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Testosterone Up-Regulates Androgen Receptors and Decreases Differentiation of Porcine Myogenic Satellite Cells *in Vitro**

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ABSTRACT

Accumulation of DNA is essential for muscle growth, yet mechanisms of androgen-induced DNA accretion in skeletal muscle are unclear. The purpose of this study was to determine whether androgen receptors (AR) are present in cultured skeletal muscle satellite cells and myotubes and examine the effects of testosterone on satellite cell proliferation and differentiation. Immunoblot analysis using polyclonal AR antibodies (PG-21) revealed an immunoreactive AR protein of approximately 107 kDa in porcine satellite cells and myotubes. Immunocytochemical AR staining was confined to the nuclei of satellite cells, myotubes, and muscle-derived fibroblasts. Administration of 10^{-7} M testosterone to satellite cells, myotubes, and muscle-derived fibroblasts increased immunoreactive AR. In satellite cells and myotubes, AR increased incrementally after 6, 12, and 24 h of exposure to testosterone. Testosterone (10^{-10}-10^{-6} M), alone or in combination with insulin-like growth factor I, basic fibroblast growth factor, or platelet-derived growth factor-BB, had no effect ($P > 0.1$) on porcine satellite cell proliferation, and testosterone pretreatment for 24 h did not alter the subsequent responsiveness of cells to these growth factors. Satellite cell differentiation was depressed (20–30%) on days 2–4 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, and testosterone administration increases immunoreactive AR protein and reduces differentiation of porcine satellite cells *in vitro*. (*Endocrinology* **137:** 1385–1394, 1996)

SKELETAL MUSCLE DNA accretion is intimately associated with muscle protein accumulation and growth. However, nuclei contained within the sarcolemma of myotubes or myofibers do not synthesize DNA (1, 2). It is known that satellite cells proliferate, differentiate, and incorporate into muscle fibers, thereby providing the only source of postnatally added nuclei to normally growing (3, 4) or regenerating (5) skeletal muscle fibers.

The anabolic effects of androgens on skeletal muscle *in vivo* are well documented (6, 7). These effects are characterized by increased protein synthesis and accretion (8–10) as well as increased satellite cell proliferation (11, 12) and DNA accumulation (13–15). Despite obvious *in vivo* effects of androgens on skeletal muscle mass, several reports have shown no effect of androgens on DNA synthesis in myoblasts (16), satellite cell proliferation (15), protein synthesis (17), protein breakdown (17, 18), or α-actin accumulation (19) in cultured skeletal muscle cells. Additionally, Thompson *et al.* (15) demonstrated that the synthetic androgen, trenbolone acetate, had no direct effect on the proliferation of cultured satellite cells, although satellite cells isolated from trenbolone acetate-treated rats were more responsive to the mitogenic effects of insulin-like growth factor I (IGF-I) and fibroblast growth factor (FGF) than those isolated from untreated rats. A single report indicated that testosterone directly stimulates a modest (25%) increase in the DNA labeling index in cultured myoblasts (20).

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 (14, 21). More recently, immunohistochemical studies revealed AR in cardiac muscle nuclei (22, 23) and in nearly every nucleus of skeletal muscle (22).

The apparent discrepancy between *in vivo* and *in vitro* skeletal muscle responsiveness to androgens has not been explained. To our knowledge, no previous studies have identified AR in satellite cells or cultured muscle cells. Additionally, effects of androgens on clonal cultures of satellite cells grown in serum-free medium have not been reported previously. The following study used a porcine satellite cell culture system to 1) identify, localize, and study the regulation of AR in cultured satellite cells and myotubes using immunochemical methods; and 2) examine the effects of testosterone on satellite cell proliferation and differentiation *in vitro*.

Materials and Methods

Materials

αMEM, antibiotic-antimycotic, gentamicin, human recombinant basic FGF (bFGF), epidermal growth factor (EGF), IGF-I, and platelet-derived

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growth factor-BB (PDGF-BB) were purchased from Life Technologies (Grand Island, NY). FBS was purchased from Hazelton (Lenexa, KS). Tissue culture dishes and plates were obtained from Corning Glass Works (Corning, NY). Pronase, MCDB-110 medium, BSA (RIA grade), dexamethasone, bovine insulin, bovine transferrin, testosterone, water-soluble linoleic acid, porcine skin gelatin, goat serum, biotinylated antirabbit IgG, extravidin-peroxidase, alkaline phosphatase-conjugated goat antimouse IgG, 3,3'-diaminobenzidine (DAB), 5-bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium, calf thymus DNA, and Hoechst no. 33258 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 Corp. (Bedford, MA).

Cell culture

Satellite cells were isolated from the semimembranosus muscles of 6- to 8-week-old pigs, as described by Doumit and Merkel (24). This is a modification of the procedure outlined by Bischoff (25) for rodent skeletal muscle. Primary satellite cells were cultured as previously described (24). Satellite cell clones were isolated using a cloning ring technique outlined by Doumit *et al.* (26). The two satellite cell 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 that did not exhibit myotube formation. These fibroblast-like clones were prepared as described for satellite cell clones. No myotube formation, creatine kinase induction, or myosin-positive cells were observed in these cultures under conditions that induce differentiation of porcine satellite cells (our unpublished observations).

Clonally derived satellite cells (fourth passage) were seeded into gelatin-coated culture wells as described previously (26). Cells used for immunocytochemistry and Western blotting were seeded on 24-well plates (5,000 cells/16 mm-diameter well) and 6-well plates (20,000 cells/35 mm-diameter well), respectively. Unless otherwise specified, fresh growth medium (MEM and 10% FBS) was supplied at 48-h intervals. When cells reached confluence (approximately day 5), myotube formation was induced by exposure to serum-free medium (0.3 ml/16-mm diameter well or 2 ml/35-mm diameter well), and fresh medium was supplied daily. This medium was originally formulated to maintain the viability and support the proliferation of cultured porcine satellite cells (26) and has been modified to promote the differentiation of porcine satellite cells. The components of the serum-free proliferation and differentiation-promoting media are listed in Table 1.

