

# The Ryanodine Receptor/Junctional Channel Complex Is Regulated by Growth Factors in a Myogenic Cell Line

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**Abstract.** The ryanodine receptor/junctional channel complex (JCC) forms the calcium release channel and foot structures of the sarcoplasmic reticulum. The JCC and the dihydropyridine (DHP) receptor in the transverse tubule are two of the major components involved in excitation-contraction (E-C) coupling in skeletal muscle. The DHP receptor is believed to serve as the voltage sensor in E-C coupling. Both the JCC and DHP receptor, as well as many skeletal muscle-specific contractile protein genes, are expressed in the BC3H1 muscle cell line. In the present study, we find that during differentiation of BC3H1 cells, induced by mitogen withdrawal, induction of the JCC and DHP receptor mRNAs is temporally similar to that of the skeletal muscle contractile protein genes  $\alpha$ -tropomyosin and  $\alpha$ -actin. Our data suggest that there is coordinate reg-

ulation of both the contractile protein genes (which have been studied in detail previously) and the genes encoding the calcium channels involved in E-C coupling. Induction of both calcium channels is accompanied by profound changes in BC3H1 cell morphology including the development of many components of mature skeletal muscle cells, despite lack of myoblast fusion. Visualized by electron microscopy, the JCC appears as "foot structures" located in the dyad junction between the plasmalemma and the sarcoplasmic reticulum of the BC3H1 cells. Development of foot structures is concomitant with JCC mRNA expression. Expression of the JCC and DHP receptor mRNAs and formation of the foot structures are inhibited specifically by fibroblast growth factor.

**I**N striated muscle, depolarization of the sarcolemma is coupled to the release of intracellular calcium from the sarcoplasmic reticulum (SR).<sup>1</sup> This coupling takes place across the triad junction, the intracellular junction between the transverse tubule and the terminal cisternae of the SR, joined by way of foot structures (Franzini-Armstrong and Nunzi, 1983). In the past several years, major progress has been made in the field of excitation-contraction (E-C) coupling (Fleischer and Inui, 1989). The foot structure has been identified as the ryanodine receptor and shown to be the calcium release channel of SR (Hymel et al., 1988; Inui et al., 1987a, b; Lai et al., 1988; Smith et al., 1988). The calcium release channel of SR represents a new class of intracellular channels characterized by their fourfold symmetry and large size (260 × 260 × 160 Å). Because the foot structure is involved in junctional association and contains the machinery for calcium release, it has been termed the junctional channel complex (JCC) (Wagenknecht et al., 1989). E-C coupling has been postulated to involve an interaction between the dihydropyridine (DHP) receptor in the transverse tubule/plasmalemma and the JCC (Rios and Brum, 1987).

The JCCs have been purified from both skeletal and cardiac muscle (Inui et al., 1987a, b; Lai et al., 1988; Smith et al., 1988). The protomer size and primary structure are available from cloning studies. Two isoforms of the JCC are known, one from skeletal muscle and another from cardiac muscle (Marks et al., 1989; Otsu et al., 1990; Takeshima et al., 1989; Zorzato et al., 1990). The JCC consists of a tetramer (Saito et al., 1988) comprised of four identical subunits each with a molecular weight of 565,000 (Marks et al., 1989; Otsu et al., 1990; Takeshima et al., 1989; Zorzato et al., 1990). The DHP receptor is a heteropolymer comprised of five subunits. The  $\alpha_1$ -subunit of the skeletal muscle DHP receptor has also been cloned and its primary structure determined (Tanabe et al., 1987). The essential role of the  $\alpha_1$ -subunit has been convincingly demonstrated in that transfection of this subunit restores E-C coupling in dysgenic mouse cells (Tanabe et al., 1988).

Myogenic differentiation is associated with coordinate regulation of the contractile proteins (Devlin and Emerson, 1978). The extent to which the genes encoding proteins required for E-C coupling (the JCC and DHP receptor) participate in this coordinate regulation has not been examined. BC3H1, a nonfusing muscle cell line derived from a mouse brain tumor (Schubert et al., 1974), has received considerable attention as a model for studying muscle cell differentia-

1. *Abbreviations used in this paper:* DHP, dihydropyridine; E-C, excitation-contraction; JCC, junctional channel complex; LSM, low serum medium; SR, sarcoplasmic reticulum.

tion and the regulation of muscle-related proteins. BC3H1 cells can be induced to differentiate by removal of growth factors from the culture medium or by allowing cultures to reach confluence. Under these conditions, BC3H1 cells express many of the striated muscle contractile protein genes (Munson et al., 1982; Olson et al., 1983; Taubman et al., 1989), as well as smooth muscle  $\alpha$ -actin (Strauch et al., 1986) and the nicotinic acetylcholine (Olson et al., 1984) and insulin receptors (Standaert et al., 1984). BC3H1 cells also express the skeletal muscle-type voltage-gated calcium channel (Caffrey et al., 1987). In addition, we have recently shown that the BC3H1 cells express the JCC (Marks et al., 1989).

In the present study we have examined growth factor regulation of the JCC, DHP receptor, and muscle-specific mRNAs in BC3H1 cells. Using electron microscopy we have demonstrated a correlation between the expression of these muscle-specific mRNAs and the appearance of foot structures and other muscle-specific proteins, as well as changes in BC3H1 cell morphology which occur during reversible differentiation in response to mitogen withdrawal and readdition.

## Materials and Methods

### Growth Factors

Mouse EGF (receptor grade), bovine FGF, and PDGF (50% pure) were from Collaborative Research, Inc. (Bedford, MA). PDGF B chain homodimer (recombinant *c-sis* product) was obtained from Amgen Biologicals, Inc. (Thousand Oaks, CA).

### cDNA Probes

The JCC probe used for all Northern blots, referred to as CRC 161 (Marks et al., 1989), corresponds to nucleotides 8,465–8,996 of the full-length JCC (Takeshima et al., 1989). The skeletal muscle DHP receptor probe is a 2-kb cDNA obtained by screening a rabbit skeletal muscle cDNA library (Marks et al., 1989) with a 570-bp cDNA obtained by polymerase chain reaction amplification of rabbit RNA using methodology essentially as described previously (Marks et al., 1990). This probe corresponds to nucleotides 2,500–4,500 of the previously described DHP receptor cDNA from rabbit skeletal muscle (Tanabe et al., 1987). The actin probe is pAC 269 from chicken skeletal muscle  $\alpha$ -actin (Fornwald et al., 1982). This probe recognizes both muscle specific (smooth, skeletal, and cardiac  $\alpha$ -actins) and nonmuscle ( $\beta$  and  $\gamma$ ) actin isoforms. The  $\alpha$ -tropomyosin probe is a cDNA encoding the entire coding region of the rat  $\alpha$ -tropomyosin (Ruiz-Opazo et al., 1985; Wieczorek et al., 1988). All probes were uniformly labeled with random primers using Klenow and  $\alpha$  P<sup>32</sup> dCTP to a sp act of 10<sup>9</sup> cpm per  $\mu$ g. (The actin and  $\alpha$ -tropomyosin probes were generous gifts from Dr. Bernardo Nadal-Ginard, Howard Hughes Medical Institute, Children's Hospital, Boston, MA).

