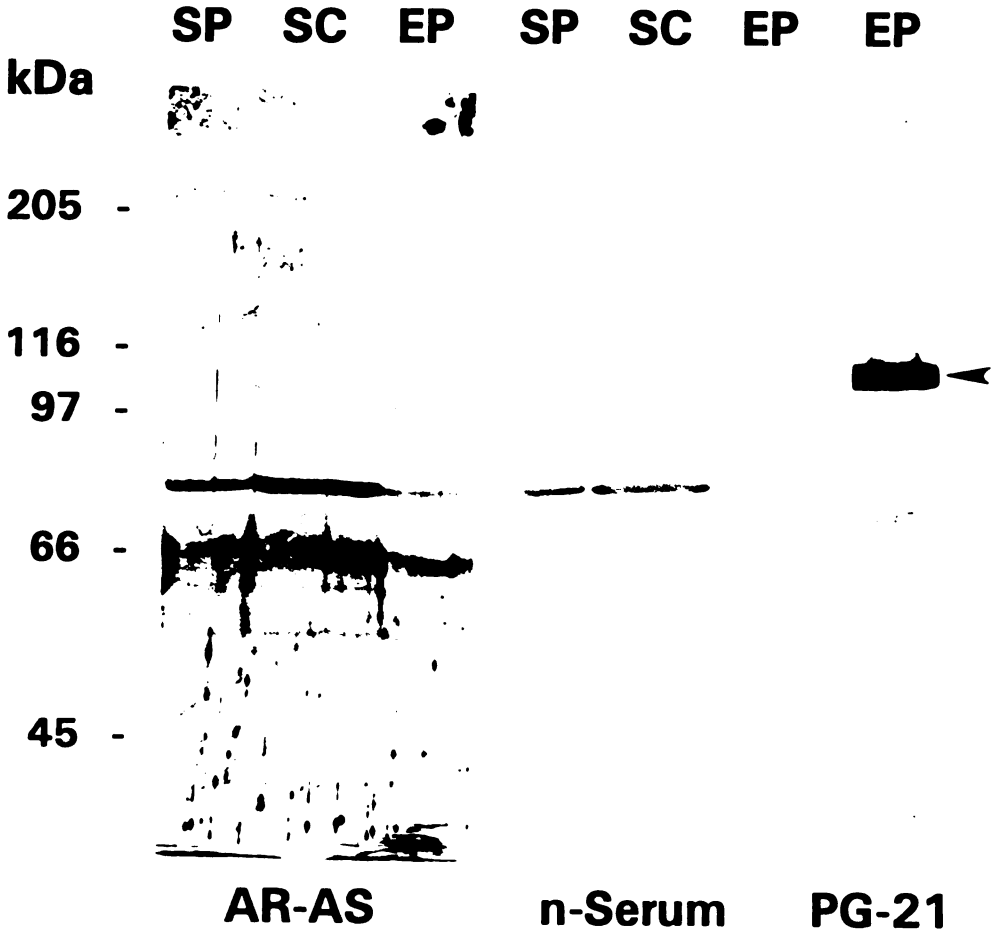

Fig. 1.3. Immunoblot analysis of porcine spleen (SP), satellite cells (SC), and epididymis (EP) using AR-antiserum. Extracts containing 250 μg protein were loaded, resolved and transferred onto nitrocellulose as outlined in Experimental Procedures. Strips were incubated with polyclonal AR antiserum (AR-AS), normal rabbit serum, or affinity purified PG-21. AR antiserum produced an immunoreactive band at ~64 kDa in all tissues (left panel), which was not observed in normal rabbit serum (center panel). As in Fig. 1.2, incubation of epididymis with PG-21, revealed a major immunoreactive AR band at 107 kDa (indicated by arrow).

1.3, lane 7). However, a 107 kDa band was detected with PG-21 in epididymis under identical conditions (Fig. 1.3, lane 7), indicating that this AR antiserum does not recognize the full-length porcine AR.

Anti-estrogen receptor antibodies (ER-21) reacted with a protein of ~64 kDa from epididymis, but not in extracts from satellite cells or spleen (Fig. 1.4). The 64 kDa protein representing the estrogen receptor corresponds in size to the 64 kDa protein observed in a parallel lane immunoblotted with AR antiserum (Fig. 1.4). However, the immunoreactive band observed with AR antiserum was more diffuse than that seen with ER-21 immunoblotting. Additionally, immunoblotting with AR antiserum produced a faint band at 64 kDa in spleen and satellite cells (Figs. 1.3 and 1.4), which was not detected with ER-21. Estrogen receptor immunostaining of fixed satellite cells and myotubes was also not detectable using ER-21.

Since PG-21 specifically recognized the porcine AR protein, these polyclonal antibodies were used for further detection of AR in cultured muscle cells. Immunoblot analysis revealed that satellite cell and myotube cultures maintained in serum-free medium (SFM) have similar quantities of AR protein (Fig. 1.5, lane 2 *vs* 3). However, 220 μg protein extracted from satellite cells grown in serum-containing medium produced a more intense AR band than a similar quantity of protein obtained from myotubes exposed to serum for 12 h

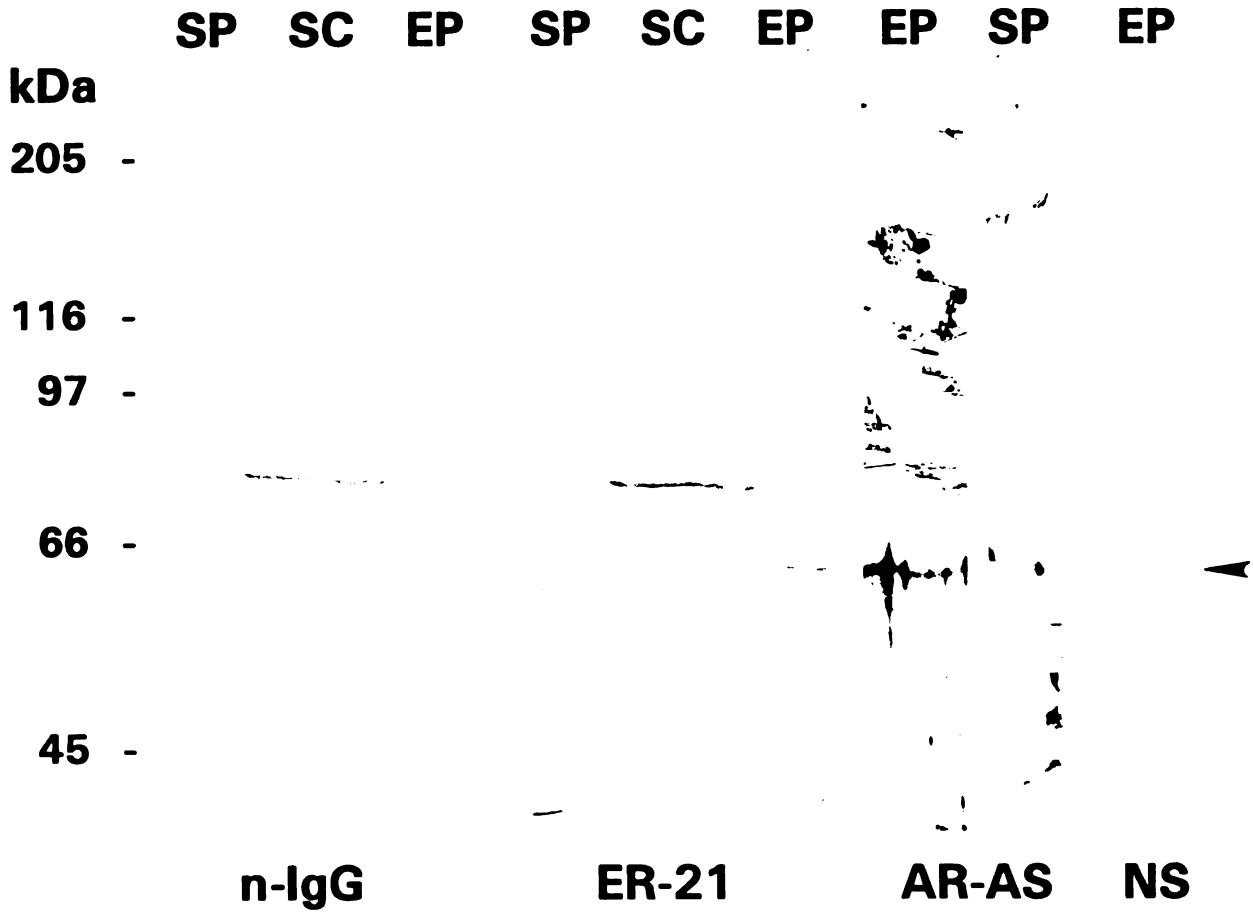

Fig. 1.4. Immunoblot of porcine estrogen receptors with ER-21. Satellite cells used in this experiment had been maintained in SFM (growth) for 4 days. Extracts containing 250 μg protein from porcine spleen (SP), satellite cells (SC), and epididymis (EP) were resolved, separated elecrophoretically and transferred to nitrocellulose. Strips were incubated with control rabbit IgG (n-IgG), ER-21, AR antiserum (AR-AS) or normal rabbit serum (NS). ER-21 and AR antiserum identify bands at ~64 kDa (indicated by arrow). AR antiserum, but not ER-21, recognizes a protein of this mass in spleen.

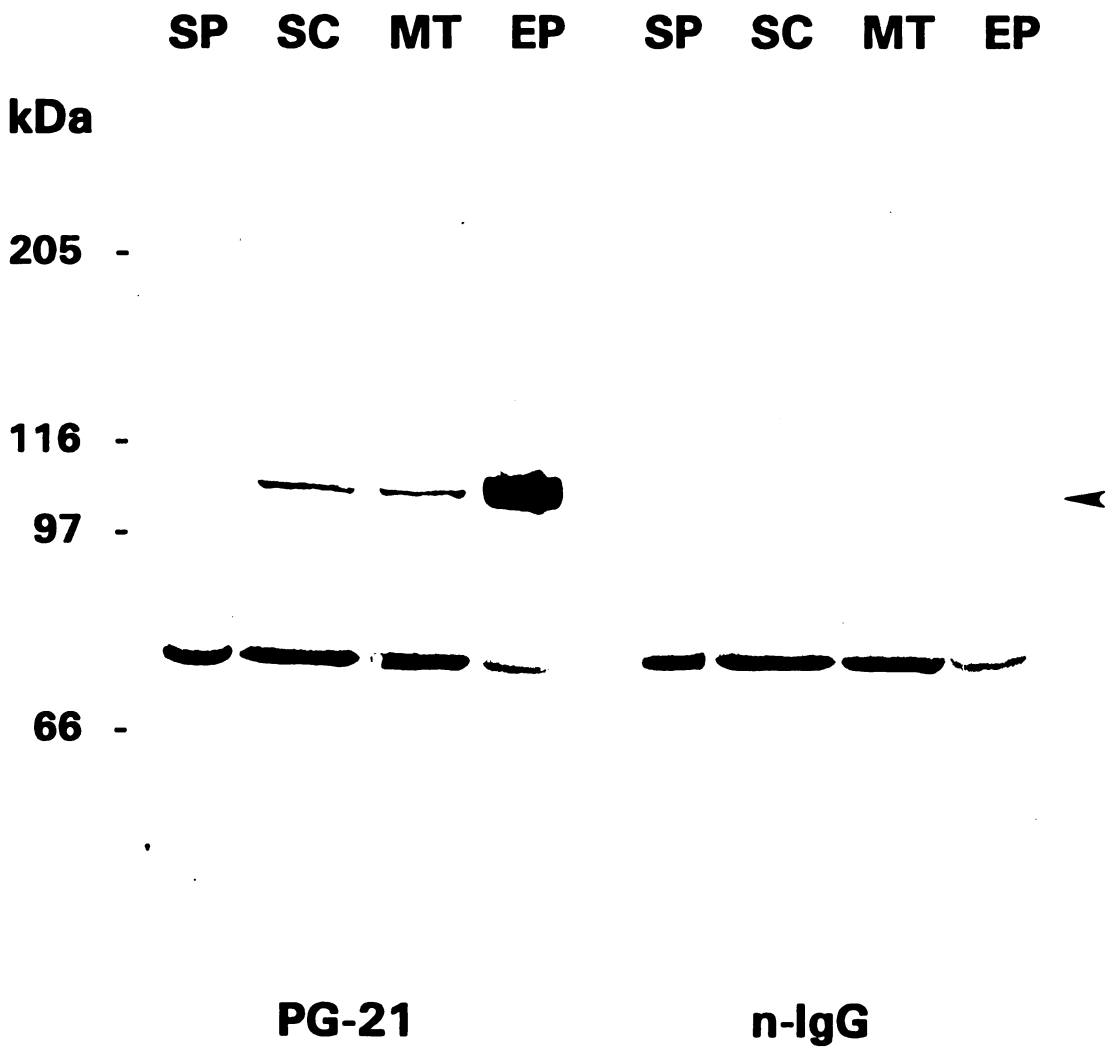

Fig. 1.5. Western blot analysis of satellite cells (SC) and myotubes (MT) maintained in SFM. Satellite cells were grown to confluence in MEM+10% FBS, then exposed to differentiation medium (Table 1.1), or this medium with 10^{-9} M instead of 10^{-6} M insulin. The latter medium typically results in <10% myotube nuclei. Lanes represent 220 μg protein from porcine spleen (SP), SC, MT and epididymis (EP) which was separated by gel electrophoresis and transferred to nitrocellulose. Incubation with PG-21 revealed a major immunoreactive AR band at ~107 kDa (indicated by arrow) in clonally-derived porcine satellite cells, myotubes (~60% myotube nuclei), and epididymis. No immunoreactive AR band was observed in spleen. Bands observed in spleen and in right panel are non-specific.

(Fig. 1.6, lane 2 *vs* 3). This apparently results from a dilution effect of myofibrillar proteins on the AR, since myotube cultures exposed to 10% FBS contain ~2-fold more protein than satellite cell cultures of similar nuclear density. Myotubes maintained in SFM generally have 30-40% greater protein than satellite cells in SFM.