To examine the effects of testosterone on satellite cell proliferation, cells were seeded and allowed to attach for 24 h, then washed twice with MEM, and treatment media were applied. Testosterone was administered as a 24-h pretreatment 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 Table 1, except no growth factors were included, and the 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 medium changes. At appropriate times, cultures were washed with PBS, and DNA was quantified spectrofluorometrically, using Hoescht 33258, as described by West *et al.* (27). Calf thymus DNA served as the standard.

TABLE 1. Porcine satellite cell SFM

Component	Final conc.	
	Growth	Differentiation
MEM:MCDB-110 medium	4:1	4:1
bFGF (ng/ml)	10.0[a]	
BSA (mg/ml)	0.5	0.5
Dexamethasone (M)	10^{-7}	10^{-10}
Insulin (M)	10^{-6}	10^{-6}
Linoleic acid (μg/ml)	0.5	0.5
PDGF-BB (ng/ml)	5.0[a]	
Transferrin (μg/ml)	100.0	100.0

[a] Concentration used was previously determined to stimulate the maximal proliferation of porcine satellite cells (26).

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 containing testosterone treatment. Testosterone (T-5641; Sigma) and water-soluble testosterone (T-5035; Sigma) produced similar results, and addition of the water-soluble carrier, β-cyclodextrin, had no effect on differentiation relative to serum-free medium alone. In initial studies, differentiation was quantified by morphological assessment of myogenic cell fusion. Cells were fixed in absolute methanol and stained with 0.03% Giemsa to enable visual evaluation of nuclei, and 6–10 observations/well were made to determine total nuclei and myotube nuclei. A minimum of 3000 total nuclei were scored for each data point shown, and cells containing 3 or more nuclei were considered myotubes. This evaluation method potentially underestimates cell differentiation by not detecting cells that have differentiated but not fused. Therefore, two additional methods were 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. After three washes in PBS, nonspecific 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 with NA4 for 1 h at 22 C produced similar results. Monoclonal antibody NA4 (kindly provided by Dr. Everett Bandman, University of California-Davis) recognizes all isoforms of sarcomeric myosin heavy chain (28). After three washes in PBS, cells were exposed to goat antimouse IgG conjugated to alkaline phosphatase (1:40) in blocking reagent for 1 h at room temperature. Cells were washed and myosin-positive cells were visualized using 5-bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium 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. Nuclei were stained with Giemsa and typically more than 3500 total nuclei/data point were scored to determine the fraction that was present in myosin-positive cells. 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).

Assay of creatine kinase

At the indicated times, cells were washed three times with PBS, overlaid 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 and sonicated for 4 sec, and 30 μl sample/well were assayed for creatine kinase (CK) activity using a kit (47-UV, Sigma). DNA was quantified as described above, except a 20-μl aliquot of sample was used for each determination. CK and DNA assays were performed in duplicate for eight wells per treatment/day.

Western immunoblots

At the times specified in the figure legends, cells were solubilized by the 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 the 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 with a mortar and pestle. Powdered tissues were solubilized in 9 vol boiling sample buffer for 5 min, then clarified by centrifugation at 2000 × *g* for 10 min. Protein concentration was determined by the bicinchoninic acid method of Smith *et al.* (29). Immediately before electrophoresis, 2-mercaptoethanol was added to 5% (vol/vol), and samples were boiled for 5 min. Proteins were separated by electrophoresis in 8% polyacrylamide separating gels with 4% stacking gels, as described previously (30). Resolved proteins were transferred to nitrocellulose or Immobilon-P at

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4 C for 5 h at 0.8 amp in a buffer containing 25 mM Tris, 193 mM glycine, and 15% methanol. Lanes containing mol wt standards were stained with Coomassie blue. To prevent nonspecific antibody binding, membranes were blocked with 10% Carnation nonfat dry milk (Nestle Food Co., Glendale, CA) and 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 membranes were washed three times with blocking buffer after each incubation. Membranes were incubated for 2 h with 1 μg/ml PG-21, a polyclonal antibody raised against a peptide (AR_{21}) containing the first 21 amino acids of the rat and human AR (31). 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. Membranes were then exposed to biotinylated goat antirabbit IgG (2 μg/ml) for 1 h, followed by 2.5 μg/ml extravidin-peroxidase in PBS containing 10% blocking solution (vol/vol) for 1 h. Blots were washed three times with PBS, and immunoreactive bands were visualized by treatment with DAB (0.5 mg/ml PBS) in the presence of 0.003% H_2O_2. We attempted to detect estrogen receptors in porcine satellite cells and myotubes using polyclonal antibodies (ER-21) under conditions identical 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 AR

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 PBS containing 0.3% Triton X-100 for 30 min. Nonspecific 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 preincubated for 1 h with a 10-fold molar excess of AR_{21} (kindly provided by Dr. Geoffrey Greene) served as a negative control for each assay. Cells were washed three times in PBS and exposed to biotinylated goat antirabbit IgG (1:200) for 1 h. After three washes in PBS, cells were incubated with extravidin-peroxidase (1:100) in PBS for 40 min. Sites with enzyme activity produced a brown reaction product when exposed to DAB (1 mg/ml PBS) containing 0.006% H_2O_2.

Statistical analysis

Data were analyzed using the general linear model procedures of the Statistical Analysis System (32). Treatment means were separated using Bonferonni *t* tests. Unless otherwise specified, stated differences are significant at the 0.05 level of probability.

Results

Identification, regulation, and localization of AR

Polyclonal AR antibodies (PG-21) were used to analyze extracts of porcine satellite cells, satellite cell-derived myotubes, epididymis, and spleen for AR. Western immunoblots of protein extracts from satellite cells, myotubes, and epididymis revealed a major immunoreactive band of ~107 kDa (Fig. 1), consistent with the known molecular mass of human, rat, and bovine AR (31, 33). AR immunostaining was more intense in epididymis than in satellite cells and was absent in spleen, an AR-negative tissue.