### RNA Preparation and Northern Blot Analysis

RNA was prepared using standard guanidinium-isothiocyanate lysis buffer and centrifugation over a cesium chloride gradient. RNA was size separated on formaldehyde/agarose gels and Northern blot transfer was carried out overnight using 10 $\times$  SSC. Hybridization was at 42°C overnight and washing was at 55°C using 0.2 $\times$  SSC. Films were autoradiographed with a single intensifying screen at -70°C. To assure that equivalent amounts of RNA were present in each lane, Northern blots were also hybridized with cDNA encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from chicken muscle (data not shown). After autoradiography individual mRNA bands were quantitated using a laser densitometer. The optical density of each of the bands was divided either by the GAPDH or control sample band optical density. Each data point presented in Fig. 8 represents data from at least two and in most cases three separate experiments. Data are expressed as mean values. The statistical significances of differences in mean values between mRNA levels for induced BC3H1 cells and samples from deinduced

cells after treatment with growth factors was assessed by the unpaired Student *t*-test. The *P* < 0.05 level was considered to be significant.

### Cell Culture and Fixation

The BC3H1 mouse cell line (Schubert et al., 1974) was a gift from Dr. Eric N. Olson (Department of Biochemistry and Molecular Biology, University of Texas, M.D. Anderson Hospital and Tumor Institute). Cultures were grown on 10-cm tissue culture dishes in DME containing 20% FCS, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin as described (Olson et al., 1983). The medium was changed every 2 d. To minimize differentiation of BC3H1 and induction of contractile protein or JCC genes, cells were passaged while no more than 75% confluent. To initiate differentiation, medium containing 20% FCS was removed and replaced with low serum medium (LSM) containing 0.5% FCS. To determine the effects of FGF on the expression of JCC "foot structures" BC3H1 cells were cultured in growth medium (20% FCS) at low confluence (~30%) and switched to either LSM or LSM plus FGF (50 ng/ml). Samples were harvested after 3 and 5 d and prepared for electron microscopy as described below, and fresh FGF was added each day.

### Electron Microscopy

Monolayers were washed three times with PBS at room temperature and fixed for 15 min at room temperature in 2.5% glutaraldehyde, 1% tannic acid (Electron microscopic grade; Polysciences, Inc., Warrington, PA) in 0.1 sodium cacodylate, pH 7.2. Cells were then scraped with a rubber policeman and pelleted at 800 g for 5 min. Cells were resuspended in fresh glutaraldehyde-tannic acid cacodylate buffer, pH 7.2, and fixed for another 2 h at 4°C. Cells were washed twice with 0.1 M sodium cacodylate, pH 7.2, treated for 2 h at 4°C in 1% OsO<sub>4</sub> in 0.1 M sodium cacodylate, pH 7.2, and then washed twice with 0.1 M sodium cacodylate, pH 7.2 (Saito et al., 1978). All samples were block stained with 0.5% uranyl acetate in veronal-acetate buffer (pH 6.0) for 2 h at room temperature, dehydrated in a series of increasing ethanol concentrations, then the ethanol was replaced with propylene oxide, and the samples were embedded in Epon 812 (Electron Microscopy Sciences, Fort Washington, PA). Thin sections were cut on an LKB Ultratome (LKB Instruments, Inc., Rockville, MD), stained with 1% uranyl acetate in 50% ethanol for 5 min, counterstained with lead citrate for 5 min, and examined in a JEOL, JEM-100S electron microscope.

