Regulation of Proliferation and Differentiation of Myoblasts Derived from Adult Mouse Skeletal Muscle by Specific Isoforms of PDGF

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Abstract. The expression of receptors and the mitogenic response to PDGF by C2 myoblasts, derived from adult mouse skeletal muscle, was investigated. Employing 125I-PDGF binding assays, we showed that the cells exhibit high level binding of PDGF-BB (≈165 × 10^3 molecules/cell at saturation) and much lower binding of the PDGF-AA and PDGF-AB (6–12 × 10^3 molecules/cell at saturation). This indicates that the C2 myoblasts express high levels of PDGF receptor β-subunits and low levels of α-subunits. PDGF-BB enhances the proliferation of C2 cells maintained in 2% FCS by about fivefold. PDGF-AB had a moderate effect on cell proliferation (less than twofold) and PDGF-AA had no effect. Inverse effects of PDGF isoforms on the frequency of differentiated myoblasts were observed; the frequency of myosin-positive cells was reduced in the presence of PDGF-BB while PDGF-AA and PDGF-AB had no effect. PDGF may thus act to increase the number of myoblasts that participate in muscle regeneration following muscle trauma by stimulating the proliferation and by inhibiting the differentiation of myogenic cells.

Throughout embryogenesis of skeletal muscle, myogenic precursors proliferate and eventually give rise to terminally differentiated, fusion-capable myoblasts. During postnatal growth, the myogenic precursor cells are still in their proliferative phase (Moss and Leblond, 1971) but in adult muscle, these cells are mitotically quiescent (Schultz et al., 1978). The myogenic precursor cells in the adult, which are often referred to as satellite cells, can reinitiate proliferative activity after muscle injury, and their progeny fuse into multinucleated fibers (Snow, 1977; Mauro, 1979; reviewed in Campion, 1984). Adult satellite cells also become mitotically active upon culturing, displaying a program of proliferation and differentiation similar to that of embryonic myoblasts (Bischoff, 1974; Yablonka-Reuveni et al., 1987). Studies on adult-derived myoblasts have identified several physiological growth factors that could regulate the quiescent and proliferative states of satellite cells. These factors include FGF, insulin-like growth factors, and transforming growth factor β (Allen et al., 1984; Allen and Boxhorn, 1989; Bischoff, 1986; Clegg et al., 1987; Jennische et al., 1987; DiMario and Strohman, 1988; reviewed in Florini and Magri, 1989).

PDGF has been implicated as an important mediator of wound healing and tissue repair (reviewed in Ross et al., 1986), but its role during skeletal muscle regeneration has not been fully investigated. PDGF is a potent mitogen for many mesenchymal cells including fibroblasts and smooth muscle cells (reviewed in Ross et al., 1986; Heldin and Westermark, 1989) and was recently also shown to play a role in the proliferation and differentiation of specific cells in the glial cell lineage (Hart et al., 1989; reviewed in Heldin and Westermark, 1989). PDGF can also elicit other biological responses, including increased metabolic rates and chemotaxis (reviewed in Ross et al., 1986; Heldin and Westermark, 1989). Two homodimeric forms of PDGF (AA and BB) and one heterodimeric form (AB) have been purified from mammals and are functionally active (Bowen-Pope et al., 1989). Although PDGF was initially isolated from platelets and megakaryocytes, it is now well established that other cell types are also capable of producing this growth factor (reviewed in Ross et al., 1986; Heldin and Westermark, 1989). There are at least two separate PDGF receptor proteins which differ in their specificities for the two chains of PDGF (Hart et al., 1988; Heldin et al., 1988). According to the model proposed by Seifert et al. (1989), the two different receptor proteins function as subunits (designated α and β) which dimerize to form three distinct receptors (αα, αβ, and ββ) (Seifert et al., 1989; Heldin et al., 1989; Hammacher et al., 1989). PDGF-AA can only bind to the αα receptor, PDGF-AB can bind to both αα and αβ receptor, and PDGF-BB can bind to all three receptor subunit combinations: αα, αβ, and ββ.