Satellite cell and myotube cultures maintained in serum-free medium (SFM) had similar quantities of AR protein (Fig. 1, 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. 2, lane 2 *vs.* 3). Although this may involve an increase in AR abundance in satellite cells exposed to serum, a dilution of the AR protein, which results from rapid accumulation of

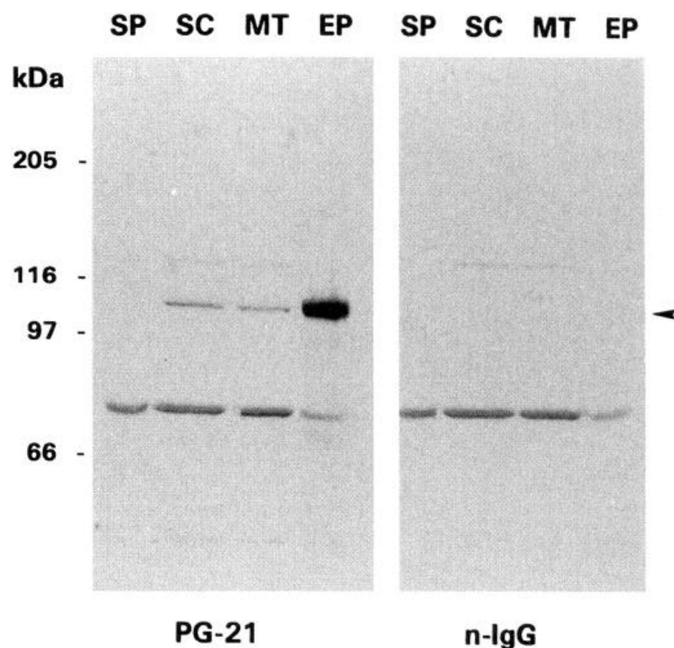

FIG. 1. Western blot analysis of satellite cells (SC) and myotubes (MT) maintained in SFM. Cells were grown to confluence in MEM and 10% FBS. Satellite cells were maintained in medium containing 10^{-9} M insulin, which results in minimal myotube formation. Differentiation and myotube formation were induced by medium containing 10^{-6} M insulin. 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 the *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 the *right panel* are nonspecific.

myofibrillar proteins in myotubes, is likely. In this study, myotube cultures exposed to 10% FBS contained ~2-fold more protein than satellite cell cultures of similar nuclear density, yet myotubes maintained in SFM had only 30–40% greater protein than satellite cells in SFM (data not shown). 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 nonspecific. The major nonspecific band at 75 kDa resulted from binding of extravidin peroxidase, as elimination of biotinylated secondary antibody did not result in the disappearance of this band, and no endogenous peroxidase activity was observed when membranes were exposed to substrate alone (data not shown).

Administration of 10^{-7} M testosterone for 6, 12, and 24 h induced a time-dependent increase in immunoreactive AR protein in both satellite cell (Fig. 3) and myotube (Fig. 4) cultures. Immunocytochemical staining for AR revealed that nuclei in all cells of primary porcine satellite cell and myotube cultures stained positive for AR in the absence of testosterone (Fig. 5). Nuclear AR staining intensity increased after exposure of clonally derived satellite cells (Fig. 6, A *vs.* B) and fibroblasts (Fig. 7, A *vs.* B) to 10^{-7} M testosterone. Withdrawal of testosterone for 24 h did not reduce AR staining to the basal level (Fig. 6, A *vs.* C). Up-regulation of AR in satellite cells was specific for androgens, as treatment of cells with 10^{-7} M dexamethasone or 10^{-8} M 17β-estradiol had

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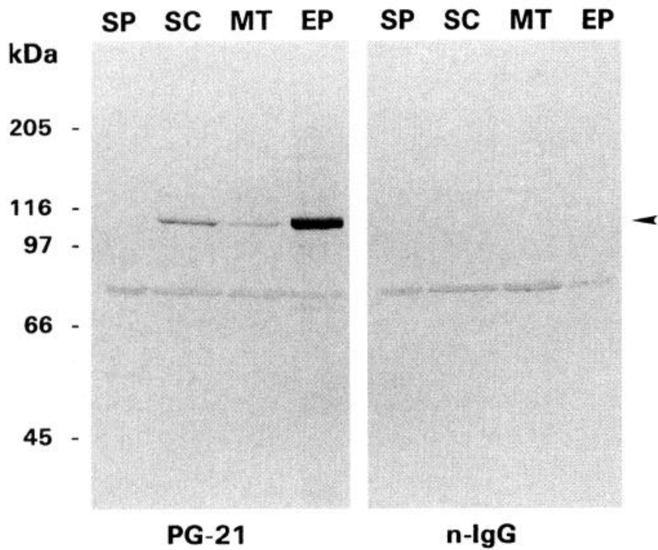

FIG. 2. Western blot analysis of satellite cells (SC) and myotubes (MT) exposed to MEM and 10% FBS. Satellite cells were grown to 90% confluence in MEM and 10% FBS, then harvested in SDS sample buffer or exposed to differentiation medium for 4 days. Myotubes were treated with MEM and 10% FBS for 12 h, 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. Membranes were incubated with PG-21 or normal IgG, as outlined in *Materials and Methods*. The major immunoreactive AR band is indicated by an *arrow*.

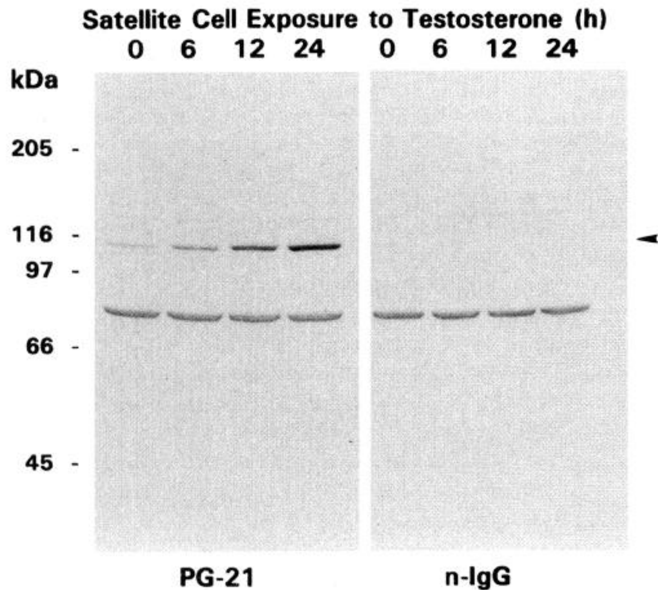

FIG. 3. Immunoblot analysis of testosterone-induced increases in satellite cell AR concentration. Satellite cells were grown to confluence in MEM and 10% FBS, then exposed to control SFM (0) or SFM containing 10^{-7} M testosterone for 6, 12, and 24 h. All cells were in SFM for 24 h. Lanes represent 220 μg protein separated by gel electrophoresis and transferred to Immobilon-P. Membranes were incubated with PG-21 (*left*) or normal IgG (*right*), as outlined in *Materials and Methods*.