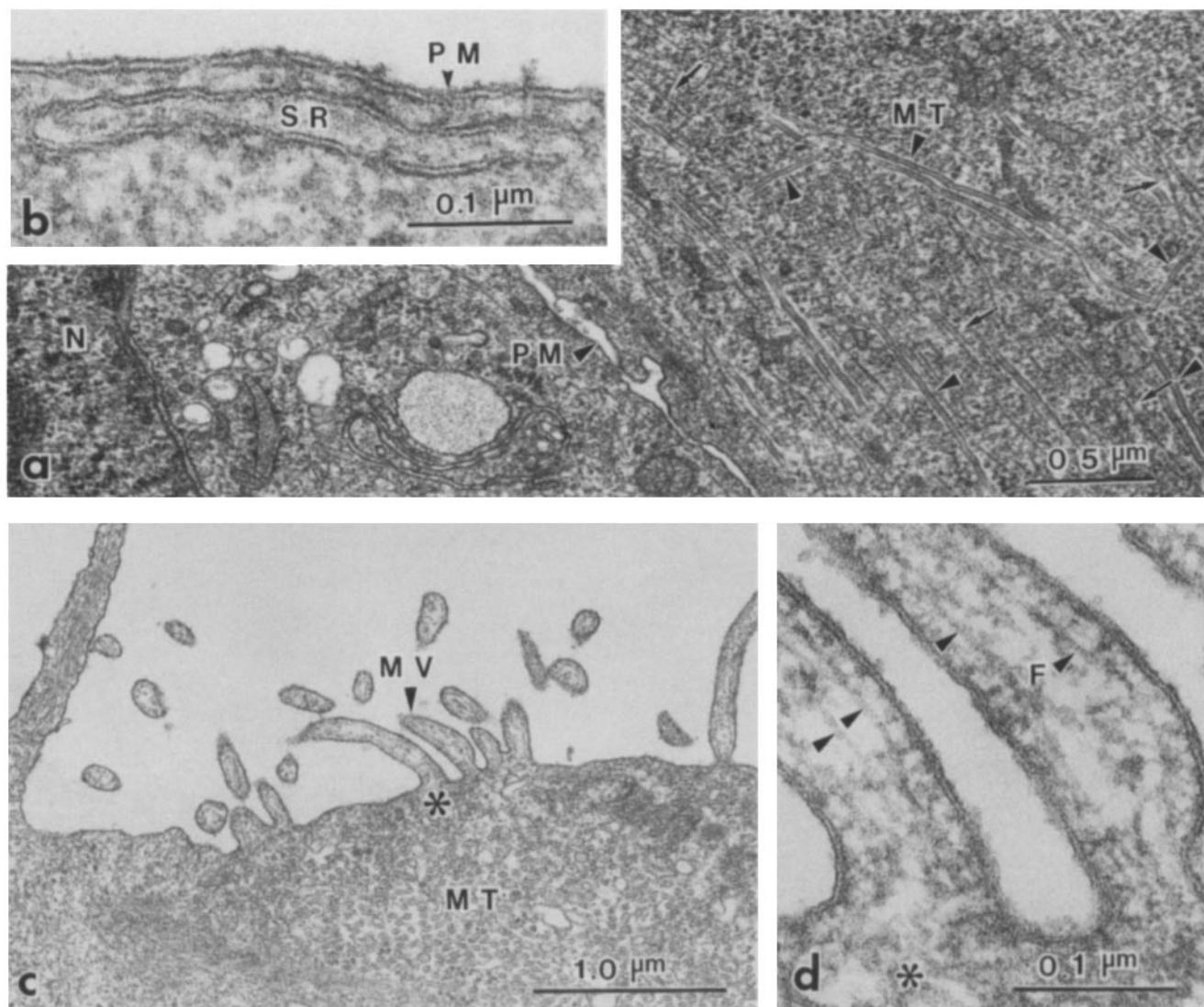
## Results

### Electron Microscopy of "Foot Structures" in Differentiating BC3H1 cells

BC3H1 cells, cultured in growth medium containing 20% FCS, had a typical flat, round-edged morphology with small processes extending from the cell as previously described (Schubert et al. 1974). When the cells were subjected to mitogen withdrawal (culturing in 0.5% serum [LSM]), there was a marked change in cell morphology. The cells became more elongated and spindly shaped. Differentiation to this "muscle phenotype" occurred within 3–5 d under these conditions. In this cell line, muscle filaments do not become oriented into striations typical of skeletal muscle and myotubes do not form.

As viewed by electron microscopy, undifferentiated, proliferating BC3H1 cells cultured in 20% FCS were devoid of myofibrils and foot structures (Fig. 1). Upon differentiation in LSM (Fig. 2) BC3H1 cells develop myofibrils as previously reported (Schubert et al., 1974). Unique to this study, we observed the development of foot structures at day 2. These structures became prominent by day 3 of serum deprivation (Fig. 2). Both myofibrils and foot structures appeared coordinately and were abundant by 5 d of culture in LSM (Fig. 2).

A feature of the BC3H1 cells is the ability to "deinduce" muscle-specific gene expression by readdition of growth fac-



**Figure 1.** Undifferentiated proliferating BC3H1 cells in 20% FCS; (a) A portion of the cell showing plasmalemma (PM), the nucleus (N), and rich proliferation of microtubules (arrowheads) and actin (thin) filaments (arrows). (b) Higher power showing the plasmalemma (PM) and sarcoplasmic reticulum (SR) apposed by a  $\sim 150$ -Å gap but devoid of foot structures. (c) Surface of the cells with characteristic microvilli (MV). Microtubules (MT) can be seen in cross-section. (d) Enlarged section near the surface from c as indicated by asterisk. The microvilli contain actin filaments (F; arrowhead).

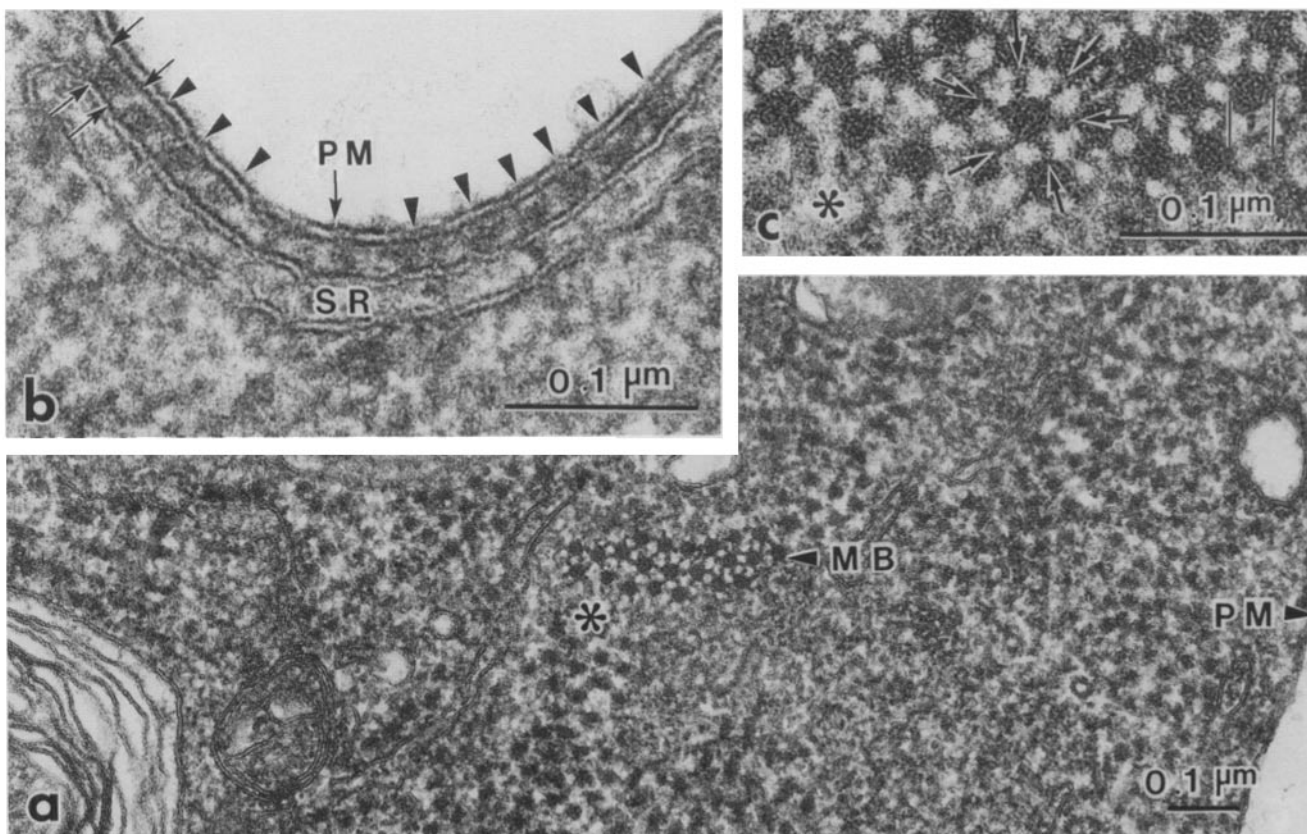
tors to induced monolayers, or by replating cultures in growth medium (Lathrop et al., 1985; Munson et al., 1982; Schubert et al., 1974; Taubman et al., 1989). This phenomenon of reversibility was also observed for the JCC structure. Differentiated cell cultures with the muscle phenotype were trypsinized and replated in 20% FCS at low density (10% confluence). As shown in Figs. 3–5, replating resulted in a progressive and ultimately complete loss of JCC and myofibrils by day 5 (Fig. 5).