In this study, we analyzed the effect of the three PDGF dimers on the myogenic cell line C2, which was derived from normal adult mouse muscle (Yaffe and Saxel, 1977) and is presumably derived from satellite cells. We chose to study a cell line rather than primary cells in order to avoid the complexity introduced by fibroblast-like cells which are present
in primary cultures and are capable of binding PDGF (Yablonska-Reuveni et al., 1988, 1990; manuscript in preparation). We used the C2 cell line because it has been demonstrated to be able to participate in muscle regeneration in vivo (Partridge et al., 1988). Nevertheless, we recognize that the behavior of the C2 cells in vitro may not completely reproduce the behavior of the satellite cells in vivo.

We were able to demonstrate significant binding of all three PDGF dimers, with a much higher number of PDGF-BB molecules bound at saturation compared to PDGF-AA and PDGF-AB. PDGF-BB also significantly promoted cell proliferation and depressed the frequency of differentiated myoblasts. PDGF-AB had only a small effect on cell proliferation, and no effects of PDGF-AA could be documented. PDGF-BB may thus play a role during muscle regeneration by increasing the number of proliferating myoblasts available for repair following muscle trauma.

**Materials and Methods**

**Cell Culture**

C2 cells, originally isolated from adult mouse skeletal muscle (Yaffe and Saxel, 1977), were kindly provided by D. Yaffe (The Weizmann Institute of Science, Rehovot, Israel). Cells were maintained at 37.5°C in a temperature-saturated air containing 5% CO2, using gelatin-coated, 100-mm tissue culture dishes. Proliferation medium consisted of MEM (Gibco Laboratories, Grand Island, NY) containing 20% FCS (HyClone Laboratories, Logan, UT), 0.5% chicken embryo extract, penicillin, and streptomycin at 105 U per liter each. It should be noted that the cells can differentiate in this medium when they reach very high cell density. For subculturing, sparse cells were exposed to 1.25% trypsin (Gibco Laboratories) for ~30 min at 37°C. Cells were then mechanically triturated, filtered through lens paper to eliminate cell aggregates (Yablonska-Reuveni and Namenoff, 1987), counted in a hemocytometer, and further cultured as described below.

**PDGF Binding and Competition Assays**

Highly purified PDGF-AB free of contaminating PDGF-AA or PDGF-BB was kindly provided by E. Raines and R. Ross (Department of Pathology, University of Washington) and was prepared as previously reported (Raines and Ross, 1982; Seifert et al., 1989). Highly purified recombinant PDGF-AA and PDGF-BB were produced from yeast as previously described (Bowen-Pope et al., 1989; Seifert et al., 1989). These recombinant molecules were kindly provided by M. Murray and J. Forstrom (ZymoGenetics, Seattle, Washington). Radioiodination of the three PDGF forms, saturation binding of 125I-PDG-F to the cells, and competition assays were performed as previously described (Bowen-Pope et al., 1989; Seifert et al., 1989).

C2 cells used for the assays were passaged into gelatin-coated, 24-well trays (well diameter = 16 mm) at a density of 5 × 104 cells/well and maintained for 1–2 d in proliferation medium until ~70% confluent. About 16–20 h before assaying for PDGF binding, medium was changed to MEM containing 2% FCS and antibiotics. This was done in order to reduce the amount of PDGF in the medium, which could bind to the PDGF receptors before the binding assay. C2 cells maintained in low serum differentiate earlier than cells in proliferation medium (detailed in Results); however, at the time of the binding assays over 98% of the cells were not differentiated, and no myotubes were observed. For binding measurements, cultures were exposed at 4°C for 3 h to increasing amounts of 125I-PDG-F in binding buffer (Ham’s medium F-12 buffered at pH 7.4 with 25 mM HEPES and supplemented with 0.25% BSA). Cultures were then rinsed with cold binding buffer, and cell-bound 125I-PDG was solubilized with 1% Triton X-100 containing 0.1% BSA and measured with a gamma counter. Nonspecific binding of PDGF, determined by preincubating cells with an excess of unlabeled PDGF (100 ng/ml), was subtracted to determine the amount of specifically bound label. Cell numbers were determined on wells that were treated as those for binding measurements. Cells were trypsinized and counted using an electronic particle counter. All measurements were done on triplicate wells. For competition binding, the cells were rinsed once with cold binding buffer and then incubated in 1 ml of binding buffer containing increasing amounts of unlabeled PDGF-AA or PDGF-BB, followed by incubation with binding buffer containing 2 ng/ml 125I-PDG-F-BB. Cells incubated with 2 ng/ml 125I-PDG-BB only were used to determine control binding. Measurement of nonspecific binding and further analysis were as described for binding assays.