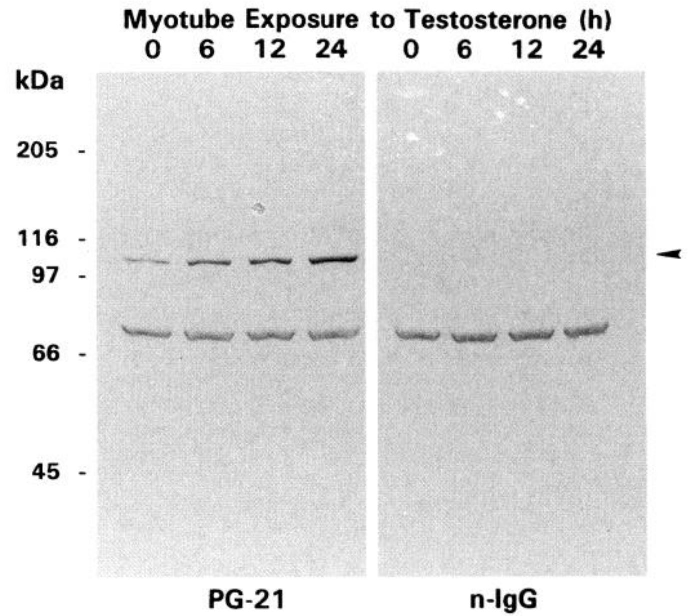

FIG. 4. Immunoblot analysis of testosterone-induced increases in myotube AR concentration. Satellite cells were grown to confluence in MEM and 10% FBS, then exposed to differentiation medium for 4 days. Thereafter, myotubes were treated with control SFM (0) or SFM containing 10^{-7} M testosterone for 6, 12, and 24 h. Lanes represent 220 μg protein separated by gel electrophoresis and transferred to Immobilon-P. Membranes were incubated with PG-21 (*left*) or normal IgG (*right*).

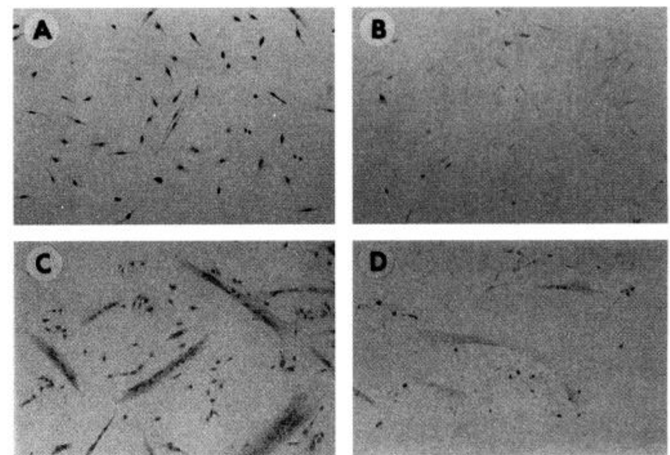

FIG. 5. Immunocytochemical staining for AR in satellite cells and myotubes from primary culture. Satellite cells were allowed to attach for 48 h in MEM and 10% FBS, then maintained in SFM for 24 h before immunostaining. Alternatively, cells were grown in MEM and 10% FBS for 6 days, then exposed to SFM for 3 days to induce differentiation. Positive immunostaining in nuclei of satellite cells (A) and myotubes (C) was abolished (B and D, respectively) by preincubation of PG-21 with a 10-fold molar excess of antigen (AR_{21}). Magnification, ×70.

no apparent effect on AR immunostaining (data not shown). Furthermore, antiestrogen receptor antibodies (ER-21) reacted with a protein of ~64 kDa from porcine epididymis, but we were unable to detect immunoreactive estrogen receptors in satellite cells by immunoblotting or immunocytochemical staining using ER-21 antibodies (data not shown).

Effects of testosterone on satellite cell proliferation

The effects of testosterone on proliferation of clonally derived porcine satellite cells were examined in both serum-

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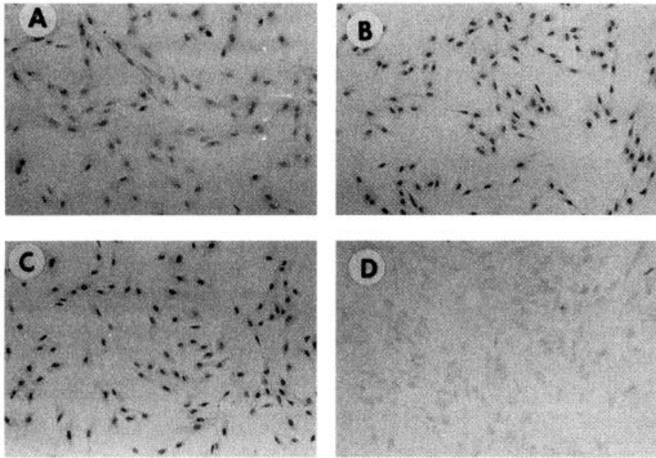