#### Calcium Channel mRNA Accumulation in Differentiating BC3H1 Cells

To determine whether the induction and deinduction of the JCC structure visualized by electron microscopy mirrored the expression of JCC mRNA, a time course of JCC mRNA

accumulation was performed (Fig. 6 A). In subconfluent, proliferating BC3H1 cells, grown in medium containing 20% FCS, the JCC mRNA was not detected (Fig. 6 A, lane 1). JCC mRNA was detected 48 h after cells were switched to LSM (Fig. 6 A, lane 3). JCC mRNA levels continued to increase up to 7 d (Fig. 6 A, lane 6) and was reversible. When the fully induced cells were switched to growth medium containing 20% fetal calf serum, there was a rapid deinduction of the JCC mRNA within 24 h (Fig. 6 A, lane 7). This deinduction was also seen when the induced cells were replated at low density and allowed to grow for 48 h in medium containing 20% FCS (Fig. 6 A, lane 8). The induction and deinduction of the DHP receptor occurred with a time course similar to that seen for the JCC mRNA. (Fig. 6 B).

It has been previously shown that during BC3H1 differentiation the mRNA encoding the nonmuscle actin isoform ( $\beta$ -ac-



**Figure 2.** BC3H1 cells 5 d after being placed in LSM (0.5% FCS). (a) Muscle filaments showing myosin M bands (MB) can be observed throughout a cross-section. (b) Foot structures are now clearly visible (arrowheads) in the junction between the plasma membrane (PM) and apposed sarcoplasmic reticulum (SR), double arrows indicate the trapezoidal appearance of the foot structure. (c) Higher magnification of a section from a (asterisk) showing myosin M bands connected via bridges in a hexagonal array (arrows).

tin) is deinduced and the muscle-specific form ( $\alpha$ -actin) accumulates (Strauch et al., 1986). We compared the time course for the actin isoform switch in BC3H1 cells with that observed for the induction and deinduction of the JCC mRNA. The time course for the actin isoform switch is shown in Fig. 6 C. In subconfluent, proliferating cells (Fig. 6 C, lanes 1–3),  $\beta$ -actin (upper band) was the predominant form, but after 72 h of incubation in LSM (lane 4), there was a switch in actin isoforms, with  $\alpha$ -actin (lower band) predominating over  $\beta$ -actin. While the probe used in this study cannot distinguish between the three  $\alpha$ -actin isoforms, previous studies (Taubman et al., 1989) have shown that the smooth, cardiac, and skeletal muscle  $\alpha$ -actin isoforms are all induced by this protocol. The switch from  $\beta$ -actin to  $\alpha$ -actin was also reversed by addition of 20% fetal calf serum to the culture medium (Fig. 6 C, lane 7) or by replating cells at low density in growth medium (lane 8).

One of the earliest changes in contractile protein gene expression in BC3H1 cells is the switch from the nonmuscle to the skeletal muscle isoform of  $\alpha$ -tropomyosin (Taubman et al., 1989). We therefore compared the expression of JCC mRNA with that of  $\alpha$ -tropomyosin. As shown in Fig. 6 D (lanes 1–6), the time course for the switch from the nonmuscle (upper band) to the skeletal muscle (lower band) isoforms of  $\alpha$ -tropomyosin was similar to that seen for the induction and deinduction of the JCC and DHP receptor mRNAs (Fig. 6, A and B) and occurred concomitantly with the iso-

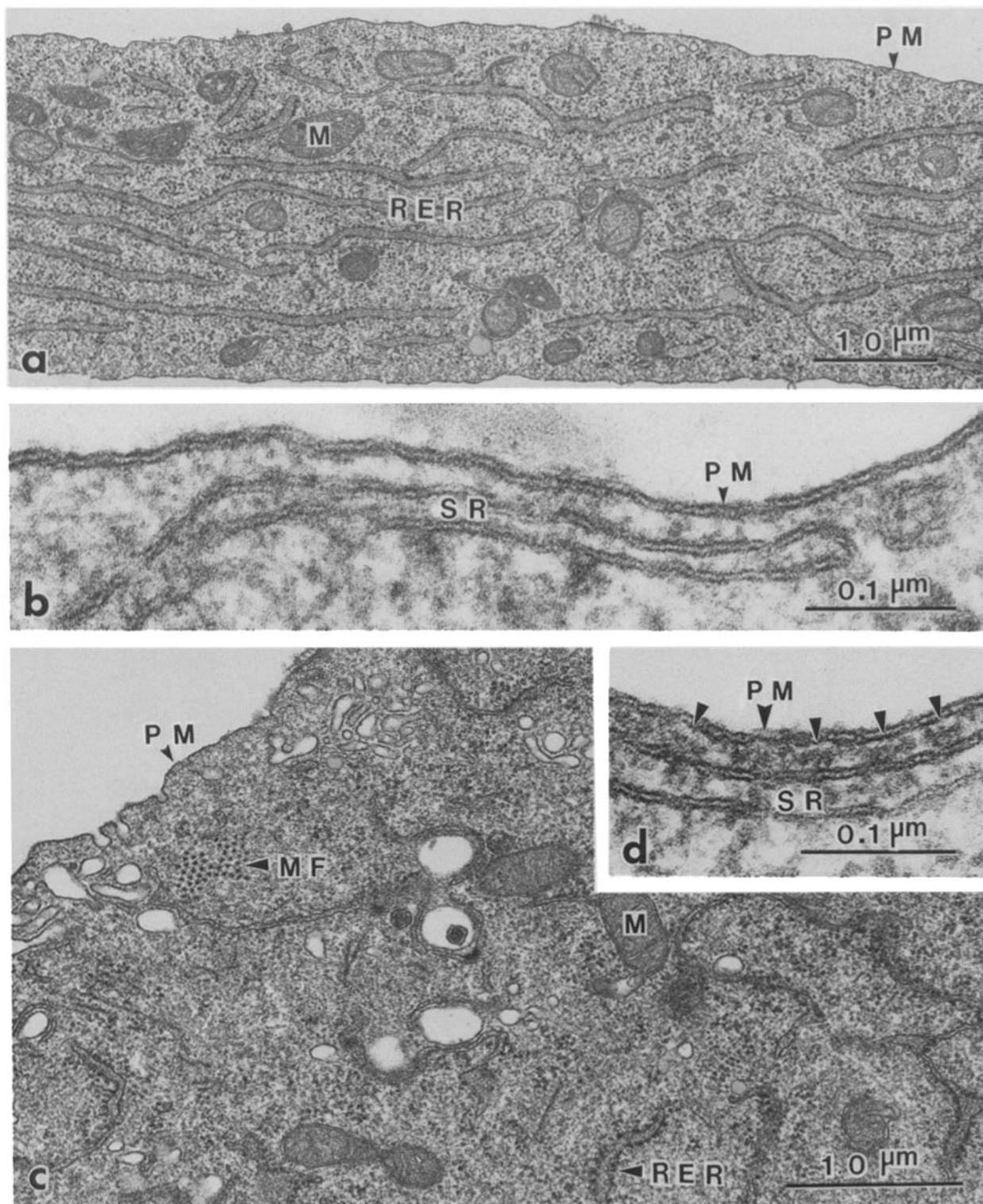
form switch observed for actin (Fig. 6 C). The isoform switch for  $\alpha$ -tropomyosin was also reversed by addition of 20% fetal calf serum to the culture medium (Fig. 6 C, lane 7) or by replating cells at low density in growth medium (lane 8).