**H-thymidine Incorporation**

To assay the effect of PDGF on proliferation, cells were passaged into gelatin-coated, 24-well trays and treated, unless noted otherwise, at a density of 105–2.5 × 106 per well. Cells were maintained for 1 d in proliferation medium and then received a medium consisting of 2% FCS plus antibiotics in MEM (2% FCS medium). Control wells received no further additives, whereas experimental wells received either PDGF-AA at 20 ng/ml, PDGF-AB at 5 ng/ml, or PDGF-BB at 20 ng/ml. To ensure ample supply of PDGF, the medium and additives were replaced every 24 h. At daily intervals (before the next medium change), cells were pulsed for 2 h with 1 μCi/ml 3H-thymidine (3H-TdR; New England Nuclear, Boston, MA specific activity 6.7 Ci/mmol). At the end of the labeling period, cells were rinsed 3× with ice-cold MEM. This was followed by the addition of 1 ml of 5% ice-cold TCA, in which the cells were maintained overnight at 4°C. Cells were then rinsed 2× with ice-cold 5% TCA and dissolved in 0.5 ml of 0.5 N NaOH at 37°C. Plates were then subjected to vigorous rotating for 15–30 min to ensure complete detachment of cells, and the NaOH suspensions were transferred into scintillation vials. Radioactivity was measured using 10 ml of ScintiVerse II (Fisher Scientific Co., Pittsburgh, PA). All measurements were made on triplicate wells.

**Detection of Terminally Differentiated Myoblasts**

Cells were cultured on gelatin-coated, 35-mm dishes, employing proliferation medium, and then exposed to 2% FCS medium with or without PDGF, as described above for 3H-TdR incorporation. Cells were initiated at a density of 5 × 105 cells per dish. Terminally differentiated cells were detected employing indirect immunofluorescence as previously described (Yablonska-Reuveni and Namenoff, 1987; Yablonska-Reuveni et al., 1987) and using the mAb MF20 which is specific for sarcomeric myosin. This antibody, which was originally prepared by Bader et al. (1982) and discussed in further publications (Zadeh et al., 1986; Saad et al., 1987), was obtained from the Developmental Studies Hybridoma Bank (maintained by a contract from NICHD (NOI-HD-6-2915)). Growth medium of hybridoma-producing MP20, the source of the antibody, was diluted 1:5 in 0.05 M Tris, 0.9% NaCl, 1% normal goat serum, pH 7.4. The secondary antibody was fluorescein-labeled goat anti-mouse IgG (Organon Teknika-Cappel, Malvern, PA) and was diluted to 1:50. After the antibody reaction, nuclei were stained with ethidium bromide and counted, employing fluorescence microscopy to determine total cell numbers (including cells that fused into myotubes) and the proportion of terminally differentiated cells (including cells that fused into myotubes). Nuclei were labeled by exposing cultures for 5 min to two to three drops of ethidium bromide (2 μg/ml). Cultures were then rinsed extensively with 0.05 M Tris, 0.9% NaCl, 0.05% Tween 20, pH 7.4, and received a final rinse with 0.05 M Tris, pH 7.4. Cultures were mounted with 90% glycerol containing antifading agent (Yablonska-Reuveni et al., 1987) and observed with a Zeiss photomicroscope equipped for epifluorescence. The bright red fluorescence of the nuclei could be visualized alone using the rhodamine filters or simultaneously with the antibody stain, employing the fluorescein filters. Ten random fields per 35-mm dish were analyzed, using duplicate dishes.

**Results**

**C2 Myoblasts Express Different Numbers of Binding Sites for the Three PDGF Isoforms**

Using 125I-PDG-F, we determined the saturation binding curves for the three different PDGF dimers, as shown in Fig. 1. The saturation binding of PDGF-AA is 12 × 105 bound molecules per cell, that of PDGF-AB is 6.5 × 105 bound molecules per cell, and that of PDGF-BB is 165 × 103 bound molecules per cell. Although high-affinity binding of all three dimers is detected, the number of PDGF-BB binding sites for repair following muscle trauma.
According to the PDGF receptor model proposed by Seifert et al. (1989), PDGF-BB can bind to all three receptor types, whereas PDGF-AA can only bind to one receptor type (αα). This model predicts that the portion of PDGF-BB binding to αα and αβ receptors can be eliminated by incubation with PDGF-AA to occupy the α-subunits. This allows us to use a competition binding protocol to test whether most of the PDGF receptors consist of ββ dimers, as implied by the results of the saturation binding. The results of such competition assays (Fig. 2) indicate that PDGF-AA competes for <20% of the 125I-PDGF-BB binding sites and confirm that most of the PDGF receptors present on C2 cells are ββ receptors.