FIG. 6. Immunocytochemical staining for AR in satellite cells exposed to testosterone. Clonally derived satellite cells were seeded and allowed to attach for 24 h in MEM and 10% FBS. Cells were exposed to SFM for 48 h, followed by control SFM (A) or SFM containing 10^{-7} M testosterone (B) for 24 h before 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 (C). No nuclear staining was observed in satellite cells incubated with PG-21 preincubated with a 10-fold molar excess of AR_{21} (D). Magnification, ×70.

containing and SFM. The responsiveness of these cells to polypeptide growth factors has been previously reported (26, 34). Testosterone (10^{-10}-10^{-6} M) had no effect on the proliferation of satellite cells grown in either 2% FBS or 3% porcine serum (Fig. 8A). Treatment of satellite cells with testosterone for 24 h, which is sufficient to up-regulate AR, 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. 8B). Previous results from our laboratory (26) indicate that EGF alone is not mitogenic for porcine satellite cells, and that bFGF masks 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 on satellite cell proliferation when applied as a continuous 72-h treatment to SFM containing FIP or EIP (Fig. 8C). Additionally, 24 h preexposure of satellite cells to testosterone in basal SFM, followed by 72-h continuous testosterone exposure in growth factor-supplemented medium (FIP), had no effect on cell proliferation (data not shown). Collectively, these data indicate that testosterone has no measurable effect on satellite cell proliferation under the conditions described.

Effects of testosterone on satellite cell differentiation

Testosterone depressed myotube formation in a dose-dependent manner, with the maximal effect occurring at 10^{-7} M testosterone (Fig. 9). Sarcomeric myosin-positive cells and CK activity were measured to determine whether testosterone depression of satellite cell fusion was accompanied by a decrease in cell differentiation. Figure 10 illustrates the effects of testosterone on the incidence of myosin-positive cells in cultures of differentiating satellite cells. These photomicrographs depict cells that are representative of those counted to obtain Fig. 11 (A and C). After 2 days of treatment, testosterone (10^{-7} M) reduced both the number of nuclei in myosin-positive cells (Fig. 11A) and CK activity (Fig. 11B) by 20–30%. The magnitude of this effect was similar after 72 and 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. 11, A and B). 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 testosterone 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. 11, C and D). The gradual loss of nuclei (or DNA) in differentiating cultures resulted primarily from the detachment of spontaneously contracting myotubes. Testosterone-induced decreases in myotube formation, myosin-positive cells, and creatine kinase activity indicate that

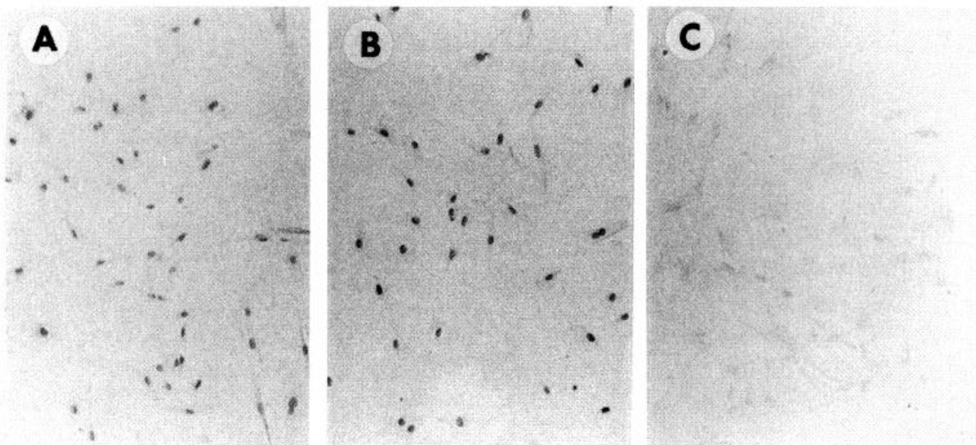

FIG. 7. Effect of testosterone on AR immunostaining of fibroblasts. Clonally derived fibroblasts were seeded and allowed to attach for 24 h in MEM and 10% FBS. Cells were exposed to SFM for 24 h, followed by control SFM for 24 h (A) or SFM containing 10^{-7} M testosterone for 24 h (B and C). Incubation 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. Magnification, ×70.

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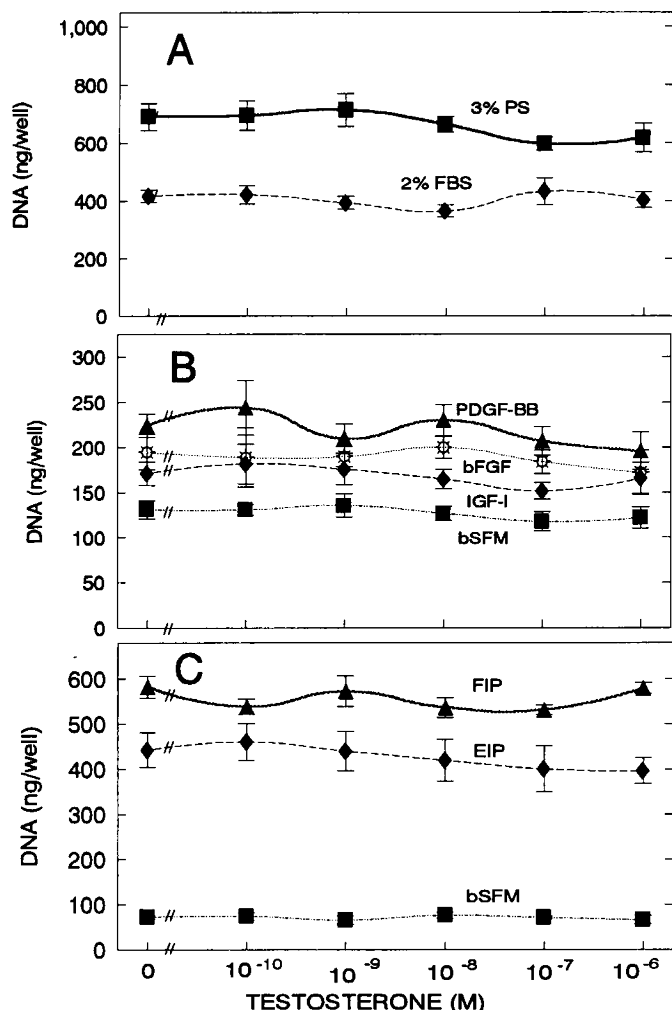