#### Effects of Growth Factors on Calcium Channel Expression

It has been previously reported that either FGF or EGF can reverse the induction of some of the muscle specific genes in BC3H1 cells (Clegg et al., 1987; Lathrop et al., 1985; Vaidya et al., 1989; Wice et al., 1987). To determine whether the JCC mRNA was also responsive to these factors, BC3H1 cells were induced to differentiate by a 3-d incubation in LSM and then treated for either 1 or 3 d with EGF (300 ng/ml, Fig. 7 A, lanes 2 and 3); FGF (50 ng/ml, Fig. 7 A, lanes 4 and 5); or PDGF (5 half-maximal units, Fig. 7 A, lanes 6 and 7). As shown in Fig. 7 A, only FGF treatment for 3 d (Fig. 7 A, lane 5) caused deinduction of the JCC mRNA.

In addition to the JCC, the regulation of the expression of the skeletal muscle isoform of the DHP receptor was also examined. As shown in Fig. 7 B, lane 5, mRNA encoding the skeletal isoform of the DHP receptor was also downregulated specifically by FGF.

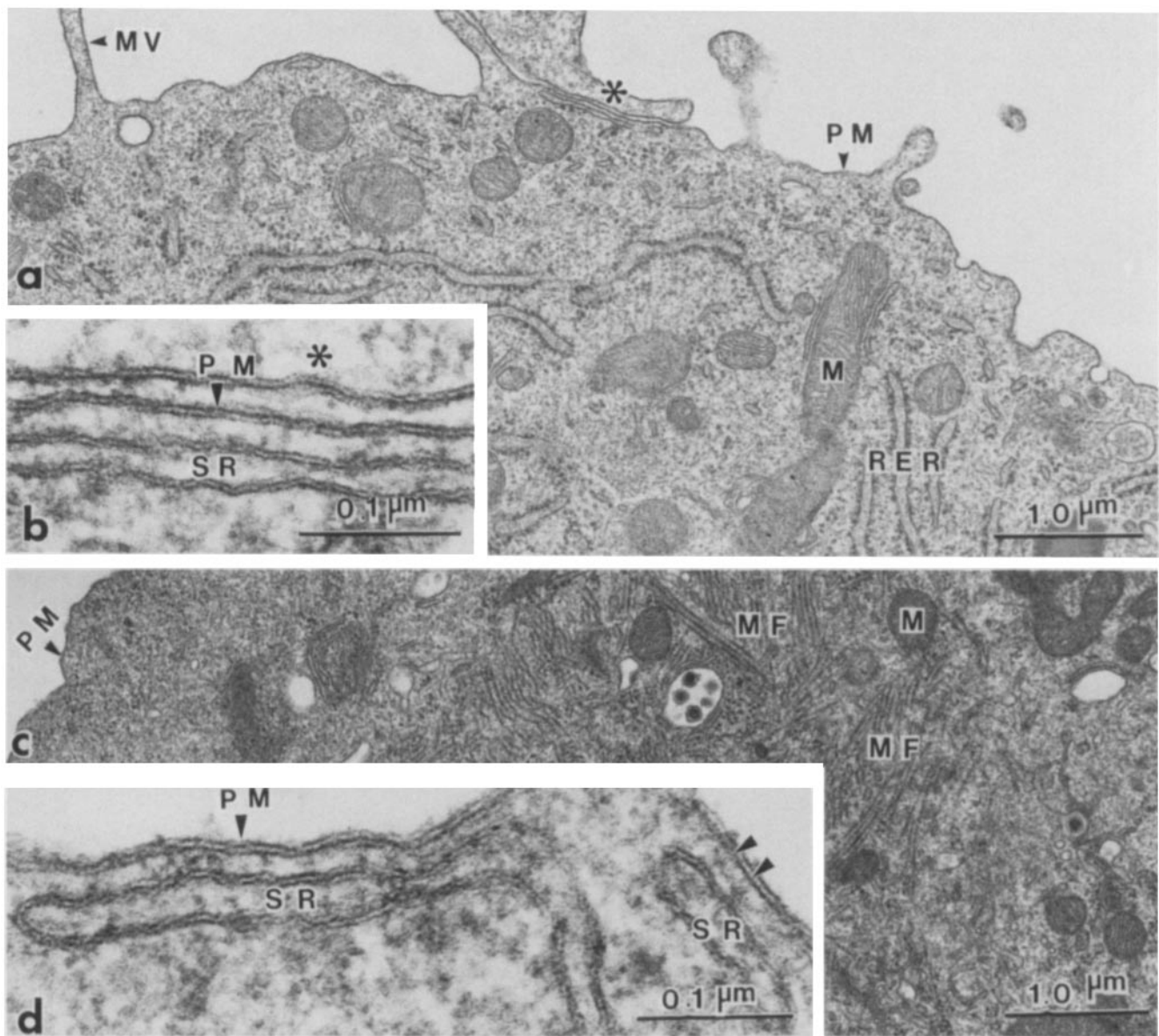
In contrast to the JCC and the DHP receptor,  $\alpha$ -tropomyo-



**Figure 3.** Replating of differentiated BC3H1 cells in 20% FCS-containing medium. After 1 d, two populations of cells are observed in *a* and *b* vs. *c* and *d*. (*a*) Cell that is devoid of myofibrils. (*b*) Foot structures are not detected between plasmalemma (*PM*) and apposed sarcoplasmic reticulum (*SR*). (*c*) Cell that retains some myofibrils (*MF*). (*d*) Foot structures are observed between plasmalemma (*PM*) and sarcoplasmic reticulum (*arrowheads*).

sin mRNA accumulation was not altered by FGF in BC3H1 cells under identical conditions. As shown in Fig. 7 C, lane 5, even after 72 h of FGF treatment, high levels of the skeletal isoform of  $\alpha$ -tropomyosin persisted without induction of

the nonmuscle isoform. This is in contrast to the results shown in Fig. 6 D, where the nonmuscle isoform of  $\alpha$ -tropomyosin was “reinduced” within 48 h of replating BC3H1 at low density in growth medium. Fig. 8 summarizes the data