Bands visible in the presence of normal rabbit IgG (~122 kDa in muscle cells and 75 kDa in all lanes) or in spleen (~50 kDa) were determined to be non-specific. The major non-specific band at 75 kDa was found to result from binding of Extravidin peroxidase, since elimination of secondary antibody had no effect on appearance of this band (Fig. 1.7, lane 2). No endogenous peroxidase activity was observed in extract from epididymis (Fig. 1.7, lane 3).

Immunocytochemical staining for AR, using PG-21, revealed that nuclei in all cells of primary porcine satellite cell cultures stained positive for AR, as did primary myotubes (Fig. 1.8). Immunostaining of clonally-derived satellite cells and fibroblasts is discussed below.

Regulation of Androgen Receptor Protein-Exposure of myotube cultures to 10^{-7} M testosterone for 12 h appeared to more than double the immunoreactive AR protein (Fig. 1.9). Likewise, 6, 12 and 24 h treatment with 10^{-7} M testosterone induced a time-dependent increase in AR protein in both myotube (Fig. 1.10) and satellite cell (Fig. 1.11) cultures. When myotubes were exposed to 10^{-8} M estradiol-17β for 12 h, no

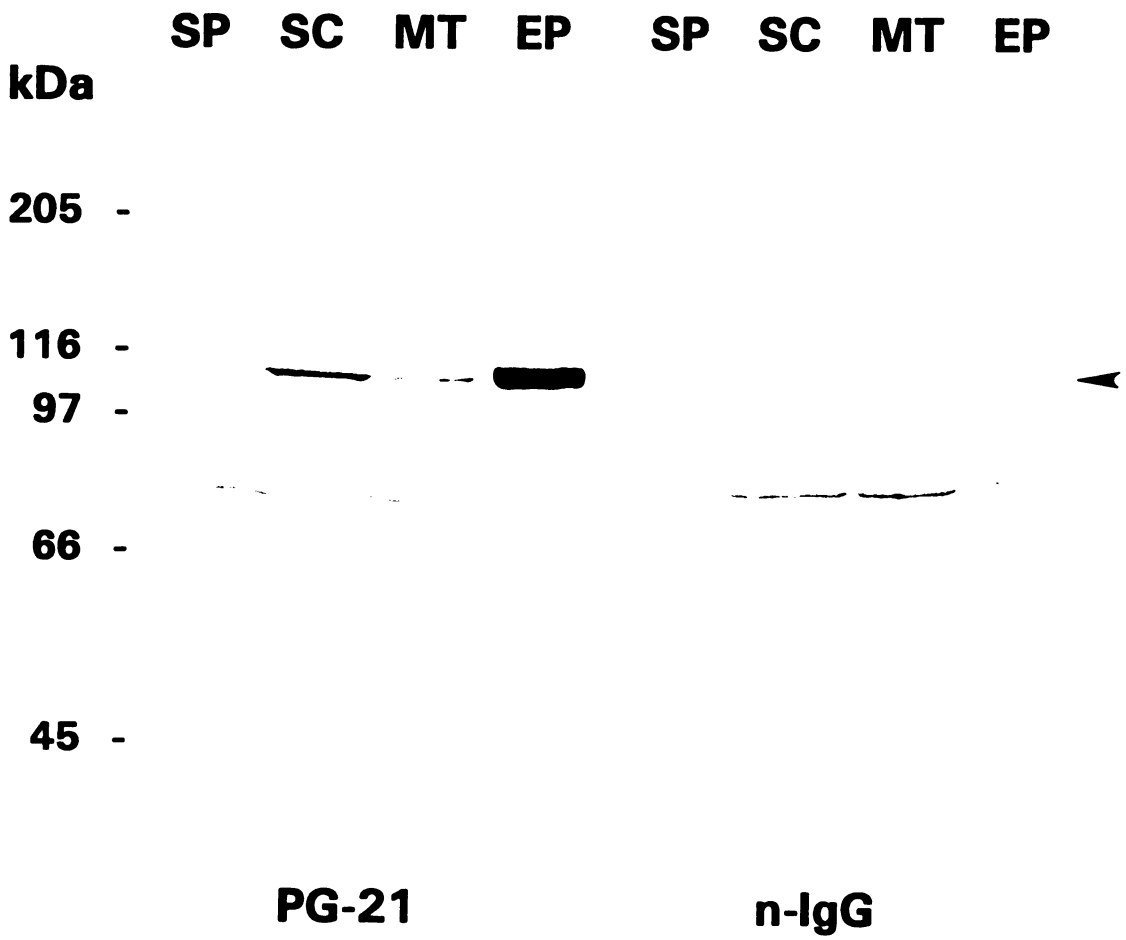

Fig. 1.6. Western blot analysis of satellite cells (SC) and myotubes (MT) exposed to MEM+10% FBS. Satellite cells were grown to 90% confluence in MEM+10% FBS, then harvested in SDS sample buffer or exposed to differentiation medium (Table 1.1) for 4 days. Myotubes were treated with MEM+10% FBS for 12 h, then harvested. Lanes represent 220 μg protein from porcine spleen (SP), SC, MT and epididymis (EP) which was separated by gel electrophoresis and transferred to nitrocellulose. Panels were incubated with PG-21 or normal IgG as outlined in Experimental Procedures. The major immunoreactive AR band is indicated by an arrow.

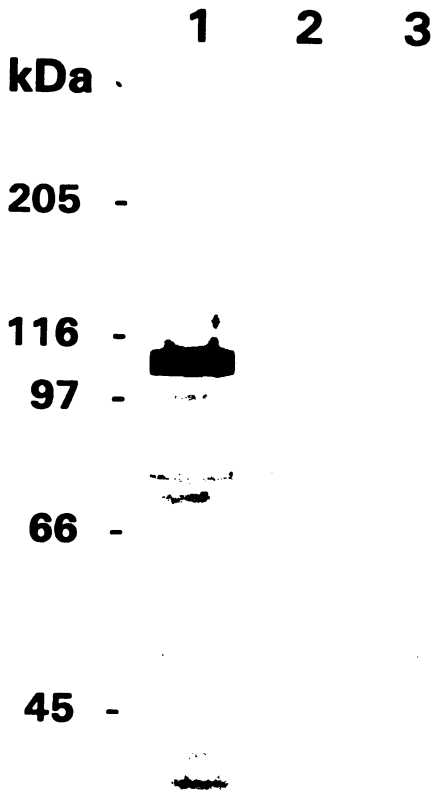

Fig. 1.7. Identification of non-specific 75 kDa band. Extracts containing 250 μg protein from porcine epididymis were loaded, resolved and transferred to nitrocellulose as outlined in Experimental Procedures. Strips were blocked, then incubated with PG-21 (lane 1), or normal IgG (lanes 2 and 3). Thereafter, lane 1 was treated as described in Experimental Procedures, lane 2 was not incubated with secondary antibody, and lane 3 was only incubated with DAB substrate.

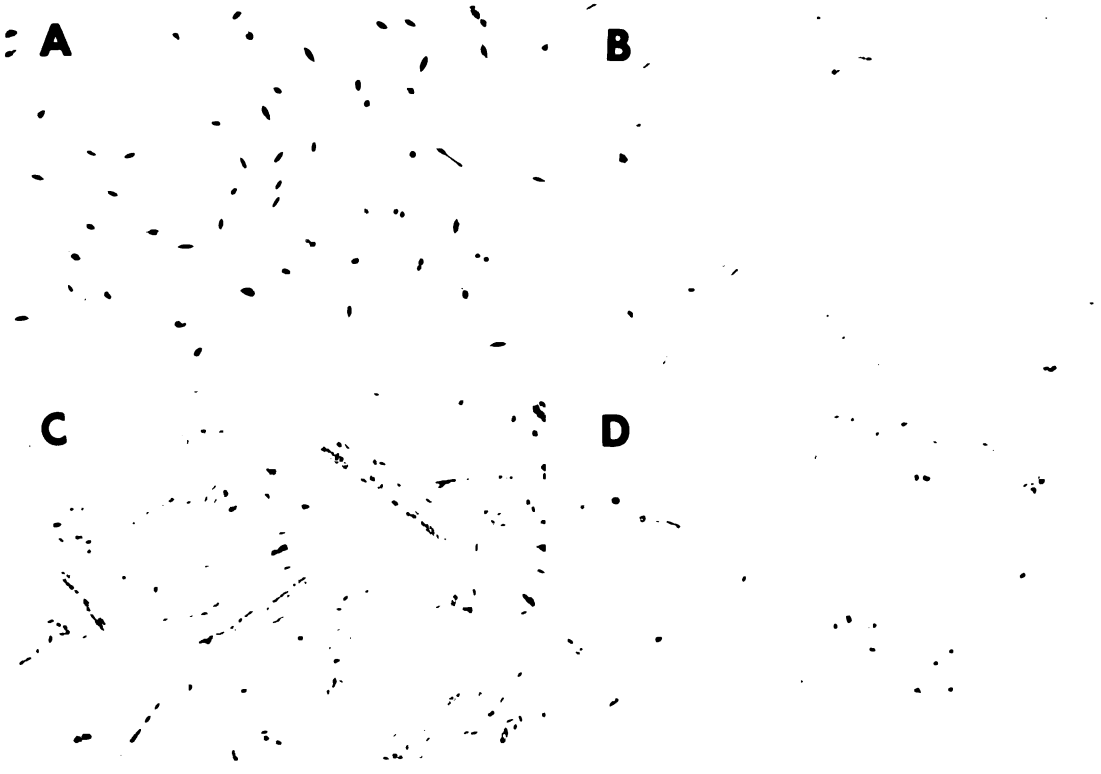

Fig. 1.8. Immunocytochemical staining for AR in satellite cells and myotubes from primary culture. Satellite cells were allowed to attach for 48 h in MEM+10% FBS, then maintained in SFM for 24 h prior to immunostaining. Alternatively, cells were grown in MEM+10% FBS for 6 days, then exposed to serum-free medium for 3 days to induce differentiation. Positive immunostaining in nuclei of satellite cells (A) and myotubes (C) was abolished (B and D, respectively) by pre-incubation of PG-21 with a 10-fold molar excess of antigen (AR_{21}). Incubations were as described in Experimental Procedures. Magnification, x70.

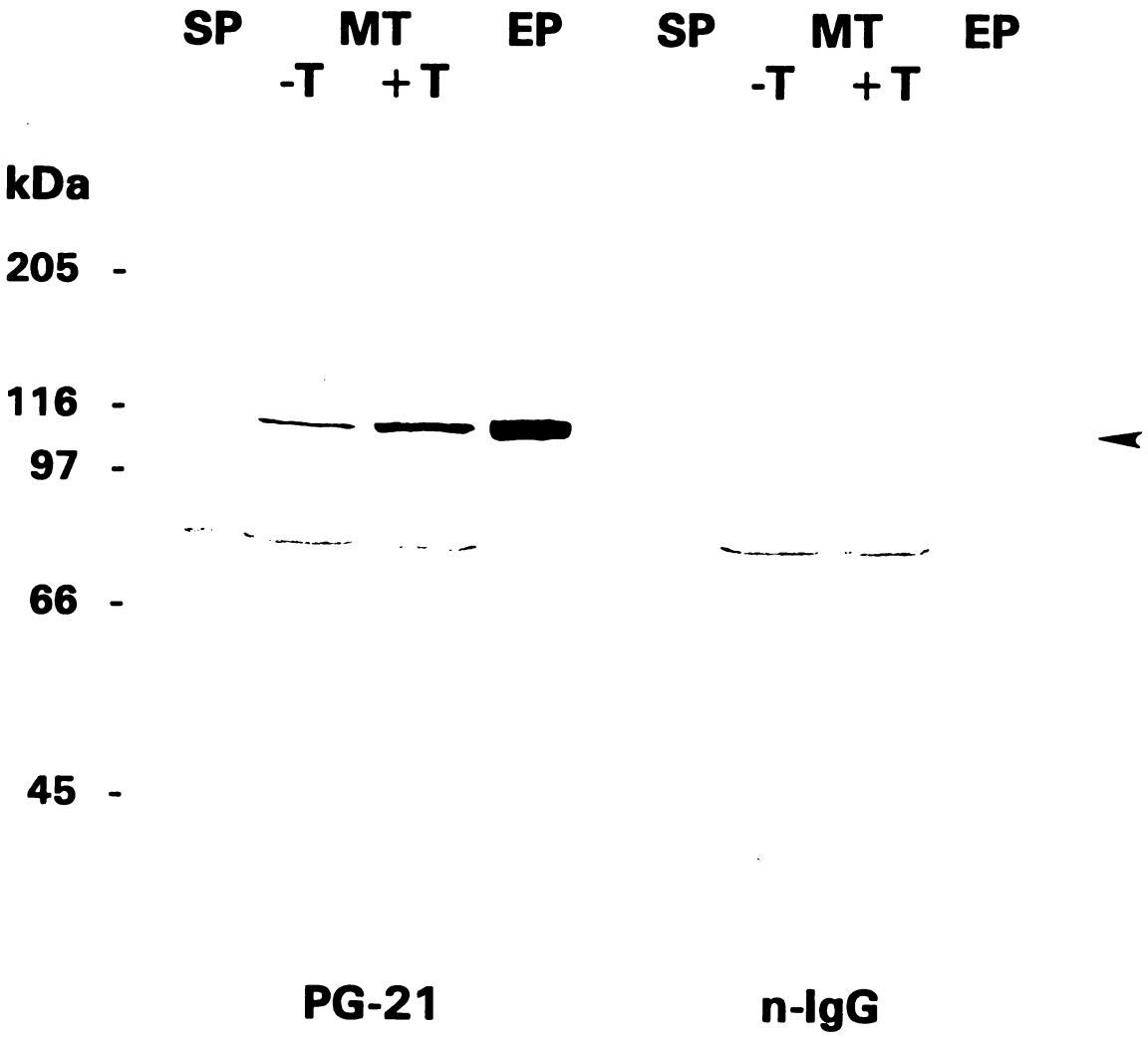

Fig. 1.9. Western blot analysis of myotubes (MT) exposed to testosterone. Satellite cells were grown to confluence in MEM+10% FBS, then exposed to differentiation medium (Table 1.1) for 4 days. Myotubes were treated with control SFM (-T) or SFM containing 10^{-7} M testosterone (+T) for 12 h, then harvested. Lanes represent 220 μg protein from porcine spleen (SP), MT and epididymis (EP) which was separated by gel electrophoresis and transferred to nitrocellulose. Panels were incubated with PG-21 (right) or normal IgG (left) as outlined in Experimental Procedures. The major immunoreactive AR band at ~107 kDa is indicated by an arrow.