FIG. 8. Effects of testosterone concentration on satellite cell proliferation. Clonally derived satellite cells were seeded and allowed to attach for 24 h in MEM and 10% FBS. Cells were washed with MEM and exposed to testosterone in A) MEM containing 2% FBS or 3% porcine serum (PS); B) testosterone in basal SFM for 24 h, followed by SFM containing PDGF-BB (5 ng/ml), bFGF (10 ng/ml), IGF-I (25 ng/ml), or basal SFM (bSFM); or C) testosterone in basal SFM, or SFM containing bFGF or EGF (10 ng/ml) combined with IGF-I and PDGF-BB (FIP and EIP, respectively). Serum-containing medium and growth factor-supplemented SFM were applied for 72 h, and fresh media were supplied daily. Values represent the mean ± SEM of DNA determinations from four culture wells.

testosterone depressed the differentiation of porcine satellite cells.

Discussion

AR have been identified in skeletal muscle by binding assay (14, 21) and more recently by immunocytochemical methods (22, 23). AR messenger RNA (mRNA) has also been detected in skeletal muscle (35, 36), although Chang *et al.* (35) reported that the level of AR mRNA per unit polyadenylated 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, as satellite cells typically represent only 2–10% of the

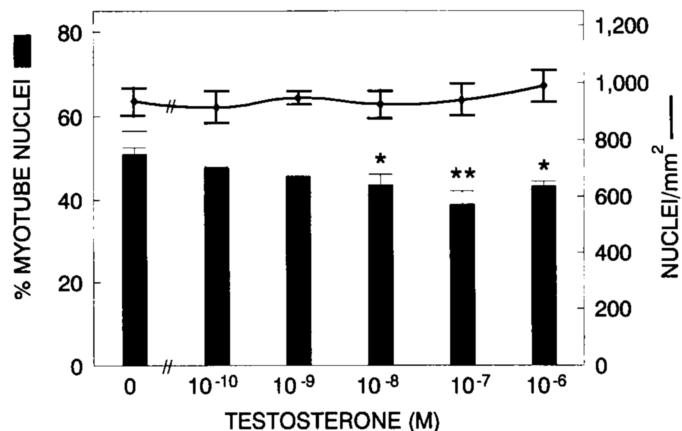

FIG. 9. Effects of testosterone on myotube formation. Clonally derived satellite cells were grown to confluence in MEM and 10% FBS. Cells were washed with MEM and exposed to serum-free differentiation medium containing the indicated testosterone concentrations. Fresh media were supplied daily for 4 days, then cells were fixed and evaluated as described in *Materials and Methods*. Values for percentage of myotube nuclei (*bars*) and nuclei per mm² (*line*) represent the mean ± SEM of four culture wells. Similar results were obtained in four independent experiments. *Asterisks* indicate that treatment means differ from control (*, $P < 0.05$; **, $P < 0.01$).

nuclei found within the basement membrane of adult skeletal muscle tissue (4), previous studies did not ascertain whether satellite cells possess AR. Additionally, the presence of AR in cultured skeletal muscle cells has not previously been reported. The current study clearly demonstrates the presence and autoregulation of AR in satellite cells, satellite cell-derived myotubes, and fibroblasts isolated from porcine skeletal muscle. Furthermore, we show that testosterone decreases the differentiation of porcine satellite cells, but has no effect on satellite cell proliferation *in vitro*.

Polyclonal antibodies (PG-21) that recognized a 108- to 110-kDa AR protein in extracts from rat and human tissues (31) were used for AR detection. 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 (35).

Immunostaining with PG-21 localized AR in the nuclei of rat prostatic cells (31). In our study, 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 several other tissues (22, 23, 37).

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, myotubes, and fibroblasts exposed to testosterone. To our knowledge, an immunological approach has not previously been used to study AR regulation in skeletal muscle cells, although Takeda *et al.* (22) observed that AR immunostaining in skeletal muscle of female rats was slightly weaker than that in male rats. This is consistent with the testosterone-induced up-regulation of AR observed in our study. Although the mechanisms of AR autoregulation were not addressed in the current study, ho-

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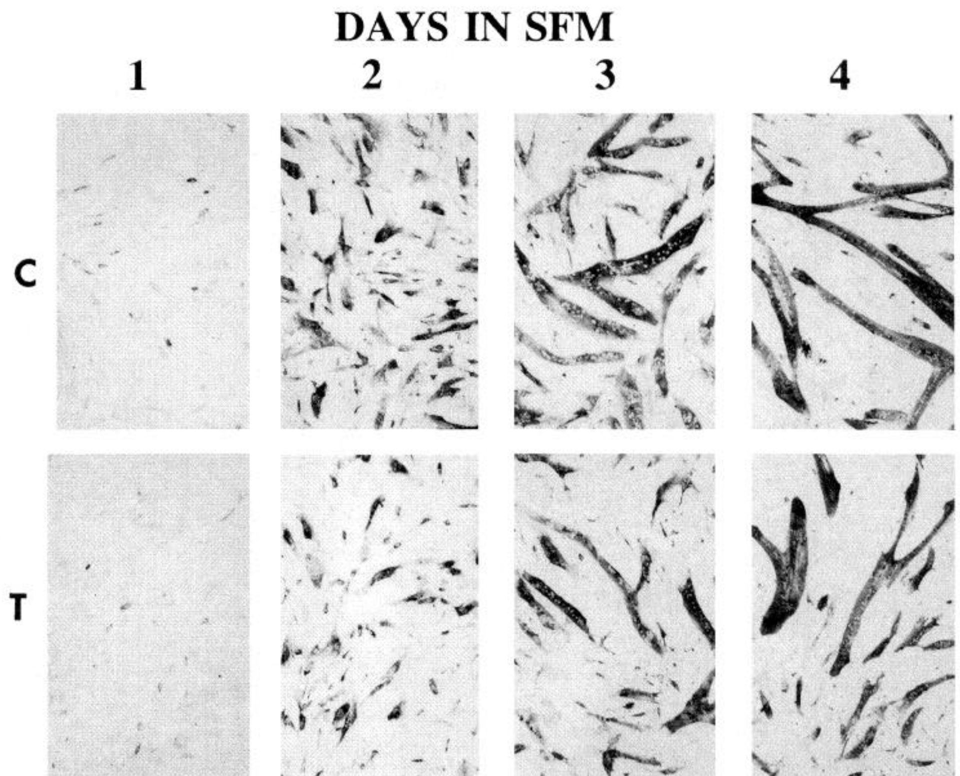