PDGF-AA, PDGF-AB, and PDGF-BB Differentially Affect the Proliferation of C2 Myoblasts

Mitogens contained in proliferation medium could obscure the specific effects of PDGF isoforms on myoblast proliferation. Therefore, to analyze such effects, we maintained the cells in 2% FCS medium to reduce the mitogens contributed by the basal medium. Incorporation of 3H-TdR after 24 h in this medium is reduced by about three- to fourfold and is further reduced by five- to sixfold by 48 h compared to cultures grown in proliferation medium (data not shown). Fusion into myotubes occurs in cultures maintained in the 2% FCS medium earlier than in cultures maintained in proliferation medium, even though cells in 2% FCS medium are less crowded (Fig. 3, a and c). However, differentiation of C2 cells in the 2% FCS is cell density dependent and can be delayed by reducing cell density. Therefore, to minimize differentiation while studying proliferation, cultures were initiated with low cell density as described under Materials and Methods, unless otherwise noted.
Figure 3. Effect of serum and PDGF-BB on proliferation and fusion of C2 cells. Cells were cultured at 5 x 10^3 cells per 35-mm dish in proliferation medium. After 24 h, medium was replaced with fresh proliferation medium (a and b), 2% FCS medium (c and d), and 0.25 mg/ml BSA medium (e and f). Only cultures b, d, and f received 20 ng/ml PDGF-BB. Medium, without or with PDGF, was replaced every 24 h thereafter. Representative phase contrast micrographs of live cultures were taken 96 h after initial culturing. Bar, 30 μm.

A summary of the effect of PDGF isoforms on ³H-TdR incorporation is shown in Fig. 4. In these experiments, cultures were initiated with standard cell density (2.5 x 10^4 cells per well), and the three PDGF isoforms were added at the time that cultures were exposed to the 2% FCS. During the first day in 2% FCS, no differences in ³H-TdR incorporation were observed in the proliferation of control cells or cells that received PDGF. However, during consecutive days in culture, the effect of PDGF-BB on cell proliferation became clear: proliferation increased by over twofold on the second day of culture and by almost fivefold on the third day, compared to control cultures. PDGF-AB had a small effect on cell proliferation and enhanced proliferation by 1.2- and 1.5-fold on the second day and the third day of culture, respectively. No effect of PDGF-AA on the level of proliferation could be documented under these conditions.

We could not document any significant effect of PDGF-BB on ³H-TdR incorporation when added to C2 cells maintained in 20% FCS (for morphological appearance of cells maintained in 20% FCS without or with PDGF-BB, see Fig. 3, a and b). However, the effect of PDGF-BB on the proliferation of C2 cells was not limited to cells maintained in 2% FCS. A very dramatic response to this isoform was observed when C2 cells were maintained in MEM containing only 0.25 mg/ml BSA (Fig. 3, e and f). Under these conditions, PDGF-BB increased the cell number and resulted in a 3.5-4.5-fold increase in ³H-TdR incorporation in cultures maintained in BSA plus PDGF-BB, compared to cultures maintained in BSA alone (data not shown). Most of the cells in cultures lacking PDGF were bipolar (presumably, differentiated cells) or fused into small, multinucleated myotubes. Cultures that received PDGF-AA morphologically resembled cultures lacking PDGF. Cultures that received PDGF-AB were intermediate in cell number and morphology (data...
Figure 4. Effect of the different isoforms of PDGF on 3H-TdR incorporation. Cells were cultured at 2.5 x 10³ cells/16-mm well in proliferation medium for 24 h, followed by an exposure to 2% FCS in MEM for various lengths of time in the absence or presence of the different PDGF isoforms. PDGF-AA, PDGF-AB, and PDGF-BB were added at 20, 5, and 20 ng/ml, respectively. The 2% FCS medium with or without PDGF was replaced every 24 h. 2 h before cell harvesting, the cultures were exposed to 3H-TdR at 1 μC/ml, and the extent of 3H-TdR incorporation into TCA precipitable material was determined. Plotted values represent the average of triplicate wells. Standard error was <5%.