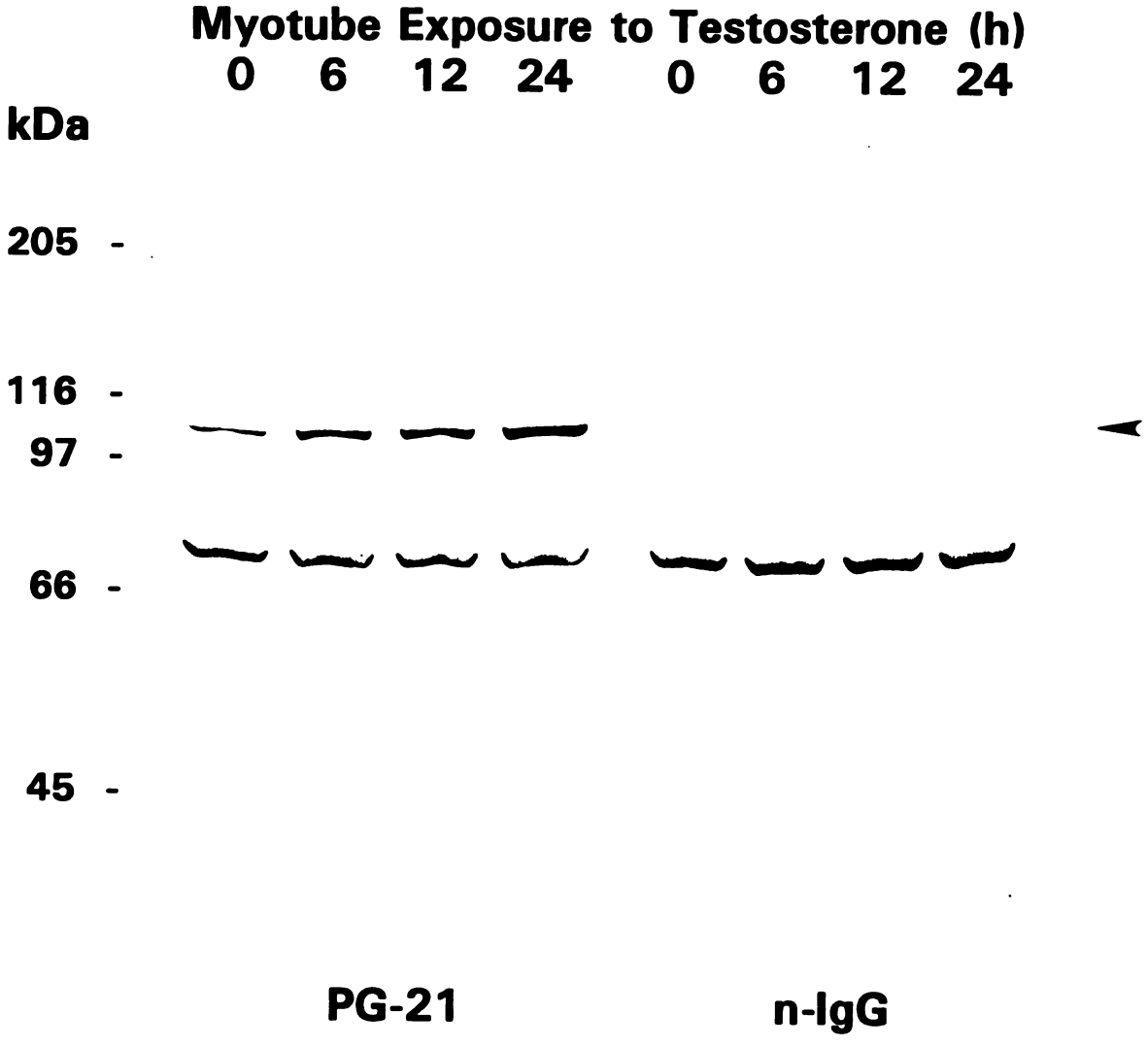

Fig. 1.10. Immunoblot analysis of testosterone-induced increases in myotube AR concentration. Satellite cells were grown to confluence in MEM+10% FBS, then exposed to differentiation medium (Table 1.1) for 4 days. Thereafter, myotubes were treated with control SFM (0) or SFM containing 10^{-7} M testosterone for 6, 12 and 24 h. Lanes represent 220 μg protein which was separated by gel electrophoresis and transferred to Immobilon-P. Panels were incubated with PG-21 (left) or normal IgG (right) as outlined in Experimental Procedures. The major immunoreactive AR band at ~107 kDa is indicated by an arrow.

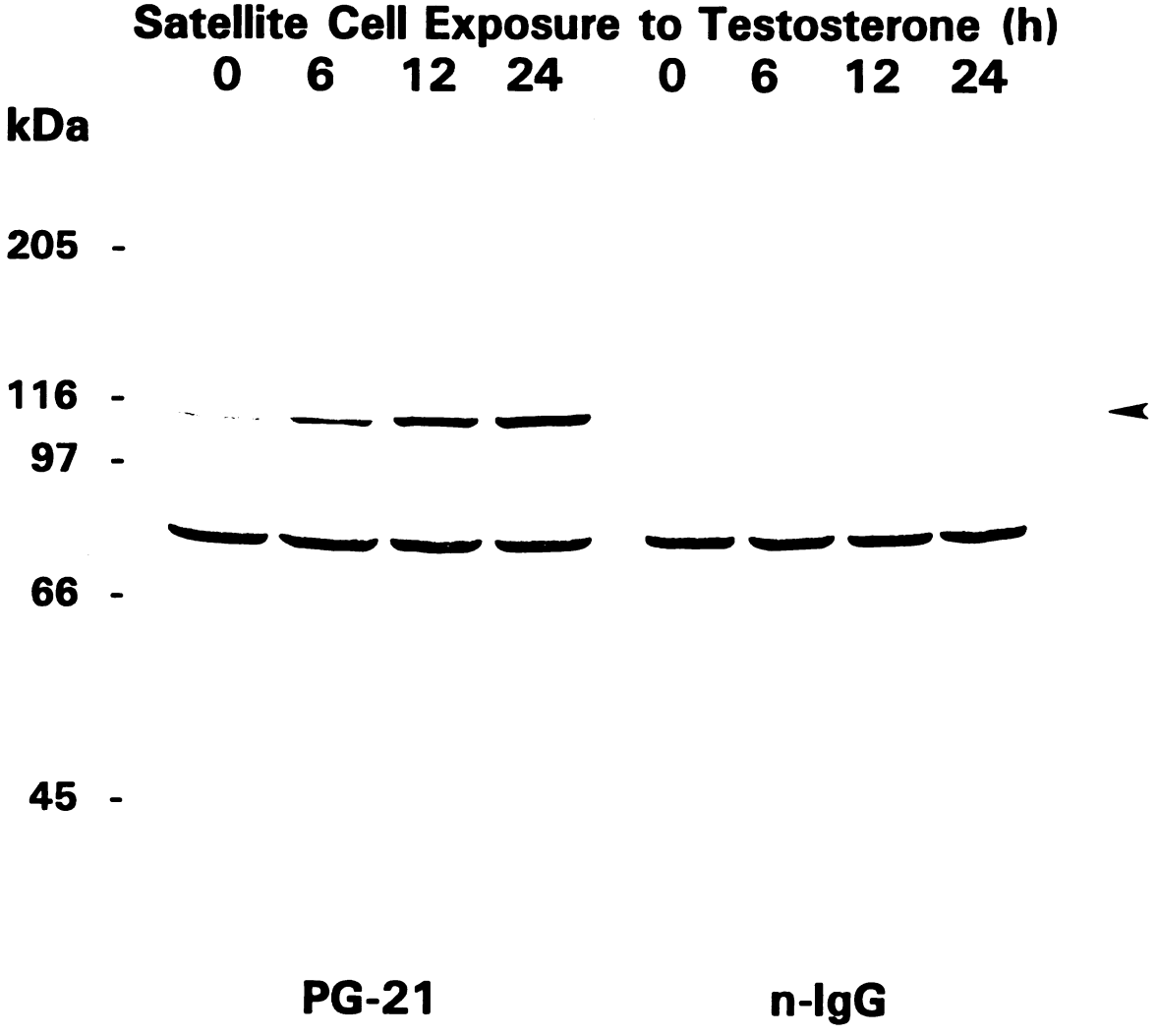

Fig. 1.11. Immunoblot analysis of testosterone-induced increases in satellite cell AR concentration. Satellite cells were grown to confluence in MEM+10% FBS, then exposed to control SFM (0) or SFM containing 10^{-7} M testosterone for 6, 12 and 24 h. All cells were in SFM for 24 h. Lanes represent 220 μg protein which was separated by gel electrophoresis and transferred to Immobilon-P. Panels were incubated with PG-21 (left) or normal IgG (right) as outlined in Experimental Procedures. The major immunoreactive AR band at ~107 kDa is indicated by an arrow.

apparent effect on either androgen or estrogen receptor proteins was observed (Fig. 1.12). Immunocytochemical evaluation of AR showed an increase in nuclear staining intensity following exposure of clonally-derived satellite cells (Fig. 1.13) and fibroblasts (Fig. 1.14) to testosterone. Withdrawal of testosterone for 24 h did not reduce AR staining to the basal level (Fig. 1.13). Treatment of satellite cells with 10^{-7} M dexamethasone had no apparent effect on AR immunostaining (Fig. 1.15).

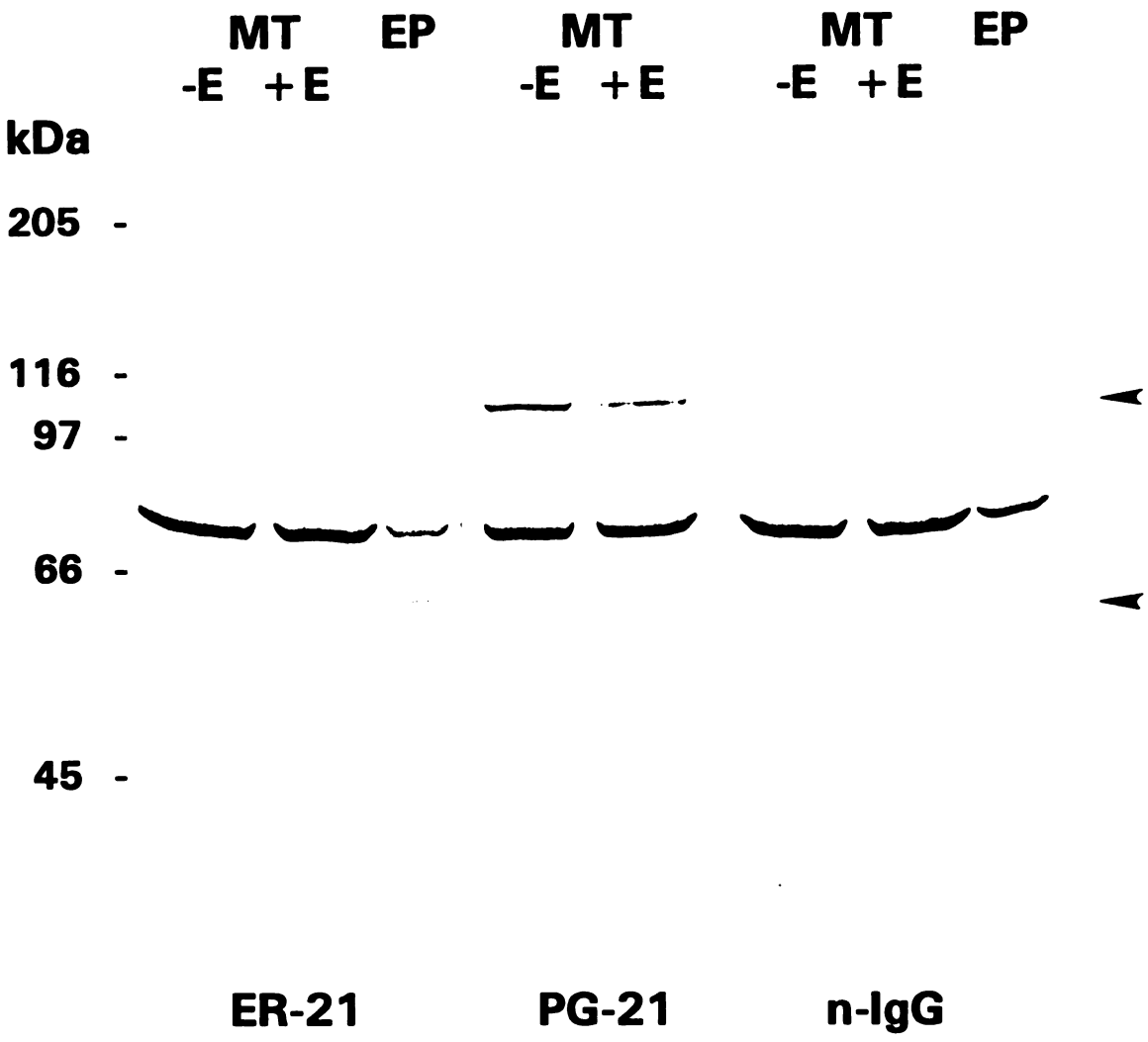

Fig. 1.12. Immunoblot analysis of myotubes (MT) exposed to estradiol-17β. Satellite cells were grown to confluence in MEM+10% FBS, then exposed to differentiation medium (Table 1.1) for 4 days. Thereafter, myotubes were treated with control SFM (-E) or SFM containing 10^{-8} M estradiol-17β (+E) for 12 h. Lanes represent 220 μg protein extracted from MT or epididymis (EP) which was separated by gel electrophoresis and transferred to Immobilon-P. Panels were incubated with ER-21 (left), PG-21 (center) or normal IgG (right) as outlined in Experimental Procedures. Specific immunoreactive AR and estrogen receptor bands are indicated by arrows (~107 and 64 kDa, respectively).