**Figure 4.** 3 d after replating of differentiated BC3H1 cells in 20% FCS containing medium. Two populations are observed *a* and *b* vs. *c* and *d*. (*a*) Elongate cells with ruffled surface are devoid of muscle characteristics. (*b*) Surface membrane and SR are devoid of JCC. (*c*) Rounded cells with smooth surface plasma membrane retaining myofibrils. (*d*) Surface membrane and apposed SR. Most of the JCC are gone although there is the suggestion of remaining JCC (arrowheads).

on the effects of growth factors on the expression of the JCC and DHP receptor genes and the contractile protein genes in BC3H1 cells. Comparison of Fig. 8, *A–C* demonstrates the coordinate regulation of the JCC and DHP receptor genes and the genes encoding  $\alpha$ -tropomyosin and  $\alpha$ -actin. Fig. 8, *A* and *B* also demonstrate the downregulation of the JCC and DHP receptor genes by 20% FCS and specifically by FGF, but not by EGF or PDGF.

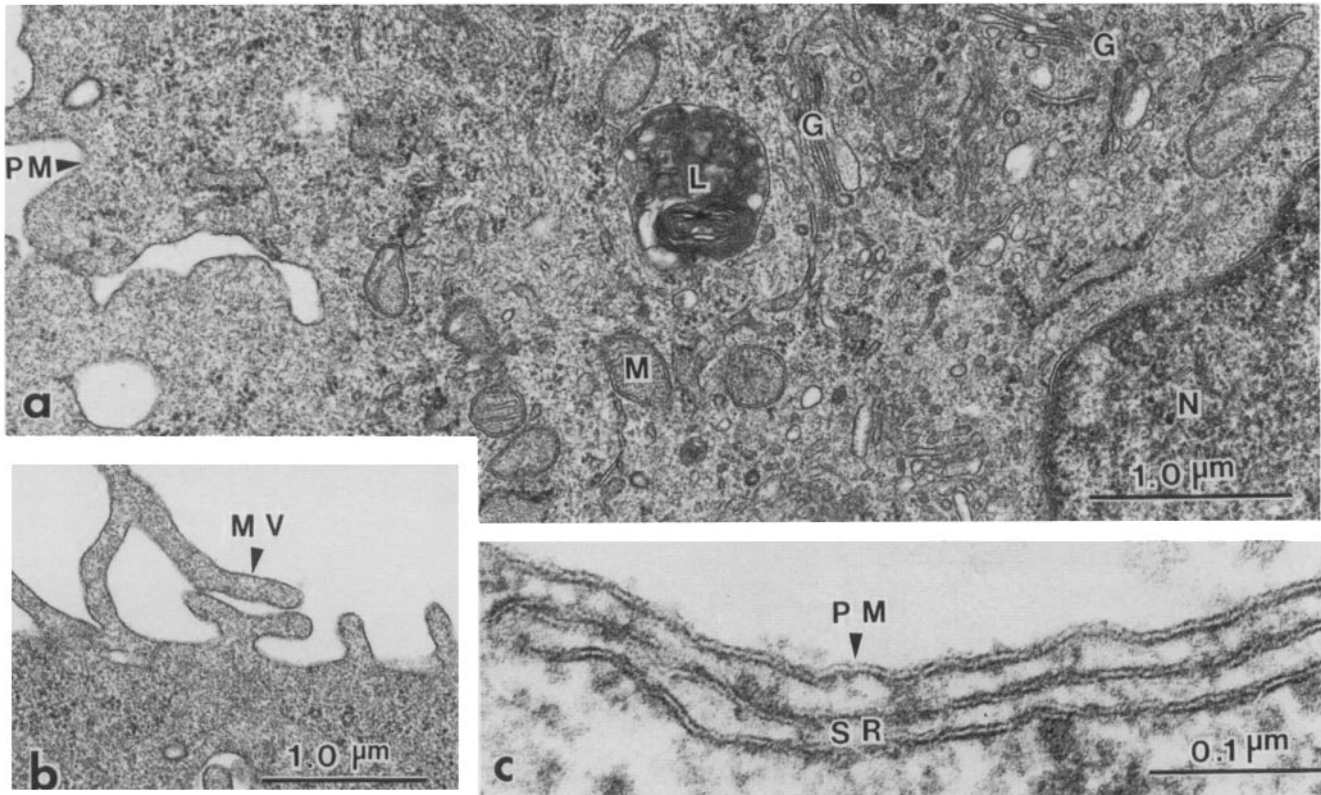
#### **FGF Blocks the Formation of Foot Structures**

We also examined the effects of FGF on the formation of foot structures induced by withdrawal of growth medium. Undifferentiated BC3H1 cells (grown in 20% FCS) were switched to LSM or LSM plus FGF (50 ng/ml). After 3 and 5 d, cells were fixed and stained for electron microscopy. Af-

ter 3 d cells cultured in LSM alone had well defined foot structures and by 5 d these cells appeared as shown in Fig. 2. However, treatment with FGF blocked the formation of foot structures at both 3 and 5 d (data not shown) while these same cells did exhibit other features of differentiation including myofibrils.

#### **Discussion**

The terminal cisternae of the SR, containing the JCC, and the transverse tubule containing the DHP receptor are apposed to one another in the triad junction of skeletal muscle. Both molecules are essential to calcium signalling and release processes and consequently to contraction in striated muscle (Fleischer and Inui, 1989; Tanabe et al., 1988). Dur-



**Figure 5.** 5 d after replating of differentiated BC3H1 cells into 20% FCS containing medium. (a) These cells are devoid of muscle filaments and JCC (see c). (b) Section close to the surface showing microvilli (MV). (c) The JCC is no longer observable between the plasmalemma and the SR.

ing E-C coupling, depolarization of the transverse tubule results in activation of the JCC with the subsequent release of intracellular calcium from stores within the SR. The availability of cDNA clones encoding the 16-kb mRNA of the JCC (Marks et al., 1989) and the skeletal isoform of the DHP receptor has made it possible to study the regulation of calcium channel gene expression. This study is the first to use these probes to correlate the induction/deinduction of the mRNA encoding the calcium channels involved in E-C coupling with the formation of the foot structure at the triad junction during muscle cell differentiation.