For further studies, we used 2% FCS medium and not BSA medium since many cells detached (presumably died) in the BSA medium, making it unsuitable for longer term studies of proliferation and differentiation.

The Manifestation of PDGF-BB Effect on Proliferation Is Cell Density Dependent

In the experiments described in Fig. 4, the effect of PDGF-BB on cell proliferation was not observed until the second day in 2% FCS. This could mean that the 2% FCS medium contains enough PDGF or other mitogens to support the initial cell proliferation, but that when cells increase in numbers, they deplete the endogenous mitogen(s), and only at this stage would the effect of exogenous PDGF be detected. To test this possibility, we examined 3H-TdR incorporation in response to PDGF in cultures initiated with high and low cell densities. As shown in Fig. 5, when starting cell densities were higher than those described for Fig. 4, the effect of PDGF-BB was already apparent after 36-48 h in 2% FCS. By contrast, the effect of the added PDGF-BB was delayed even further (to day 4) in experiments where the cultures were initiated with fewer cells (Fig. 6). This supports the notion that 2% FCS can sustain proliferation of sparse cells but that PDGF becomes limiting when cells reach higher densities and deplete endogenous PDGF or other mitogens.

Table 1. Frequency of Myosin-positive Cells in C2 Cultures Treated with PDGF

| PDGF added | 48 h in 2% FCS | 72 h in 2% FCS |
|------------|---------------|---------------|
|            | Total nuclei  | Nuclei in myosin + cells | % Differentiation | Total nuclei  | Nuclei in myosin + cells | % Differentiation |
| None       | 812           | 65             | 8.0            | 906           | 278             | 30.6            |
| AA         | 720           | 55             | 7.6            | 872           | 292             | 33.4            |
| AB         | 889           | 70             | 7.8            | 983           | 269             | 27.3            |
| BB         | 949           | 29             | 3.0            | 1231          | 126             | 10.2            |

Cells were fixed at indicated time points and identification of differentiated cells was done immunohistochemically, using an antibody against sarcomeric myosin. Nuclei within myosin-positive cells (and multinucleated myotubes) and myosin-negative cells were visualized using ethidium bromide. Nuclear counts were made on duplicate 35-mm dishes, screening 10 random fields per dish.
The Frequency of Differentiated Myoblasts Is Reduced in the Presence of PDGF-BB

Various studies have indicated that mammalian myoblasts differentiate in response to reducing the serum concentration in the growth medium (i.e., Yaffe, 1973; Yaffe and Saxel, 1977; Clegg et al., 1987; Fig. 3, this study). Our preliminary studies indicated that C2 cultures maintained in 2% FCS in the presence of PDGF-BB exhibit fewer myotubes than in the absence of the factor (Fig. 3, c and d). To further investigate the effect of PDGF on C2 cells, we analyzed the frequency of terminally differentiated cells in control and PDGF-treated cultures maintained in 2% FCS medium. Cultures were initiated in proliferation medium then shifted to 2% FCS medium with or without PDGF as for ^H-TdR incorporation assays. Differentiated cells were detected with an antibody against sarcomeric myosin (MF20). Total cell numbers, the number of differentiated mononucleated cells, and the number of cells fused into multinucleated fibers were determined by counting nuclei. A summary of this study is shown in Table I. In all cases, PDGF-BB increased the total number of nuclei and decreased the number of nuclei in differentiated cells.