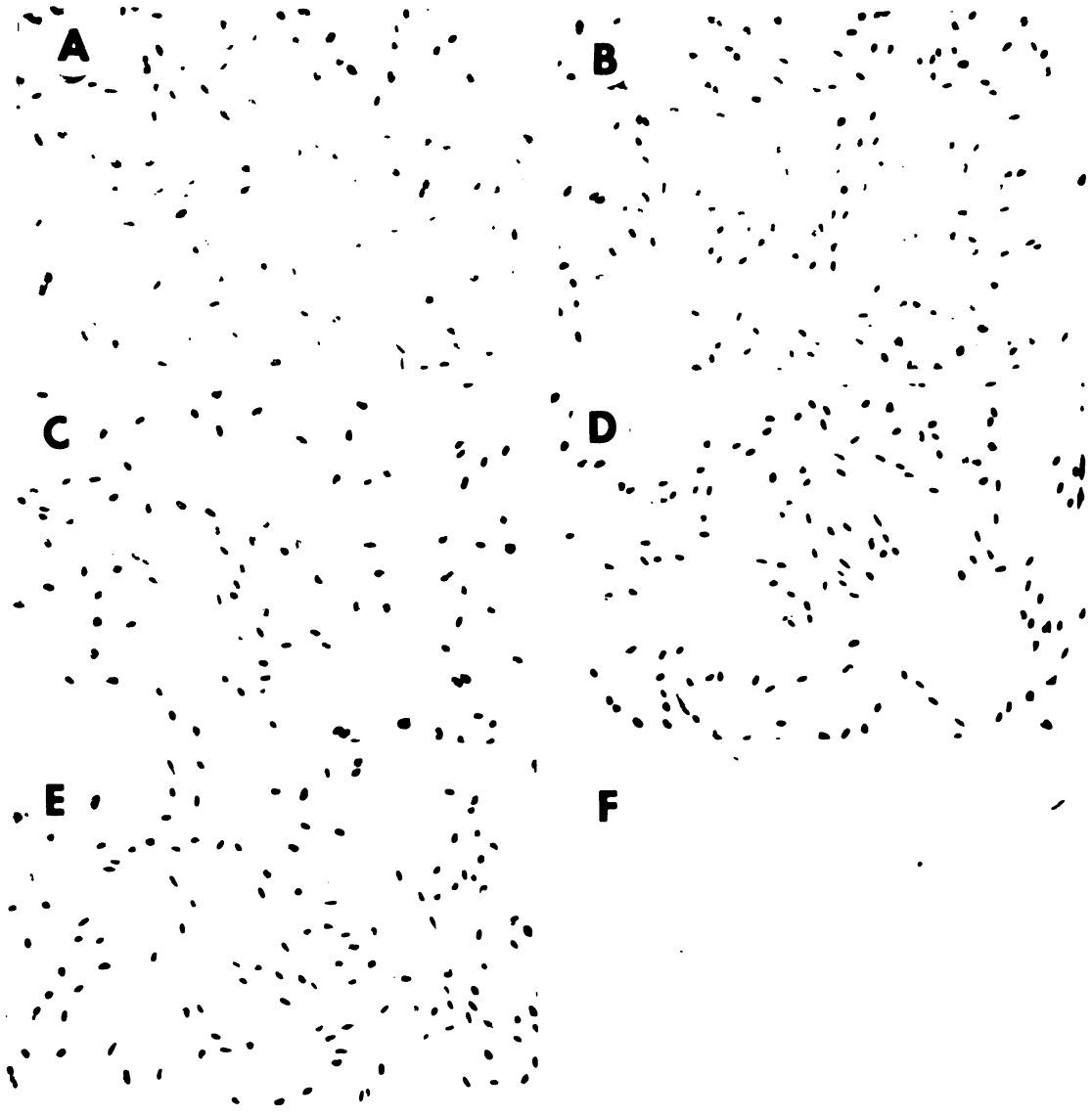

Fig. 1.13. Immunocytochemical staining for AR in satellite cells exposed to testosterone. Clonally-derived satellite cells were seeded and allowed to attach for 24 h in MEM+10% FBS. Cells were exposed to SFM for 48 h, followed by (A) control SFM or SFM containing 10^{-7} M testosterone for (B) 6 h, (C) 12 h, or (D) 24 h prior to immunostaining with PG-21. Alternatively, cells were exposed to SFM for 24 h, treated with SFM containing 10^{-7} M testosterone for 24 h, then returned to control SFM for 24 h (E). No nuclear staining was observed in satellite cells incubated with (F) PG-21 pre-incubated with a 10-fold molar excess of AR_{21}. Immunocytochemistry was as described in Experimental Procedures. Magnification, x70.

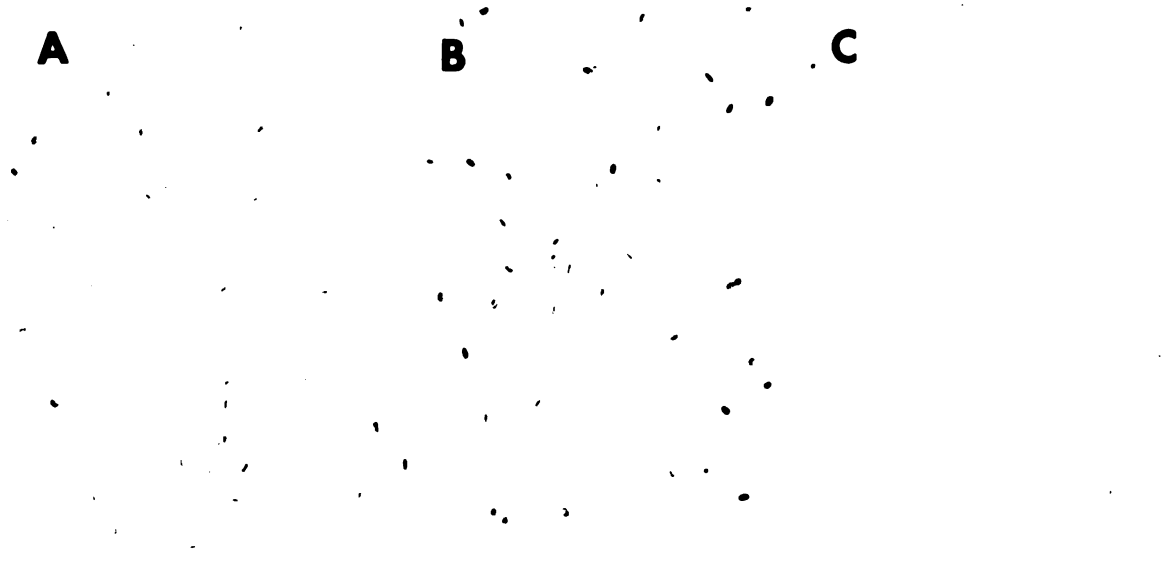

Fig. 1.14. Effect of testosterone on AR immunostaining of fibroblasts. Clonally-derived fibroblasts were seeded and allowed to attach for 24 h in MEM+10% FBS. Cells were exposed to SFM for 24 h, followed by (A) control SFM for 24 h, or (B and C) SFM containing 10^{-7} M testosterone for 24 h. Incubation with PG-21 (A and B) revealed specific nuclear staining whereas incubation with control rabbit IgG (C) showed no nuclear stain, but light background stain. Immunocytochemistry was as described in Experimental Procedures. Magnification, x70.

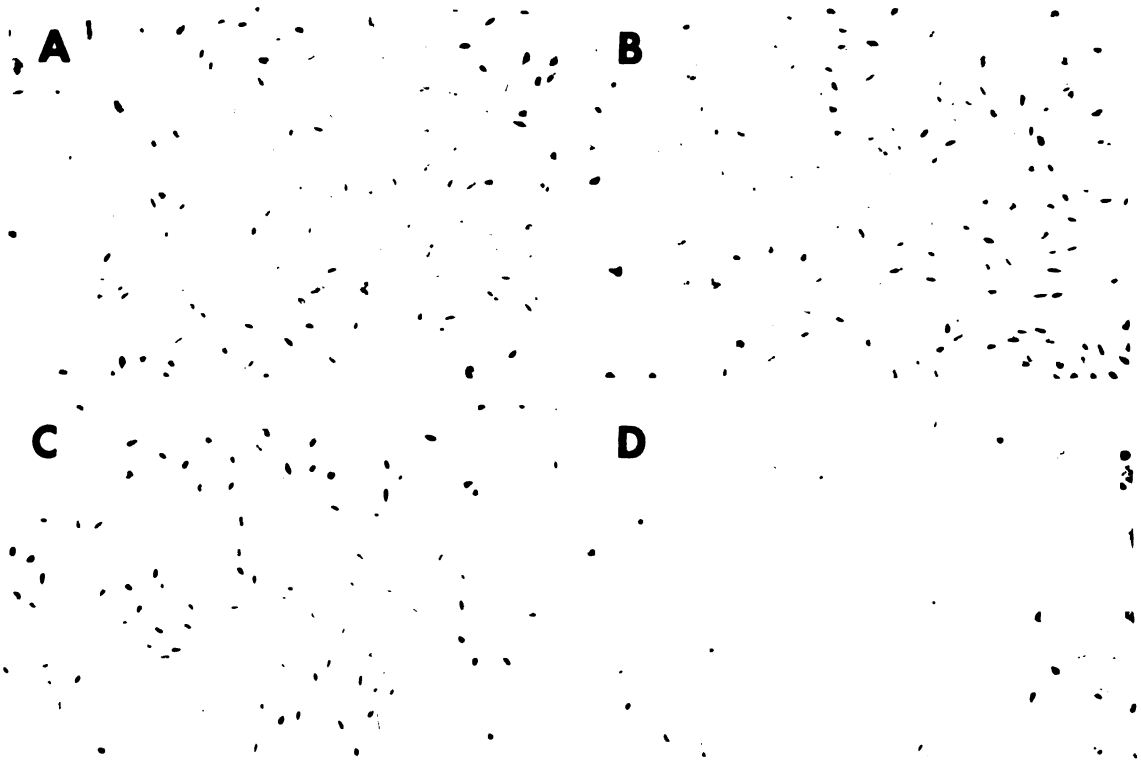

Fig. 1.15. Effect of dexamethasone on AR immunostaining of satellite cells. Clonally-derived satellite cells were seeded and allowed to attach for 24 h in MEM+10% FBS. Cells were exposed to SFM for 24 h, followed by (A) control SFM, (B) SFM containing 10^{-7} M dexamethasone, or (C) SFM containing 10^{-8} M testosterone for 24 h. Immunostaining with PG-21 (A-C) or normal IgG (D) was as described in Experimental Procedures. Magnification, x70.

DISCUSSION

The current study clearly demonstrates the presence of AR in satellite cells, satellite cell-derived myotubes, and fibroblasts isolated from porcine skeletal muscle. Polyclonal antibodies (PG-21) were used for AR detection in this study. PG-21 was raised against a peptide containing amino acids 1-21 of the human and rat AR, and has been shown to recognize a 108-110 kDa AR protein in extracts from rat and human tissues (Prins et al., 1991). In the current study, this antibody specifically recognized a protein of ~107 kDa from AR-positive tissues of the pig. These findings suggest that the porcine AR shares epitopes with N-terminal amino acids 1-21 of the human and rat AR, which are identical (Chang et al., 1988).

Immunostaining with PG-21 localized AR in the nuclei of rat prostatic cells (Prins et al., 1991). Immunocytochemical staining of satellite cells, myotubes, and muscle-derived fibroblasts revealed that AR are confined to the nuclei of these cells as well. This is consistent with the nuclear localization of AR described in immunological studies of other tissues (Takeda et al., 1990; Sar et al., 1990; 1991; Kimura et al., 1993).

Immunoblot analysis revealed that AR of satellite cells

and myotubes are up-regulated in response to testosterone. Additionally, immunocytochemical staining for AR was more intense in nuclei of satellite cells, fibroblasts and myotubes exposed to testosterone. To my knowledge, AR regulation has not previously been studied in skeletal muscle cells, although Takeda et al. (1990) observed that AR immunostaining in skeletal muscle of female rats was slightly weaker than that of male rats. The findings in this dissertation are in agreement with this observation. Although the mechanisms of AR autoregulation were not addressed in this study, homologous up-regulation of AR has been demonstrated in several cell culture systems (Syms et al., 1983; De Pergola et al., 1990; Grino et al., 1990). Syms et al. (1985) demonstrated that homologous AR augmentation in ductus deferens smooth muscle (DDT_1MF-2) cells resulted from an increase in both *de novo* synthesis and half-life. In cultured smooth muscle cells from rat penis, AR augmentation has been attributed to increased transcription of AR mRNA (Gonzalez-Cadavid et al., 1993). In contrast, several studies have shown that castration increases steady state levels of AR mRNA, and testosterone reverses this process (Chang et al., 1988; Tan et al., 1988; Quarmby et al., 1990). Androgen withdrawal also decreases AR mRNA in cultured LnCAP human prostate cancer cells (Quarmby et al., 1990c), and

human hepatoma (HepG2) cells (Shan et al., 1990). The physiological role of down-regulation in these tissues is not clear, since the concentration of AR protein and mRNA appears to reflect the degree of androgen responsiveness of a tissue (Voight et al., 1975; Chang et al., 1988; Quarmby et al., 1990a). This is also demonstrated in the present study, as extracts from epididymal tissue contain much greater AR immunoreactivity than skeletal muscle cells. Porcine spleen is devoid of AR immunoreactivity and this is consistent with previous studies showing the spleen to be an AR-negative tissue (Quarmby et al., 1990b; Takeda et al., 1990 Prins et al., 1991).

Polyclonal AR antiserum, raised against a fusion protein containing amino acids 331-572 of the human AR (Chang et al., 1989), failed to recognize the full length porcine AR in the present study. This may indicate that differences between porcine and human AR occur in this region. Recognition of a ~64 kDa protein, in addition to the AR, has been observed previously (Dr. S. Liao, personal communication), but the identity of this protein is currently unknown. It is tempting to speculate that the AR antiserum used in this study recognizes porcine estrogen receptors, since epididymal estrogen receptors detected with ER-21 have a similar

molecular mass. However, AR antiserum also recognizes a protein of ~64 kDA in spleen and satellite cells, which do not have estrogen receptors detectable with ER-21.