FIG. 10. Effects of testosterone on appearance of myosin-positive cells. Clonally-derived satellite cells were grown to confluence in MEM and 10% FBS. Cells were washed with MEM and exposed to serum-free differentiation-promoting medium (SFM) or this medium containing 10^{-7} M testosterone. Fresh media 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, ×30.

mologous up-regulation of AR has been demonstrated in several cell culture systems (38, 39). The concentration of AR protein and mRNA generally reflects the degree of androgen responsiveness of a tissue. 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 spleen to be an AR-negative tissue in other species (22, 31).

The presence and autoregulation of AR in satellite cells suggest 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, because AR are also present in fibroblasts and myotubes. In this study, testosterone did not affect satellite cell proliferation, regardless of the treatment protocol. These protocols consisted of continuous treatment in serum-containing medium or SFM for 72 h or exposure to testosterone in basal SFM for 24 h, followed by 72-h incubation in growth factor-supplemented SFM either with or without testosterone addition. When testosterone treatments were added to serum-containing medium, serum concentrations that support submaximal proliferation of porcine satellite cells were used (24). Alternatively, growth factors were used at concentrations shown previously to elicit maximal mitogenic responses (26). Therefore, 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 (15, 16).

Itagane *et al.* (40) 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 (15). These researchers suggested that prior exposure of cells to androgens may prime cells for the action of secondary agents. In the current study, pretreatment of porcine satellite cells with testosterone for 24 h, which up-regulates AR, did not alter the responsiveness of these cells to IGF-I, bFGF, or PDGF-BB. These growth factors are mitogens for porcine satellite cells (26). Therefore, our findings do not support the hypothesis 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.* (15) used primary satellite cell cultures that probably contain nonmyogenic cells (*e.g.* fibroblasts), which may have influenced their results. Indeed, we demonstrate that fibroblast-like cells isolated from primary satellite cell cultures possess AR and are potential targets for androgen action.

Recently, Joubert and Tobin (41) demonstrated that testosterone treatment results in activation of quiescent satellite cells in rat levator ani muscle. These researchers suggested that the effects of testosterone may be manifested by autocrine or paracrine factors, such as TGFs. Androgens have been shown to increase the number of EGF receptors on cultured human prostate cancer cells (33) and induce the production of TGFβ in bone cells (42). TGFβ1 has been shown to stimulate porcine satellite cell proliferation in basal SFM, enhance the mitogenic actions of FGF, and inhibit proliferation induced by PDGF-BB (34). Although we were unable

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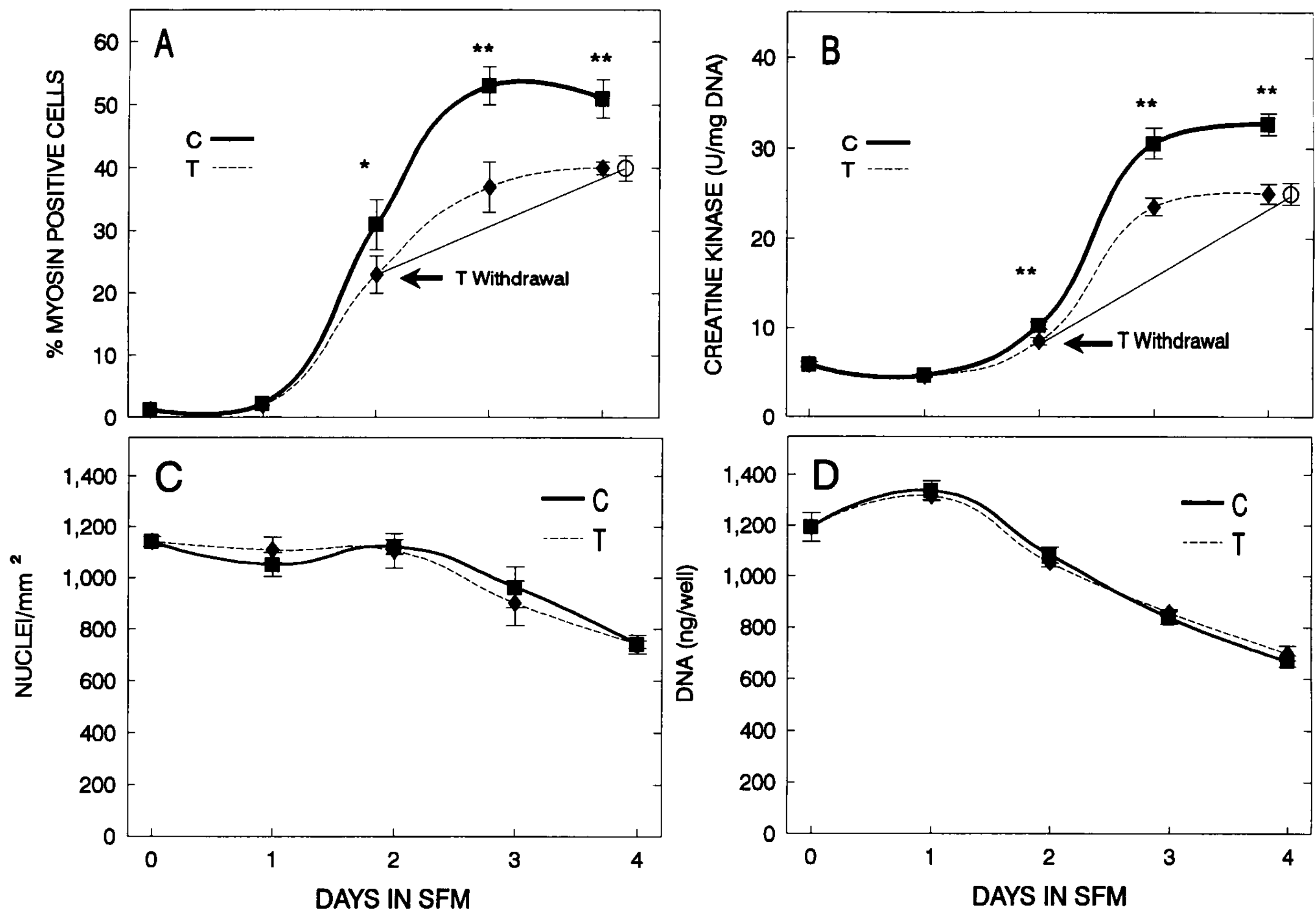