Discussion

Our studies with the C2 myogenic line derived from adult mouse skeletal muscle demonstrate that these myoblasts express receptors for PDGF and that PDGF exerts a mitogenic effect on these cells. Of the three known PDGF dimers, the BB isoform has the greatest number of binding sites on the C2 myoblasts and is the most potent in promoting ^H-TdR incorporation and in reducing the frequency of terminally differentiated myoblasts. Whereas PDGF-AA can bind to the cells, the number of binding sites is much lower than that of PDGF-BB and no mitogenic effect can be demonstrated. PDGF-AB binds to the C2 cells at a level similar to that of PDGF-AA and exerts a very slight mitogenic effect on the cells. According to the model proposed by Seifert et al. (1989), PDGF-AA can only bind to the αα receptor, PDGF-AB can bind to the αα and the αβ receptors, and PDGF-BB can bind to all three receptor combinations: αα, αβ, and ββ. The saturation binding experiments indicate that the C2 cells express much higher levels of β than α-receptor subunits. This is supported by the competition experiment (Fig. 2) which demonstrates that the α-subunit accounts for <20% of total receptor subunits. Other adult cell types also express many more β- than α-subunits and respond much better to PDGF-BB than to PDGF-AA (e.g., see Seifert et al., 1989). Whether this difference in response results from quantitative differences in signalling through the different receptor proteins or merely reflects quantitative differences in level of receptor subunit expression is not yet clear.

The saturation binding experiments demonstrate that C2 cells maintained in 2% FCS for 16–20 h express receptors to PDGF-BB. However, the response to PDGF-BB, as measured by ^H-TdR incorporation, is delayed beyond 24 h in cultures initiated at low cell densities. This delay in response may reflect the presence of enough mitogens in 2% FCS to support the initial cell proliferation but that proliferation becomes dependent on exogenous PDGF when these mitogens have been depleted by more crowded cells. In contrast to the stimulation of proliferation, the number of differentiated myoblasts is reduced in the presence of PDGF-BB. Little or no differentiation occurs during the first 24 h, even in the absence of PDGF. As cells become more crowded, mitogens become limiting, and increasing numbers of myoblasts differentiate and exit the cell cycle in the absence of PDGF. In the presence of PDGF, fewer cells differentiate, and more cells are available for the next round of cell division. If PDGF operates by suppressing differentiation rather than by promoting proliferation, then another mitogen needs to be present in sufficient amounts in the mitogen-depleted cultures in order to promote proliferation. It is possible that PDGF-BB functions both as a mitogen and as a suppressor of cell differentiation, and the effect of PDGF becomes pronounced through the combined effects of decreased frequency of withdrawal from the cell cycle and increased number of undifferentiated cells available for the next round of cell division.

The binding of PDGF to skeletal muscle cells and the response of these cells to PDGF has been studied previously, but only briefly. Linkhart et al. (1982) mentioned that the mouse myoblast cell line MM14, which, like the C2 cell line, was derived from adult muscle, did not respond to PDGF (primarily AB isoform). More recent studies on these MM14 myoblasts indicate that they do not bind PDGF-BB (Bowen-Pope and Olwin, unpublished results). Bischoff (1986), using isolated intact muscle fibers containing satellite cells in their native position under the basement membrane, concluded that the addition of PDGF did not activate the proliferation of quiescent satellite cells. This study did not investigate the effects of PDGF following the initial activation of satellite cells, and employed PDGF-AB, which is also a poor mitogen for C2 cells. By contrast, studies of cultured avian myoblasts have indicated a potential role for PDGF during certain stages of myogenesis. Venkatasubramanian and Solursh (1984) showed that PDGF (the AB type) can induce a migratory response in a myogenic cell population from avian limb buds. We have previously demonstrated a significant binding of PDGF-AB to myogenic cultures from adult chicken skeletal muscle but not to such cultures from 10 day embryos (Yablokova-Reuveni et al., 1988, 1990). Thus, it is possible that myoblasts from different developmental and physiological stages respond differently to PDGF. Another intriguing possibility is that PDGF, which is a chemoattractant for a variety of cells (Seppä et al., 1982; Grotendorst et al., 1982; reviewed in Ross et al., 1986), as well as for cells in avian limb buds (Venkatasubramanian and Solursh, 1984), may affect the migration of satellite cells. Such migration from regions distal to the injury would be an additional source for myoblasts during regeneration (Mauro, 1979; Schultz et al., 1988; Watt et al., 1987).

In summary, the experiments presented in this study using a rodent satellite cell-derived cell line provide the first direct evidence that PDGF can regulate myoblast proliferation and differentiation in vitro. It is thus possible that PDGF has a role in increasing the number of myoblasts during skeletal muscle regeneration by stimulating proliferation and/or inhibiting differentiation.

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Note Added in Proof. Jin et al (1990) have recently reported similar observations using rat myoblasts.

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