It is well documented that gonadally intact males are more muscular than females (reviewed by Seideman et al., 1982, Purchas, 1991). Gender-related differences in muscle mass are characterized by increases in both protein and DNA content of skeletal muscle (Cheek and Hill, 1970; Snochowski et al., 1981). Accordingly, androgen treatment of females and castrated males has been shown to increase *in vivo* protein synthesis (Florini and Breuer, 1966; Martinez et al., 1984) and satellite cell proliferation (Mulvaney et al., 1988; Joubert and Tobin, 1989). Despite the observed *in vivo* effects of androgens, several reports have shown no effect of androgens on DNA synthesis (Gospodarowicz et al., 1976), cell proliferation (Thompson et al., 1989), protein synthesis (Roeder et al., 1986), protein breakdown (Ballard and Francis, 1983) or alpha-actin accumulation (Allen et al., 1983) in cultured skeletal muscle cells. A single report indicated that testosterone directly stimulated a modest increase in DNA labeling index in myoblasts (Powers and Florini, 1975). While Thompson et al. (1989) found no direct effect of the synthetic androgen, trenbolone acetate (TBA), on satellite cell

proliferation, satellite cells isolated from TBA-treated rats were more responsive to the mitogenic effects of IGF-I and FGF than those isolated from untreated rats. Given the observed discrepancy between reported effects of androgens *in vivo* and *in vitro*, it appeared likely that the effects of androgens on skeletal muscle, and particularly skeletal myoblasts and satellite cells, were indirect.

Cells which possess AR are generally considered potential targets for direct androgen action. Androgen receptors have been identified in skeletal muscle by binding assay (Michel and Baulieu, 1980; Snochowski, 1981) and more recently by immunocytochemical methods (Takeda et al., 1990; Kimura et al., 1993). Androgen receptor mRNA has also been detected in skeletal muscle (Chang et al., 1988; Tan et al., 1988; Shan et al., 1990), although Chang et al. (1988) reported that the level of AR mRNA per unit of poly $(A)^+$ RNA in thigh muscle was less than 10% of that found in seminal vesicle. These findings indicate that skeletal muscle is a potential target tissue for direct androgen action. However, since satellite cells typically represent only 4 to 8% of the nuclei found within the basement membrane of adult skeletal muscle tissue (Campion, 1984), previous studies did not ascertain that satellite cells possess AR. Additionally, the presence of AR

in cultured skeletal muscle cells has not previously been reported.

The current findings clearly demonstrate that cultured skeletal muscle satellite cells, myotubes and fibroblasts possess AR. Moreover, regulation of AR in these cells is consistent with that observed in other androgen-responsive tissues. The presence and autoregulation of AR in satellite cells lends support to the possibility that these cells are direct targets for androgen action. Nevertheless, autocrine or paracrine mechanisms of androgen action on satellite cell proliferation or differentiation *in vivo* cannot be ruled out, since AR are also present in fibroblasts and myotubes. Direct effects of testosterone on satellite cell proliferation and differentiation are discussed in Chapter 2.

CHAPTER 2

EFFECTS OF TESTOSTERONE ON PROLIFERATION AND DIFFERENTIATION OF CLONALLY-DERIVED PORCINE SATELLITE CELLS

ABSTRACT

The effects of testosterone on proliferation and differentiation of porcine satellite cells were investigated. Clonally-derived porcine satellite cells were seeded onto 24-well plates in Minimum Essential Medium containing 10% fetal bovine serum (MEM+10% FBS) and allowed to attach for 24 h. Cells were then washed and exposed to testosterone (0, 10^{-10} to 10^{-6} M) in basal serum-free medium (bSFM) or SFM containing various combinations of growth factors (bFGF, IGF-I, PDGF-BB and EGF). Testosterone alone or in combination with growth factors had no effect ($P>0.1$) on porcine satellite cell proliferation. To evaluate the effect of testosterone on satellite cell differentiation, cells were grown to confluence in MEM+10% FBS, then exposed to SFM containing testosterone (0, 10^{-10} to 10^{-6} M). Testosterone depressed myotube formation in a dose-dependent manner with maximal inhibition occurring at 10^{-7} M testosterone. Testosterone (10^{-7} M) reduced ($P<0.05$) the number of myosin-positive cells and creatine phosphokinase activity approximately 20% by day 2 in SFM. A similar reduction was observed on days 3 and 4 of treatment and this effect was not reversible within 48 h after testosterone withdrawal. These data indicate that testosterone decreases

differentiation of cultured porcine satellite cells, and may thereby increase the population of proliferative satellite cells in the absence of a direct mitogenic effect.

INTRODUCTION

Skeletal muscle DNA accretion is intimately associated with muscle protein accumulation and growth. Over 95% of the nuclei contained within mature muscle fibers of some species accumulates postnatally (reviewed by Allen et al., 1979), however, nuclei contained within the sarcolemma of myotubes or myofibers do not synthesize DNA (Stockdale and Holtzer, 1961; Moss and LeBlond, 1971). Skeletal muscle satellite cells were first described and named by Mauro (1961), who suggested that satellite cells may be the source of nuclei added to myofibers during regeneration. It is now clear that satellite cells proliferate, differentiate, and incorporate into muscle fibers, thereby providing the only source of postnatally added nuclei to normally growing (Allen et al., 1979; Campion, 1984) or regenerating (Allbrook, 1981; Carlson and Faulkner, 1983) skeletal muscle fibers.

Numerous polypeptide growth factors, hormones, and nutrients have been implicated as regulators of myogenic cell proliferation and differentiation (reviewed by Florini et al., 1987; 1991). We have recently described the effects of several polypeptide growth factors on porcine satellite cell proliferation (Cook et al., 1993; Doumit et al., 1993). The

responses elicited by growth factors such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), Insulin-like growth factor-I (IGF-I), platelet derived growth factor-BB (PDGF-BB) and transforming growth factor-β (TGF-β) on cultured myogenic cells indicate that these factors play an integral role in muscle development and regeneration. At this time, however, the precise physiological roles of growth factors in muscle development *in vivo* are unclear.

Despite obvious *in vivo* effects of androgens on muscle mass, direct effects of androgens on cultured skeletal muscle cells have been difficult to demonstrate. Powers and Florini (1975) reported a 25% increase in DNA labeling index of myoblasts exposed to testosterone. However, androgens have generally been shown to have no direct effect on proliferation (Gospodarowicz et al., 1976; Thompson et al., 1989) or protein accretion (Allen et al., 1983; Roeder et al, 1986) in skeletal muscle cell cultures. Thompson et al. (1989) demonstrated that, while androgens have no direct effects on cultured satellite cells, cells isolated from androgen-treated rats were more responsive to the mitogenic actions of IGF-I and FGF than cells from control rats.

No previous studies have evaluated the effects of androgens on pure cultures of satellite cells grown in serum-free medium. Clonally-derived cultures of porcine satellite cells possess nuclear androgen receptors and respond to testosterone by up-regulating these receptors (data in Chapter

1). The objective of this study was to examine the effects of testosterone on satellite cell proliferation and differentiation, using pure satellite cell cultures in conjunction with serum-free media.

EXPERIMENTAL PROCEDURES

Materials-Minimum Essential Medium (MEM), antibiotic-antimycotic, gentamicin, human recombinant bFGF, EGF, IGF-I, and PDGF-BB were purchased from GIBCO BRL (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Hazleton (Lenexa, KS). MCDB-110 medium, bovine serum albumin (BSA; RIA grade, A-7888), dexamethasone (D-8893), bovine insulin (I-1882), bovine transferrin (T-8027), water-soluble linoleic acid (L-5900), porcine skin gelatin, calf thymus DNA and Hoechst No. 33258 were obtained from Sigma Chemical Co. (St. Louis, MO). Tissue culture 24-well plates were obtained from Corning Glass Works (Corning, NY).

Porcine Satellite Cell Isolation-Satellite cells were isolated from semimembranosus muscles of 8-week-old pigs using a procedure outlined for rodent muscle by Bischoff (1974) and modified by Doumit and Merkel (1992). Satellite cell clones were isolated using a cloning ring technique (Doumit et al., 1993) and cultures expanded to the fourth passage. In all experiments, plating medium consisted of MEM containing 10% FBS, 0.5% antibiotic-antimycotic and 0.1% gentamicin. Cells were seeded into gelatin-coated culture wells and maintained in a humidified CO_2 incubator containing 95% air and 5% CO_2 at

37°C. Gelatin coating (0.1%) was performed using the method of Richler and Yaffe (1970).

Satellite Cell Cultures-Clonally-derived porcine satellite cells were suspended in MEM containing 10% FBS, and approximately 5000 cells per gelatin-coated 16 mm diameter well were seeded and allowed to attach for 24 h. Cells were washed twice with MEM, and treatment media were applied. Testosterone was administered as a 24 h pre-treatment, or as a continuous treatment in basal serum-free medium or growth factor supplemented medium. Basal medium was the same as the growth medium listed in Chapter 1 (Table 1.1), except no growth factors were included, and insulin concentration was 10^{-9} M. Treatments were applied to cells in four replicate 16-mm diameter cell culture wells for 72 h, with daily media changes. At appropriate times, cultures were washed with PBS and DNA was quantified using a fluorometric assay as described by West et al. (1985). Calf thymus DNA served as the standard. Using this procedure, the DNA content of porcine satellite cells (quantified microscopically) was found to be approximately 7.4 pg/cell.

To evaluate the effects of testosterone on satellite cell differentiation, cells were grown to confluence in MEM containing 10% FBS, then exposed to serum-free differentiation medium (Chapter 1, Table 1.1) containing testosterone treatment. Testosterone (T-5641; Sigma Chemical Co.) and water soluble testosterone (T-5035; Sigma Chemical Co.)

produced similar results, and addition of the water soluble carrier, β-cyclodextrin, had no effect on differentiation relative to serum-free medium with no additions. In preliminary studies, differentiation was quantified by morphological assessment of myogenic cell fusion. Cells were fixed in absolute methanol, stained with 0.03% Giemsa to enable visual evaluation of nuclei and 6 to 10 observations per well were made to determine total nuclei and myotube nuclei. Cells containing three or more nuclei were considered myotubes. A shortfall of this evaluation method is that it potentially underestimates cell differentiation by not accounting for cells that have differentiated but not fused. Therefore, two objective methods were also used to assess differentiation and are described below.

Immunocytochemical Detection of Myosin Positive Cells- Immunostaining for sarcomeric myosin was performed to assess satellite cell differentiation. At stated times, satellite cells were fixed in absolute ethanol for 5 min at room temperature. Following three washes in PBS, non-specific binding was blocked by incubation with PBS containing 1% goat serum for at least 30 min. Cells were exposed to monoclonal antibody NA4 (1:5000 dilution in blocking reagent) or control mouse ascites fluid (M-8273 Sigma) overnight at 4°C in a humidified chamber. Incubation of NA4 for 1 h at 22°C produced similar results. Monoclonal antibody NA4 (kindly provided by Dr. Everett Bandman, University of California,

Davis, CA) recognizes all isoforms of sarcomeric myosin heavy chain. Following three washes in PBS, cells were exposed to goat anti-mouse IgG conjugated to alkaline phosphatase (1:40; A-1293 Sigma) in blocking reagent for 1 h at room temperature. Myosin positive cells were visualized using BCIP/NBT (B-5655, Sigma) as substrate to produce a dark blue reaction product. No immunostaining was observed in clonal cultures of porcine muscle-derived fibroblasts exposed to serum-free fusion medium for 4 days, or in cells exposed to control ascites fluid. Cells were counter-stained with 0.03% Giemsa to enable visual evaluation of nuclei and ten observations per well were made to determine total nuclei and nuclei in myosin positive cells.

Assay of Creatine Kinase-At the indicated times, cells were washed three times with PBS, overlayed with 0.05 M glycylglycine buffer, pH 6.75 (0.2 ml/16 mm-diameter well), and frozen at -20°C. Within 1 week, cells were thawed on ice, sonicated for 4 s and 30 μl of sample per well was assayed for creatine kinase (CK) activity using a kit (47-UV, Sigma) based on the procedure of Szasz et al. (1976). DNA was quantified spectrofluorometrically, using Hoescht 33258, as described by West et al. (1985), except a 20 μl aliquot of sample was used for each determination. Calf thymus DNA served as the standard. CK and DNA assays were done in duplicate for eight wells per treatment per day.

Statistical Analysis-Data were analyzed using general linear model procedures of the Statistical Analysis System

(SAS, 1985). Treatment means were separated using Bonferonni t-tests. Unless otherwise specified, stated differences are significant at the 0.05 level of probability.