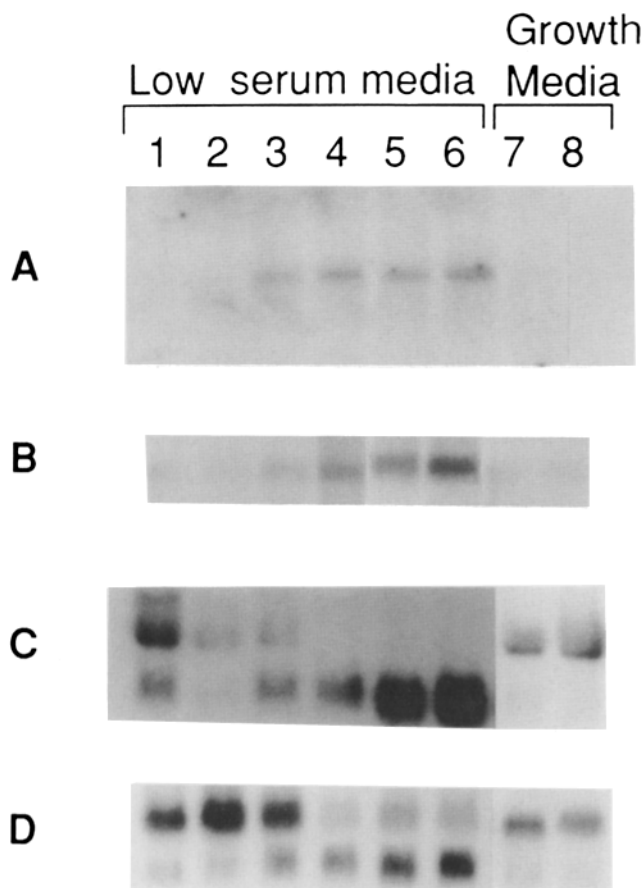
The present study demonstrates a correlation between the expression of key receptors involved in E-C coupling (JCC and DHP receptor), the contractile protein genes and profound changes in cell morphology. We find that FGF, but not EGF or PDGF, regulates the expression of the calcium handling genes. More interestingly, addition of FGF also blocks the formation of foot structures in cells induced to differentiate by serum withdrawal, while other elements of differentiated muscle cells are expressed (including myofibrils). Thus, inhibition of the formation of the foot structures required for excitation-contraction coupling appears to be an important effect of FGF in BC3H1 cells.

Changes in cellular morphology in response to growth factor withdrawal were examined using electron microscopy (Figs. 1–5). In the undifferentiated BC3H1 cells, the SR and plasmalemma are associated, although foot structures are not visualized. 2 d after growth factor withdrawal, myofibrils appear and the beginnings of foot structures are also appar-

ent. By 5 d, the BC3H1 cells have well-developed muscle filaments and the SR is associated with the plasmalemma via foot structures spanning the gap. The foot structure in the junction of the BC3H1 cells is characteristic in appearance of that associated with the terminal cisternae of the SR in the triad junction of skeletal muscle (Saito et al., 1988). In the case of the JCC, the protomer is targeted to the junctional face membrane of the SR, associates to form a foot structure, and then associates with the plasmalemma in a manner suggesting the ability to be functionally coupled.

In response to serum withdrawal, both the induction of foot structures (Figs. 1–5), and the JCC and DHP receptor mRNAs (Fig. 6, A and B, lane 3) occur within 48 h in proliferating BC3H1 cells. Deinduction of the foot structure (Figs. 3–5) and the JCC and DHP receptor mRNAs (Fig. 6, A, lanes 7 and 8) occur within 2 d. This rapid turnover of the 16-kb JCC and 7.5-kb DHP receptor mRNAs and the JCC protein with a molecular mass of 2.3 million indicates that the JCC gene and protein and the DHP receptor gene are highly regulated *in vitro*. Moreover, the regulation is coordinate with that of the contractile protein genes and the DHP receptor.

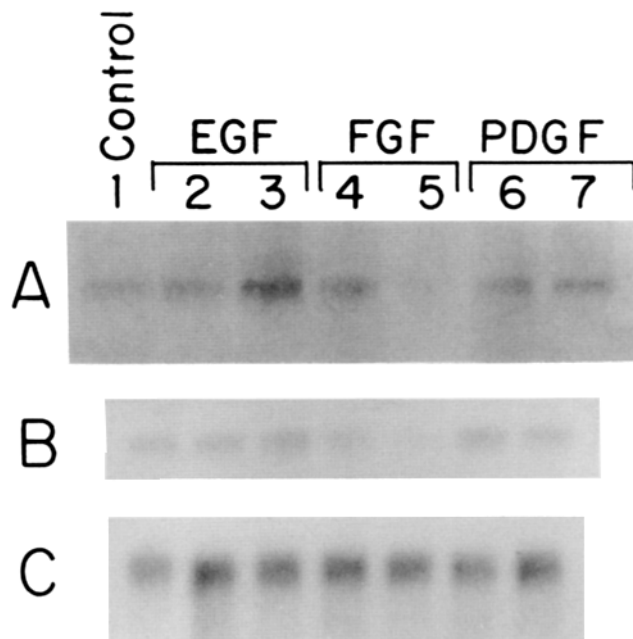
FGF has been observed to inhibit skeletal muscle differentiation (Clegg et al., 1987; Lathrop et al., 1985; Wice et al., 1987). We have found that FGF deinduces both the JCC and DHP receptor (Figs. 7 and 8, A and B). Under the same conditions, FGF has no detectable effect on the expression of  $\alpha$ -tropomyosin (Figs. 7 and 8 C). The mRNAs encoding the skeletal muscle myosin light chains 1 and 3 and troponin T



**Figure 6.** Northern blot analyses showing the time course for induction and deinduction of muscle-specific mRNAs in BC3H1 cells (See Materials and Methods for description of the cDNA probes used): (A) ryanodine receptor/JCC; (B) DHP receptor; (C) actin; and (D)  $\alpha$ -tropomyosin. BC3H1 cells were cultured in growth medium (20% FCS) until 50% confluent (lane 1) and then were switched to LSM (0.5% FCS). Cells were maintained in LSM for 1 (lane 2), 2 (lane 3), 3 (lane 4); 4 (lane 5); and 7 d (lane 6). At day 7 the cells were switched to growth medium (20% FCS) and samples were harvested at 24 h (lane 7), or replated at low density and harvested after 48 h, (lane 8). The upper band in C represents  $\beta$ -actin mRNA, the lower band,  $\alpha$ -actin. The upper band in D represents the nonmuscle  $\alpha$ -tropomyosin isoform mRNA, the lower band, the muscle-specific isoform of  $\alpha$ -tropomyosin mRNA.

are likewise unresponsive to FGF (data not shown). This suggests that in BC3H1 cells the effects of FGF on myogenesis are coordinate with blockade of calcium channel expression and may be distinct from its effects on muscle-specific gene expression. Alternatively the half-life of the muscle-specific contractile protein mRNAs may be significantly longer than those of the calcium channels and that the time points sampled (1 and 3 d after growth factor treatment) are too early to see an effect on the contractile protein gene expression.