FIG. 11. Effects of testosterone on satellite cell differentiation. Culture conditions were described in Fig. 10. In some plates, testosterone treatment (T) was replaced by control medium (C) after 2 days as indicated. The incidence of myosin-positive cells (A) or creatine kinase activity (B) was determined on replicate cultures, as described in *Materials and Methods*. Cell density, determined microscopically (C) or by DNA assay (D) for control and 10^{-7} M testosterone-treated cultures are represented by *solid and dashed lines*, respectively. The mean ± SEM for eight wells are shown. *Asterisks* indicate that treatment means differ from the control value (*, $P < 0.05$; **, $P < 0.01$).

to detect a mitogenic effect of testosterone, as would be expected to result from autocrine production of satellite cell mitogens, we do not discount the possibility that local growth factors are involved in testosterone-induced increases in myonuclei. Testosterone may stimulate the production and/or release of mitogens from muscle fibers or nonmyogenic cells, which could subsequently act in a paracrine manner to stimulate satellite cell proliferation. Further investigations are needed to explore this possibility.

Satellite cell differentiation would be expected to increase fusion into myotubes as well as increase the expression of muscle-specific proteins, such as sarcomeric myosin and creatine kinase. Creatine kinase activity and immunodetection of sarcomeric myosin were used as indexes of muscle cell differentiation in this study. The latter method affords sufficient sensitivity to detect mononucleated cells that have differentiated, but not fused. At a pharmacological concentration (10^{-6} M), insulin interacts with the type 1 IGF receptor and stimulates myogenic cell differentiation (43). Clonally derived porcine satellite cells undergo relatively little differentiation in the absence of exogenous insulin-like growth factors or pharmacological insulin concentrations (our unpublished observations). In the present study, testosterone ($\geq 10^{-8}$ M) depressed satellite cell differentiation induced by 10^{-6} M insulin. Decreased satellite cell differentiation in response to testosterone has not previously been reported. Although the effective dose of testosterone in these studies is somewhat higher than what may be expected based on the known affinities of AR for testosterone (21), it is likely that testosterone is being metabolized by porcine muscle satellite cells during these experiments. Cultured L6 myoblasts have been shown to metabolize testosterone to 5α-dihydrotestosterone, which is rapidly converted to 5α-androstane-3α,17β-diol in those myoblasts (44).

To our knowledge, a direct effect of testosterone on cultured myoblasts has been demonstrated in only one other report. In their study, Powers and Florini (20) demonstrated an increase in the thymidine labeling index of DNA in myoblasts exposed to 10^{-8} M testosterone. Myoblasts in that study were grown in medium containing 2% gelding serum, which has little mitogenic activity. Low concentrations of horse serum have since been reported to induce differentiation of a variety of cultured muscle cells (24, 45, 46). Although sensitive measures of myoblast differentiation were

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not readily available at the time of their report, the culture conditions used by Powers and Florini (20) may have been favorable for myoblast differentiation. In that case, an increased DNA labeling index may have resulted from a testosterone-induced decrease in myoblast differentiation. As differentiated muscle cells are incapable of DNA synthesis (1, 2), an apparent increase in the labeling index may reflect the number of cells capable of synthesizing DNA or those myoblasts that are undifferentiated. It is interesting to note that the only reported (20) effect of testosterone on the myoblast DNA labeling index (25% increase) is comparable to the decrease in satellite cell differentiation observed in the present study.

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 supported by the lack of effect of testosterone on the 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 the effects of testosterone on differentiation may also be sustained after treatment withdrawal. The mechanism(s) involved in the observed testosterone-induced decrease in differentiation are currently unknown. One possibility is that autocrine secretion of small quantities of a potent inhibitor of muscle cell differentiation, such as TGFβ (43), may be sufficient to depress differentiation of satellite cells while having no detectable effect on cell proliferation.

Delayed differentiation of myoblasts has been observed in several situations in which muscle mass is increased. Myoblasts from bovine double muscled fetuses (47) and satellite cells from heavily muscled commercial turkeys (48) differentiate later in culture than normal bovine myoblasts or satellite cells from lighter muscled Merriams turkeys, respectively. Additionally, delayed somite formation, expression of myogenic regulatory factors, and myosin heavy chain accretion have been observed in a heavy muscled quail line exhibiting increased myofiber number and increased DNA accretion (49). The current study demonstrates that increases in DNA accumulation and muscle mass observed in male animals may involve a testosterone-induced decrease or delay in satellite cell differentiation. This would result in a larger population of satellite cells capable of proliferation and subsequent differentiation. Furthermore, our results suggest that the effects of testosterone are AR mediated, although specific androgen-regulated genes in skeletal muscle satellite cells or myoblasts have not been identified.

In summary, we demonstrate that cultured satellite cells and myotubes possess AR, which are up-regulated in response to testosterone. Additionally, we provide evidence that testosterone decreases satellite cell differentiation, yet has no effect on satellite cell proliferation. Our data support a mechanism by which satellite cells are direct targets for androgen action, and androgen-associated increases in the population of proliferative satellite cells *in vivo* may be manifested at least in part by a reduction in cell differentiation.

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