RESULTS

Testosterone Effects on Satellite Cell Proliferation-The effects of testosterone on proliferation of clonally-derived porcine satellite cells were examined in both serum-containing and serum-free media (SFM). Satellite cells used in these studies were predominantly from one highly myogenic clone, designated M1, yet comparable results were obtained with a second clone (M2). The responsiveness of these cells to polypeptide growth factors has been reported (Cook et al., 1993; Doumit et al., 1993). Testosterone (10^{-10} to 10^{-6} M) had no effect on proliferation of satellite cells grown in either 2% FBS or 3% porcine serum (Fig. 2.1). Treatment of satellite cells with testosterone for 24 h, which has been shown to up-regulate AR (Chapter 1), did not affect subsequent proliferation of satellite cells in basal SFM or the responsiveness of satellite cells to PDGF-BB, bFGF, or IGF-I (Fig. 2.2). Previous results from our laboratory (Doumit et al., 1993) indicated that EGF alone was not mitogenic for porcine satellite cells, and that bFGF masked the synergistic effects of EGF with PDGF and IGF-I. Therefore, testosterone was applied to combinations of bFGF, IGF-I and PDGF-BB (FIP) or EGF, IGF-I, and PDGF-BB (EIP). Testosterone had no effect

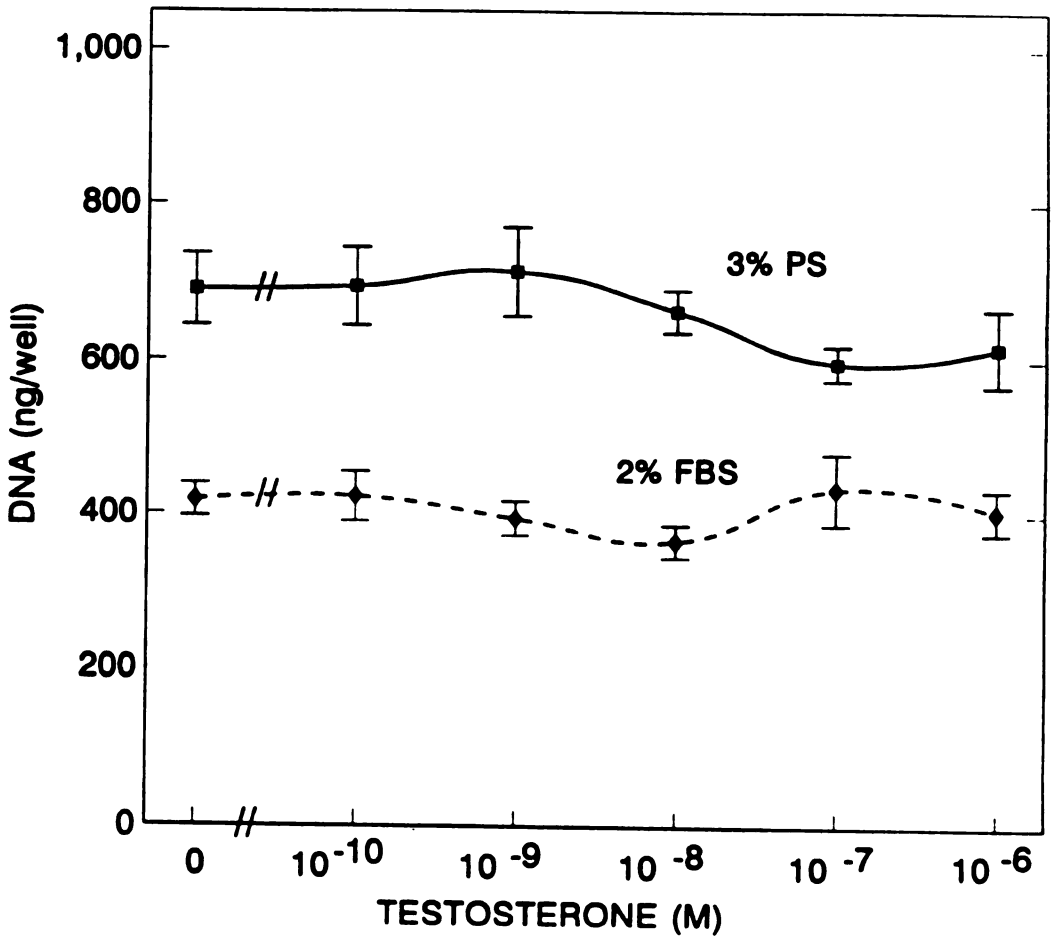

Fig. 2.1. Effects of testosterone concentration on satellite cell proliferation in serum-containing media. Clonally-derived satellite cells were seeded and allowed to attach for 24 h. Cells were washed with MEM and exposed to testosterone in MEM containing 2% FBS or 3% porcine serum (PS). Treatments were applied for 72 h, with daily media changes, then DNA determined. Values represent mean ± S.E.M. of four culture wells.

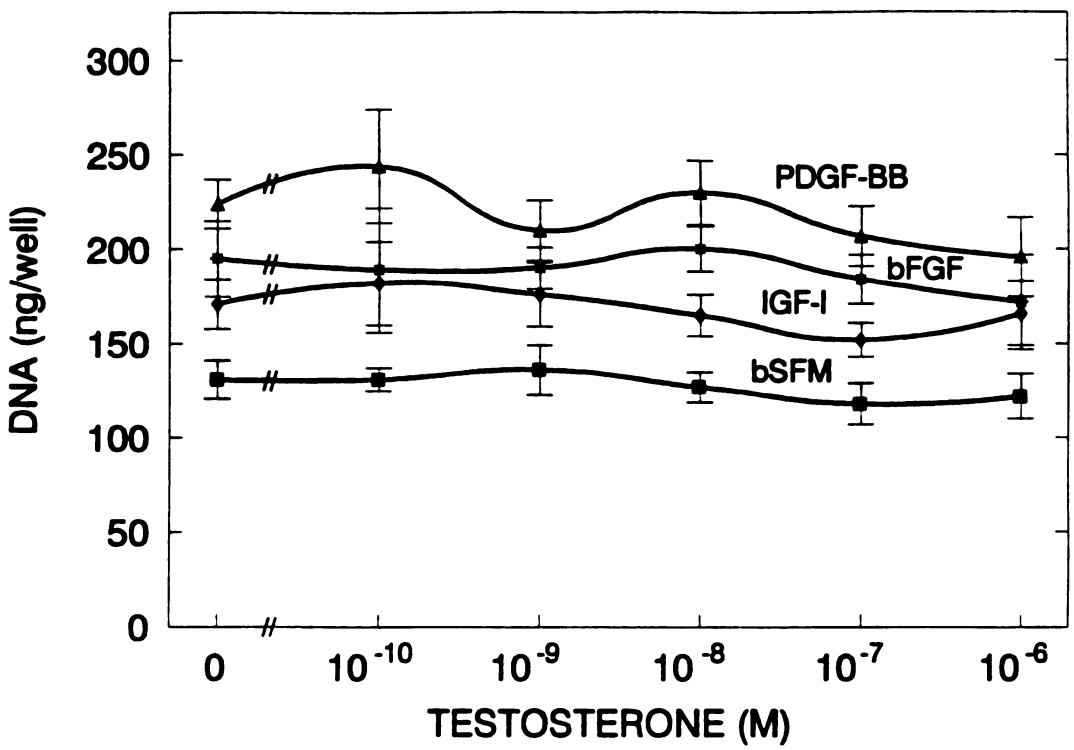

Fig. 2.2. Effects of testosterone pre-treatment on satellite cell responsiveness to growth factors. Clonally-derived satellite cells were seeded and allowed to attach for 24 h. Cells were washed with MEM and exposed to testosterone in basal SFM for 24 h. Cells were again washed with MEM and exposed to SFM containing PDGF-BB (5 ng/ml), bFGF (10 ng/ml), IGF-I (25 ng/ml) or basal SFM (bSFM). Growth factor media were applied for 72 h, with daily media changes, then DNA determined. Values represent mean ± S.E.M. of four culture wells.

when applied as a continuous 72 h treatment to SFM containing FIP or EIP (Fig. 2.3). Additionally, pre-exposure of satellite cells to testosterone in basal SFM, followed by continuous testosterone exposure in growth factor-supplemented medium, had no effect on cell proliferation (Fig. 2.4). Data in Figure 2.4 are expressed relative to control cultures to correct for differences in cell density of the 4 experiments represented. Collectively, these data indicate that testosterone has no measurable effect on satellite cell proliferation under the conditions described.

Testosterone Effects on Satellite Cell Differentiation-To evaluate the effects of testosterone on satellite cell differentiation, cells were grown to confluence in MEM containing 10% FBS, then exposed to treatments in serum-free differentiation medium (Chapter 1, Table 1.1). Testosterone depressed myotube formation in a dose-dependent manner with the maximal effect occurring at 10^{-7} M testosterone (Fig. 2.5). Sarcomeric myosin-positive cells and CK activity were measured to determine if testosterone depression of satellite cell fusion was accompanied by a decrease in cell differentiation. Figure 2.6 illustrates the effects of testosterone on the incidence of myosin positive cells in differentiating satellite cell cultures. After 2 days of treatment, testosterone (10^{-7} M) reduced both the number of nuclei in myosin positive cells (Fig. 2.7) and CK activity (Fig. 2.8) by 20-30%. The magnitude of this effect was similar after 72 and

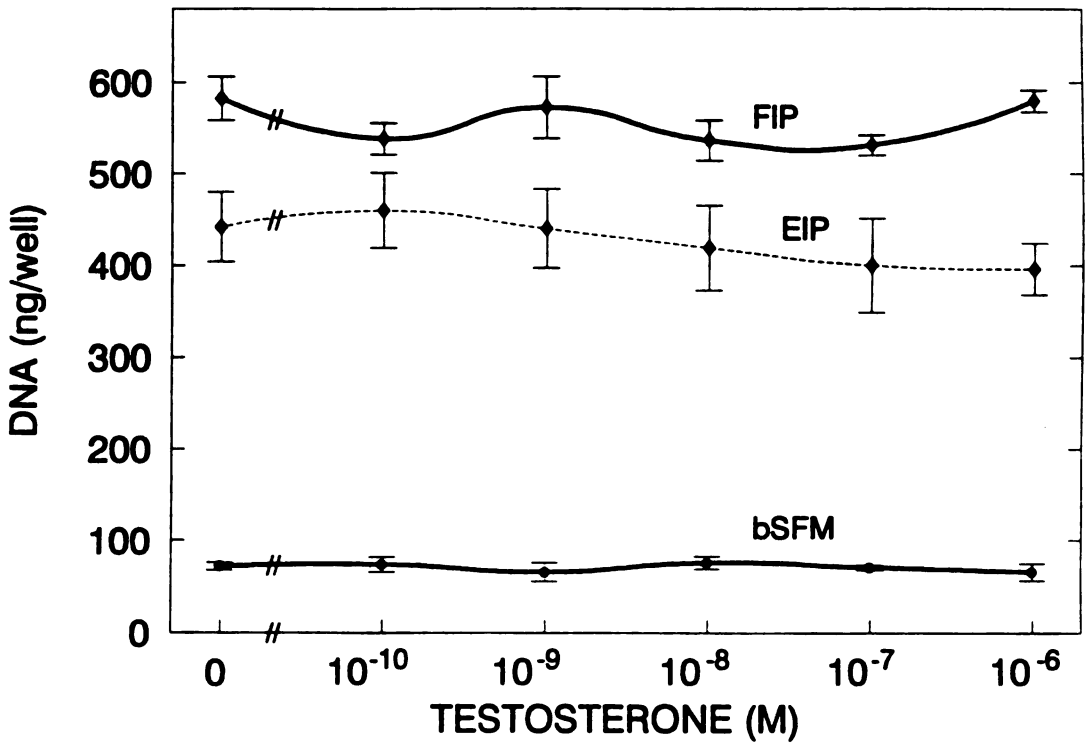

Fig. 2.3. Effects of testosterone on satellite cell proliferation in serum-free medium. Clonally-derived satellite cells were seeded and allowed to attach for 24 h. Cells were washed with MEM and exposed to testosterone in basal SFM, or SFM containing bFGF or EGF (10 ng/ml) combined with IGF-I and PDGF-BB; (FIP) and (EIP), respectively. Concentrations of bFGF, PDGF and IGF-I were the same as stated for Fig. 2.2. Media were applied for 72 h, with daily media changes, then DNA determined. Values represent mean ± S.E.M. of four culture wells.

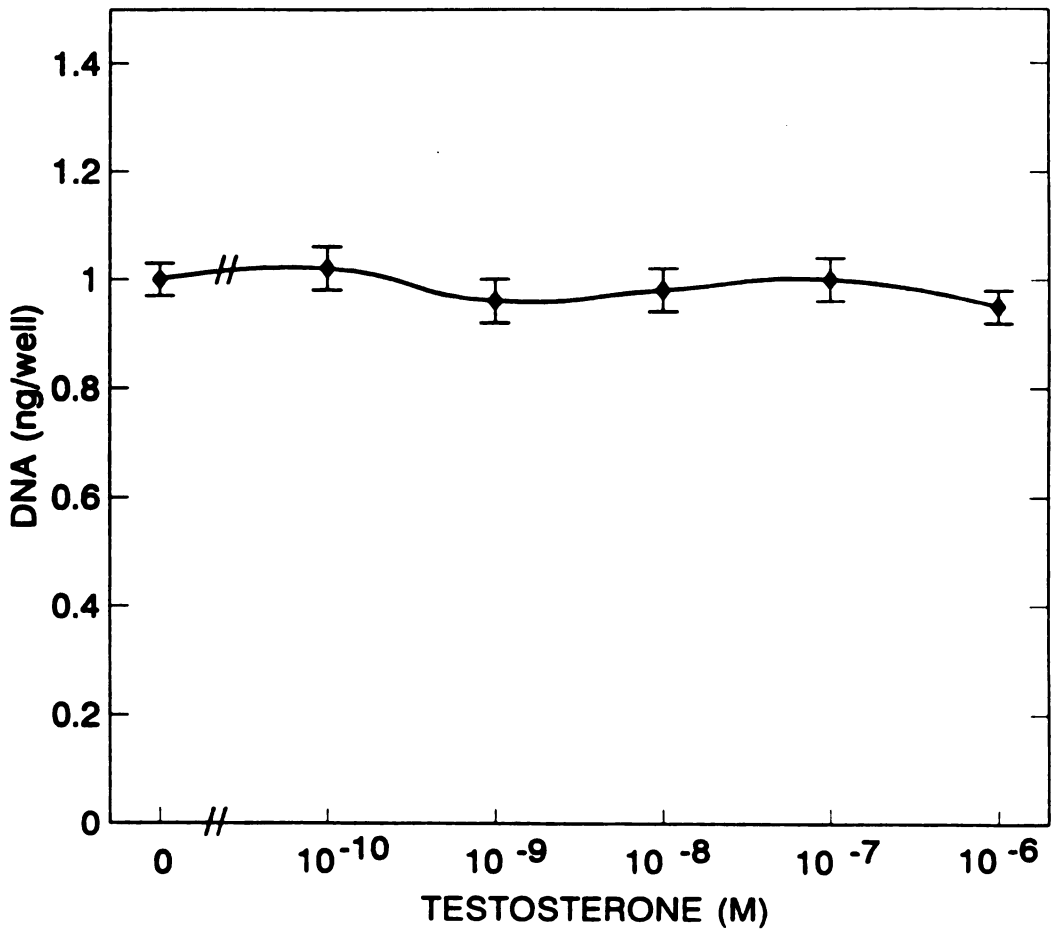

Fig. 2.4. Effects of testosterone pre-treatment on satellite cell proliferation in serum-free medium. Clonally-derived satellite cells were seeded and allowed to attach for 24 h. Cells were washed with MEM and exposed to testosterone in basal SFM for 24 h, followed by testosterone treatments in SFM containing bFGF, IGF-I and PDGF-BB at concentrations stated in the legend of Fig. 2.2. Media were applied for 72 h, with daily media changes, then DNA determined. Values represent mean ± S.E.M. of sixteen wells from 4 experiments.