The mechanism of FGF blockade of myogenic differentiation requires further study. Growth-stimulating hormones which involve tyrosine kinases and allow cells to pass from  $G_0$  to  $G_1$  are associated with an increase in *c-myc* levels (Coughlin et al., 1985). FGF transiently increases *c-myc* levels in BC3H1 but constitutive expression of *c-myc* does not



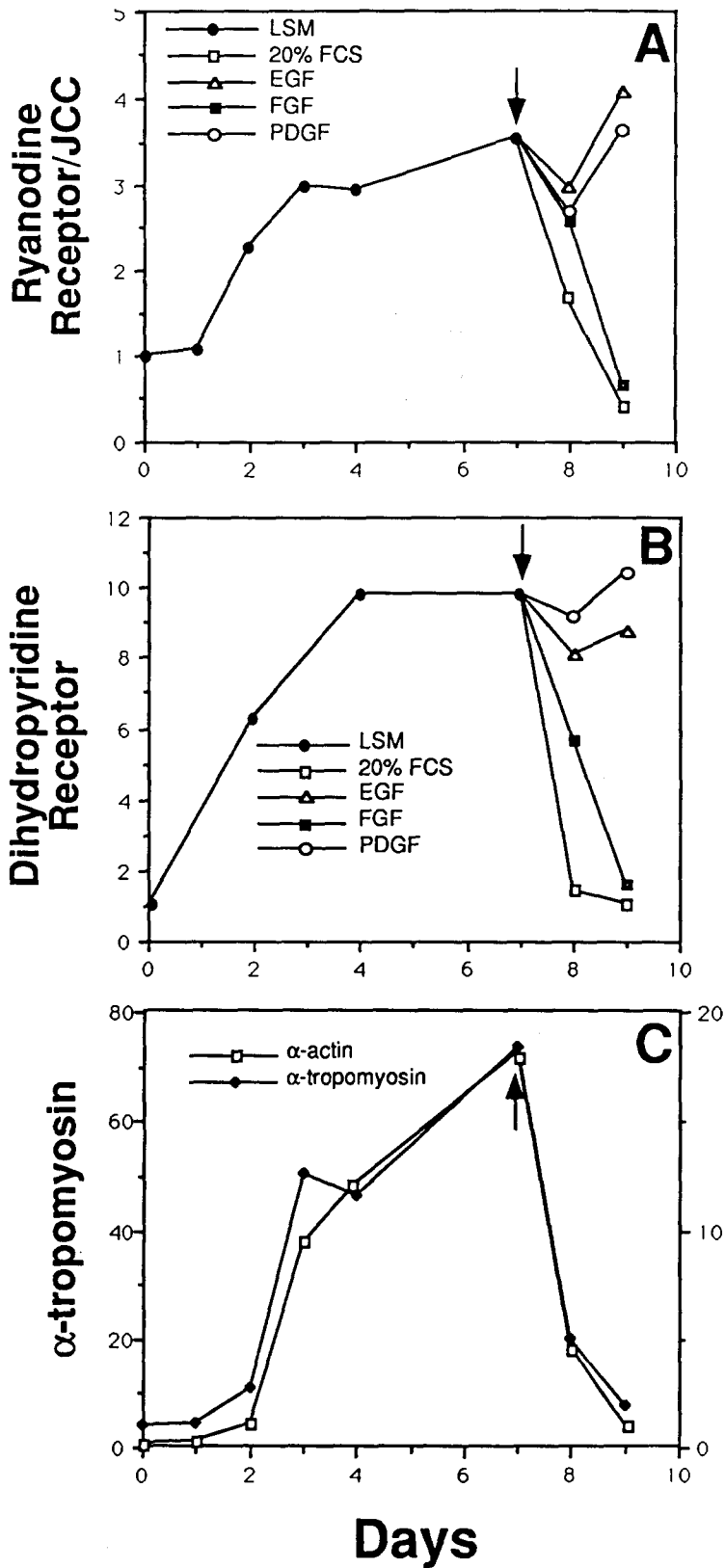
**Figure 7.** Northern blot analyses showing the deinduction of (A) the ryanodine receptor/JCC, (B) DHP receptor, and (C)  $\alpha$ -tropomyosin mRNAs in BC3H1 cells by growth factor. Lane 1, induced cultures (grown in LSM for 3 d); lanes 2 and 3, duplicate cultures treated with 300 ng/ml EGF for 1 and 3 d, respectively; lanes 4 and 5, duplicate cultures treated with 50 ng/ml FGF for 1 and 3 d, respectively; lanes 6 and 7, duplicate cultures treated with PDGF (5 half-maximal units of 50% human PDGF) for 1 and 3 d, respectively. Similar results to those in lanes 6 and 7 were obtained with 20 ng/ml recombinant PDGF B-chain homodimer. These results are representative of three separate experiments.

fully block muscle cell differentiation (Schneider et al., 1987). Constitutive expression of the muscle-specific factor MyoD1 cDNA is unable to block the inhibiting effects of FGF on myogenic differentiation (Konieczny et al., 1989). However, MyoD1 constitutively expressed in the presence of the related muscle-specific factor myogenin promotes differentiation and fusion in BC3H1 cells (Brennan et al., 1990). Thus MyoD1 and myogenin appear to function cooperatively to regulate myogenesis (Vaidya et al., 1989). The mechanism of FGF inhibition of muscle cell differentiation thus appears to be multifactorial.

Our data also suggests that FGF's effects on myogenic differentiation are multifactorial and involve regulation specifically of the formation of foot structures, and the calcium handling genes, in addition to direct effects on the muscle-specific factors myogenin and MyoD1. In the rat myogenic cell line  $L_6E_9$ -B, translation of muscle-specific genes is calcium dependent (Endo and Nadal-Ginard, 1987). The effect of FGF on the calcium-handling genes may alter intracellular calcium concentrations and such changes in calcium might act as signals for subsequent events controlling cellular differentiation. Thus, in addition to directly regulating transcriptional events leading to myogenic differentiation, another role for FGF may include indirect regulation of muscle-specific mRNA translation via its effects on regulation of calcium-handling genes.



# mRNA Levels (arbitrary units)



**Figure 8.** Effects of growth factors on JCC and DHP receptor gene expression in differentiated BC3H1 cells. BC3H1 cells were cultured in growth medium (20% FCS) until 50% confluent and then were switched to LSM (0.5% FCS) at day 0. Cells were maintained in LSM for 7 d. At day 7 (arrow) cells were switched either to growth medium (20% FCS) or to LSM plus growth factors (EGF, FGF, or PDGF as indicated). For both  $\alpha$ -tropomyosin and  $\alpha$ -actin no decrease in mRNA levels was seen with addition of any of the growth factors (data not shown). Each data point represents at least two and in most cases three separate experiments. Deinduction of the JCC, and DHP receptor mRNA levels, compared to fully induced levels (at day 7 after switching to LSM), by either 20% FCS or FGF, was significant at  $P < 0.05$  level.

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