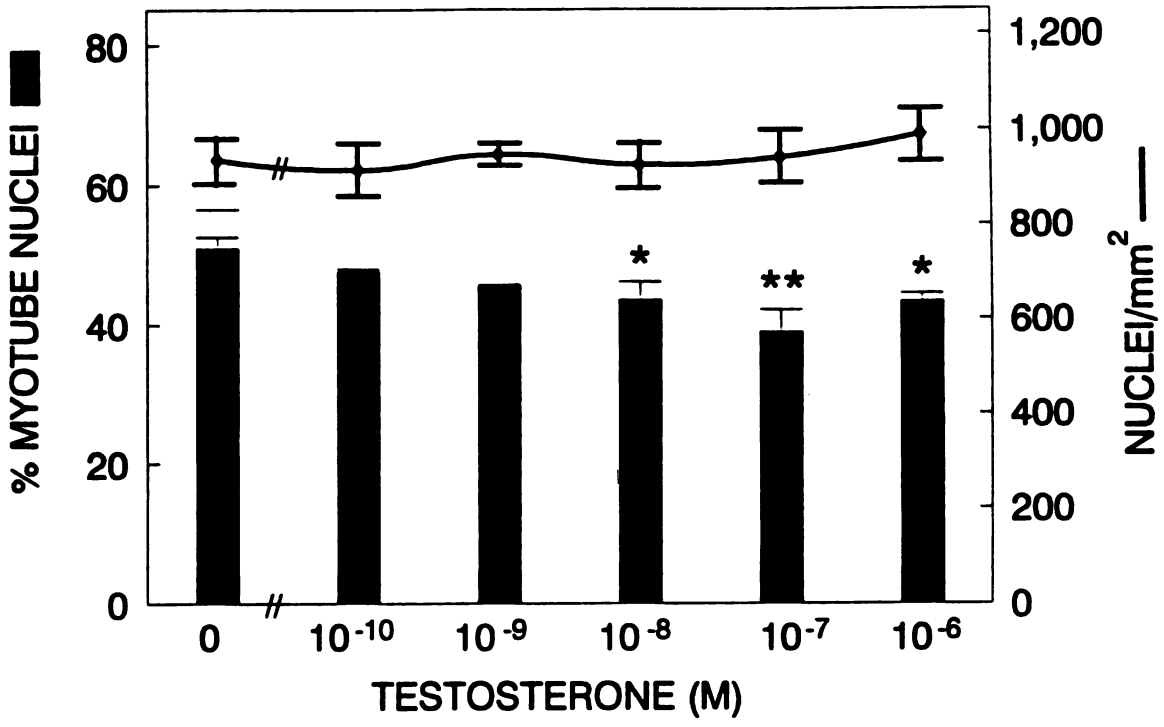

Fig. 2.5. Effects of testosterone on myotube formation. Clonally-derived satellite cells were grown to confluence in MEM+10% FBS. Cells were washed with MEM and exposed to differentiation-promoting medium (Table 1.1) containing the indicated testosterone concentrations. Fresh media were supplied daily for 4 days, then cells were fixed and evaluated as described in Experimental Procedures. Values for % myotube nuclei (bars) and nuclei/mm^2 (line) represent mean $\pm$ S.E.M. of one experiment. Similar results were obtained in 4 independent experiments. Asterisks indicate that treatment means differ from control (*P<0.05; **P<0.01).

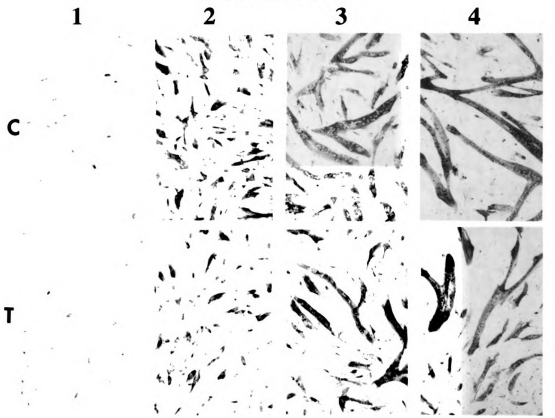

Fig. 2.6. Effects of testosterone on appearance of myosin-positive cells. Clonally-derived satellite cells were grown to confluence in MEM+10% FBS. Cells were washed with MEM and exposed to serum-free differentiation-promoting medium (SFM) or this medium containing 10^{-7} M testosterone. Fresh media were supplied daily, and replicate plates of cells were fixed and stained daily. The appearance of myosin positive cells is shown for days 1-4 in control (C) or testosterone-treated (T) cultures, respectively. Magnification, x30.

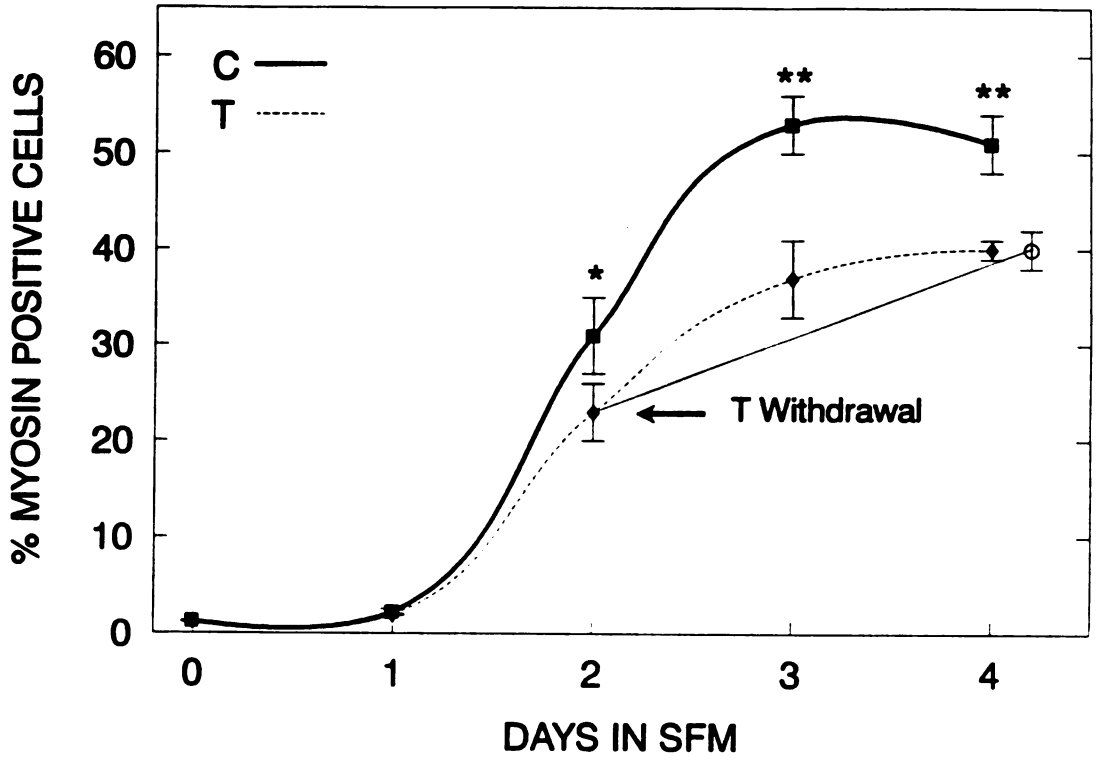

Fig. 2.7. Effects of testosterone on incidence of myosin-positive cells. Culture conditions were as described in the legend of Fig. 2.6. Replicate plates of cells were fixed and stained daily. In four wells, testosterone treatment was replaced by control medium after 2 days as indicated. Myosin positive cells were evaluated as described in Experimental Procedures, and are expressed as a percent of total nuclei. Means ± S.E.M. for (C) control and (T) testosterone-treated cultures are shown. Asterisks indicate that treatment means differ from control (*P<0.05; **P<0.01).

96 h. Testosterone treatment for 48 h, followed by exposure to control SFM for 48 h, reduced cell differentiation to the same extent as continuous exposure to testosterone for 96 h (Fig. 2.8). Thus, the effect of testosterone on differentiation appears to be mediated within the first 48 h of treatment, and is not reversible within 48 h after withdrawal. No apparent morphological changes resulted to indicate an adverse effect of 10^{-7} M testosterone on cell viability in either proliferating or differentiating cultures. Likewise, no differences in nuclear density or DNA content were observed between testosterone treated and control cultures (Fig. 2.9). The gradual loss of nuclei (or DNA) in treated and control cultures resulted primarily from the detachment of myotubes. These results indicate that testosterone depresses porcine satellite cell differentiation in the absence of a mitogenic effect.

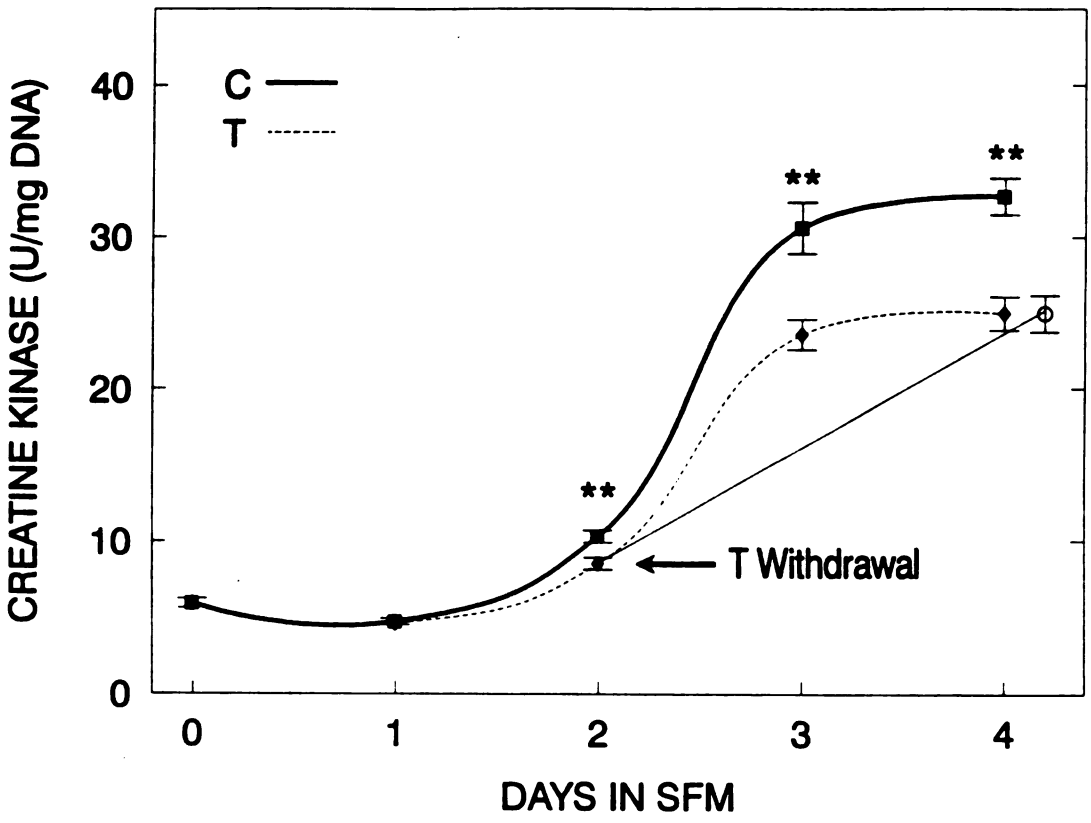

Fig. 2.8. Effects of testosterone on creatine kinase activity. Culture conditions were as described in the legend of Fig. 2.6. Replicate plates of cells were washed and frozen daily. In eight wells, testosterone treatment was replaced by control medium after 2 days as indicated. Creatine kinase activity was determined as described in Experimental Procedures. Means ± S.E.M. for eight wells of (C) control and (T) testosterone-treated cultures are shown. Asterisks indicate that treatment means differ from control (**P<0.01).

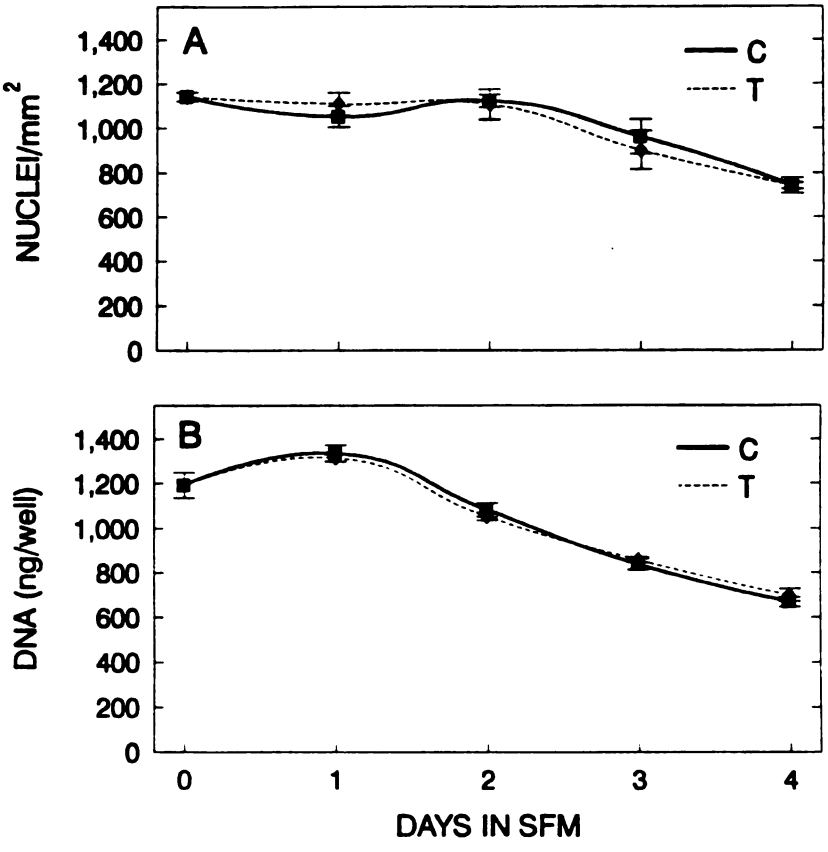

Fig. 2.9. Effects of testosterone on cell density in differentiating cultures. Culture conditions were as described in the legend of Fig. 2.6. Control (C) and 10^{-7} M testosterone-treated (T) cultures are represented by solid and dashed lines, respectively. Nuclei were evaluated (A) and DNA determined (B) as described in Experimental Procedures. Means ± S.E.M. are shown.

DISCUSSION

Immunological methods were used to demonstrate that satellite cells possess AR (data in Chapter 1). Furthermore, AR increase incrementally in satellite cells and myotubes treated with 10^{-7} M testosterone for 6, 12, and 24 h (data in Chapter 1). In the present study, testosterone had no effect on proliferation, yet decreased differentiation of porcine satellite cells *in vitro*.

The anabolic effects of androgens on skeletal muscle *in vivo* are well documented (Reviewed by Kochakian, 1975). These effects are characterized by increased protein synthesis and accretion (Florini and Breuer, 1966; Grigsby et al., 1976; Martinez et al., 1984), as well as increased satellite cell proliferation (Mulvaney et al., 1988; Joubert and Tobin, 1989) and DNA accumulation (Cheek and Hill, 1970; Snochowski et al., 1981; Thompson et al., 1989). However, addition of androgens to cultured myogenic cells elicited no effect on DNA synthesis (Gospodarowicz et al., 1976), cell proliferation (Thompson et al., 1989), protein synthesis (Roeder et al., 1986), protein degradation (Ballard and Francis, 1983; Roeder et al., 1986) or α-actin accumulation (Allen et al., 1983).

In this study, testosterone did not affect satellite cell

proliferation, regardless of treatment protocol. These protocols consisted of continuous treatment in serum-containing media or SFM for 72 h, or exposure to testosterone in basal SFM for 24 h, followed by 72 h incubation in SFM either with, or without testosterone addition. When testosterone treatments were added to serum-containing medium, serum concentrations which support sub-maximal proliferation of porcine satellite cells were used (Doumit and Merkel, 1992). Alternatively, growth factors were used at concentrations shown previously to elicit maximal mitogenic responses (Doumit et al., 1993). Hence, testosterone does not appear to affect the sensitivity or maximal responsiveness of porcine satellite cells to mitogens. These results are consistent with previous studies showing no direct effect of androgens on myogenic cell proliferation (Gospodarowicz et al., 1976; Thompson et al., 1989).

Itagane et al. (1991) demonstrated that treatment of chondrocytes with testosterone for 24 h, increased the subsequent mitogenic effect of IGF-I. Similarly, satellite cells isolated from trenbolone-treated rats were shown to be more responsive to the mitogenic effects of IGF-I and FGF than satellite cells isolated from control female rats (Thompson et al., 1989). These authors suggested that prior exposure of cells to androgens may prime cells for the action of secondary agents. In the current study, pre-treatment of porcine satellite cells with testosterone for 24 h, which up-regulates

AR (data in Chapter 1), did not alter the responsiveness of these cells to IGF-I, bFGF, or PDGF-BB. These growth factors are all mitogenic for porcine satellite cells (Doumit et al., 1993). Therefore, these findings do not support the notion that androgens imprint or prime satellite cells to become more responsive to growth factors. It is possible that other endocrine or local tissue factors are necessary to elicit the apparent effects of androgens on satellite cell proliferation. Thompson et al. (1989) used primary satellite cell cultures which likely contain non-myogenic cells that may have influenced their results. Indeed, non-myogenic cells isolated from primary satellite cell cultures possess AR and are potential targets for androgen action (Chapter 1).

Creatine kinase activity and detection of sarcomeric myosin are commonly used indices of muscle cell differentiation. The latter method affords sufficient sensitivity to detect mononucleated cells which have differentiated, but not fused. At high concentrations, insulin interacts with the type-1 IGF receptor and stimulates myogenic differentiation (Ewton et al., 1987). Clonally-derived porcine satellite cells undergo relatively little differentiation in the absence of exogenous insulin-like growth factors or pharmacological insulin concentrations.

In the present study, testosterone depressed myogenic differentiation induced by 10^{-6} M insulin. Decreased satellite cell differentiation in response to testosterone has not

previously been reported. To my knowledge, a direct effect of testosterone on cultured myoblasts has been demonstrated in only one other report. In their study, Powers and Florini (1975) demonstrated an increase in thymidine labeling index of DNA in myoblast cultures exposed to 10^{-8} M testosterone. Myoblasts in that study were grown in medium containing 2% gelding serum, which had little mitogenic activity. Low concentrations of horse serum have since been reported to induce differentiation of a variety of cultured muscle cells (Blau and Webster, 1981; McFarland et al., 1988; Dodson et al., 1990; Doumit and Merkel, 1992). It is possible that the testosterone-induced increase in DNA labeling index observed by Powers and Florini (1975), resulted from a decrease in myoblast differentiation, although sensitive measures of myoblast differentiation were not readily available at the time of that study. When DNA labeling index is used to calculate cell cycle time, it is assumed that all cells being studied are capable of DNA synthesis. Since differentiated muscle cells are incapable of DNA synthesis (Stockdale and Holtzer, 1961; Okazaki and Holtzer, 1966; Moss and Leblond, 1971), an apparent increase in labeling index may reflect the number of cells capable of synthesizing DNA, or those myoblasts which are undifferentiated. It is interesting to note that the only reported effect of testosterone on myoblast DNA labelling index (25% increase; Powers and Florini, 1975) is comparable to the decrease in satellite cell

differentiation observed in the present study.

Delayed differentiation of myoblasts has been observed in several situations in which muscle mass is increased. Myoblasts from bovine double-muscled fetuses (Quinn et al., 1990) and satellite cells from heavily muscled commercial turkeys (McFarland et al., 1993) differentiate later in culture than control myoblasts or satellite cells from Merriams turkeys, respectively. Additionally, delayed somite formation, expression of myogenic regulatory factors, and myosin heavy chain has been observed in a quail line exhibiting myofiber hypertrophy and increased DNA accretion (Coutinho et al., 1993). Results of the current study indicate that testosterone-induced increases in muscle mass may also involve a decrease or delay in myogenic cell differentiation.

The intracellular events governing the observed testosterone-induced decrease in differentiation are currently unknown. Testosterone augmentation of immunoreactive AR protein in porcine satellite cells and myotubes has been observed (data in Chapter 1). Thus, it seems likely that the effects of testosterone are AR-mediated, yet specific androgen-regulated genes in skeletal muscle satellite cells or myoblasts have not been identified. Florini (1970) found that while testosterone increased protein synthesis in skeletal muscle, no new protein species were detected using disk gel electrophoresis or isoelectric focusing. It is possible that

these procedures were not adequate to detect additional proteins synthesized in response to testosterone. Testosterone may modulate expression of genes which code for low abundance nuclear proteins. However, attempts to identify androgen-responsive genes in myoblasts using sensitive molecular techniques have not been reported.

Since testosterone did not affect DNA content of either proliferating or confluent cultures, it is unlikely that testosterone depresses differentiation by increasing the secretion of, or responsiveness to, autocrine growth factors. Although the effect of testosterone on differentiation is not reversible within 48 h after treatment withdrawal, no adverse effects of testosterone on cell viability were visually apparent. This is also supported by the lack of effect on culture DNA content. Testosterone administration for 24 h increased the intensity of AR immunostaining, and staining intensity 24 h after testosterone withdrawal did not return to the basal level. It seems reasonable to expect that effects of testosterone on differentiation may also be sustained after treatment withdrawal.

The observed response to testosterone may result from a satellite cell population which is heterogeneous with respect to androgen responsiveness, yet no obvious differences in AR staining were observed among clonally-derived satellite cells. Still, a subpopulation of cells which is more sensitive to testosterone could explain why only some cells (20-30% fewer

than in control cultures) do not differentiate in the presence of testosterone, while others apparently differentiate normally. Additional research is needed to clarify these observations.

SUMMARY AND IMPLICATIONS

Since nuclei within the sarcolemma of muscle fibers do not synthesize DNA (Moss and Leblond, 1971), satellite cells provide the only source of myonuclei to postnatal skeletal muscle. Skeletal muscle DNA accumulation is controlled by satellite cell proliferation, differentiation, and incorporation into muscle fibers. Thus, rates of satellite cell proliferation and differentiation ultimately govern the size of the satellite cell population in skeletal muscle. This, in turn, reflects the potential of muscle to accumulate DNA. To date, efforts to explain androgen-associated increases in proliferative satellite cells and skeletal muscle DNA content, have focused on mechanisms which involve direct mitogenic stimulation. Direct effects of androgens on myogenic cell proliferation have been difficult to demonstrate, despite several attempts outlined in this dissertation and other reports. Nevertheless, evidence for a direct effect of testosterone on satellite cell differentiation is provided in this dissertation.

A mechanism by which testosterone may directly increase the population of proliferating satellite cells by reducing satellite cell differentiation is proposed, and a hypothetical

model is presented (Fig. 2.10). The model illustrates that modest reductions (12.5-25%) in myogenic differentiation could lead to marked increases in satellite cell numbers and myonuclear accretion. In this model, it is assumed that control and testosterone-treated satellite cells have an identical population doubling time. When exposed to control conditions, 50% of the daughter cells resulting from each population doubling remain as satellite cells, while 50% of the cells differentiate and become myonuclei (Moss and Leblond, 1971). Testosterone is shown to reduce differentiation of satellite cells by 12.5-25% (1 cell fewer/generation), and this results in a 125% increase in satellite cells and a 25% increase in myonuclei after 5 generations. These events appear similar to those which initially follow testosterone administration *in vivo* (Joubert and Tobin, 1989). Undoubtedly, the satellite cell population *in vivo* is subject to more dynamic changes than this model affords. The absolute number of satellite cells does not appear to increase to the extent predicted by this model, indicating that periods of more extensive differentiation must exist. In theory, however, this model provides an alternative mechanism whereby androgens may increase skeletal muscle DNA content in the absence of a direct mitogenic stimulation.

Control			*+Testosterone*	
sc	*mn*	*Generation*	*sc*	*mn*
xxxx		0	xxxx	
xxxx	oooo	1	xxxx x	ooo
xxxx	oooooooo	2	xxxx xx	ooooooo
xxxx	ooooooooooo	3	xxxx xxx	ooooooooooo
xxxx	ooooooooooo oooooo	4	xxxx xxxx	ooooooooooo oooooooo
xxxx	ooooooooooo ooooooooooo	5	xxxx xxxx x	ooooooooooo ooooooooooo ooooo

Fig. 2.10. Hypothetical model for testosterone-induced increases in DNA. This model assumes a similar population doubling time for treated and control satellite cell populations. To maintain a reserve population of satellite cells (x), 50% of the daughter cells in the control population remain as satellite cells and 50% become myonuclei (o). Testosterone reduces differentiation of satellite cells by 12.5-25% (1 cell less/generation), and this results in a 125% increase in the satellite cell population and a 25% increase in myonuclei after 5 population doublings.

One possible explanation for the effect of testosterone on differentiation is founded on the observation that gonadally intact males have greater skeletal muscle calpastatin activity, yet similar calpain activity, compared to castrated males (Ou et al., 1991; Morgan et al., 1992). Calpastatin is an endogenous inhibitor for the calcium-activated neutral proteases, or calpains (reviewed by Murachi, 1989). Kaur and Sanwal (1981) found that myoblasts contain a potent inhibitor of calcium-activated neutral protease activity, and this inhibitor is lost during differentiation. Schollmeyer (1986) reported that m-calpain (active at millimolar Ca^{2+}) becomes membrane associated in fusing myoblasts and hypothesized that the protease may be involved in disassembly of the cytoskeletal-membrane linkage. Filamin, a protein known to facilitate actin microfilament assembly, has recently been shown to be cleaved by m-calpain in cultured myoblasts (Kwak et al., 1993). Exogenous calpain inhibitors, leupeptin and E64d, also inhibit the fusion of cultured myoblasts (Kumar et al., 1992).

Kwak et al. (1993) demonstrated that both the activity and protein level of m-calpain increase just prior to myoblast fusion. These authors also showed that regulation of m-calpain protein is independent of that for creatine kinase.

Mellgren (1991) has shown that m-calpain is capable of hydrolyzing proteins in isolated rat liver nuclei at Ca^{2+} concentrations as low as 3 μM. Activation of m-calpain at low Ca^{2+} concentrations appears to require interaction of DNA, calpain and calpain substrates in the nuclear matrix (Mellgren et al., 1993). Transcription factors *c-fos* and *c-jun* are extremely sensitive to calpain cleavage *in vitro* and *c-jun* is a substrate for calpains *in vivo* (Hirai et al., 1991). Furthermore, these transcription factors are known to inhibit myogenesis by repressing transcriptional activation by myogenic regulatory factors (Bengal et al., 1992; Li et al., 1992). It is tempting to speculate that testosterone depresses differentiation by increasing calpastatin activity, which in turn, would decrease activity of m-calpain and proteolysis of *c-fos* and/or *c-jun*, and ultimately depress transcriptional activation of myogenic regulatory factors. However, at this time, experiments substantiating an effect of testosterone on calpastatin activity in cultured muscle cells have not been conducted.

Results presented in this dissertation provide the first evidence for a direct effect of testosterone on satellite cell differentiation. Porcine satellite cells possess androgen receptors and testosterone decreases satellite cell

differentiation, yet has no effect on cell proliferation. This decrease in satellite cell differentiation may help explain the observation that androgens increase skeletal muscle DNA content. Collectively, these data provide a new perspective on the mechanism of androgen-induced increases in skeletal muscle growth.

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