Expression of PDGF A-Chain and β-Receptor Genes during Rat Myoblast Differentiation

Pei Jin,* Magnus Rahm,* Lena Claesson-Welsh,‡ Carl-Henrik Heldin,‡ and Thomas Sejersen*

*Department of Medical Cell Genetics, Medical Nobel Institute, Karolinska Institutet, S-104 01 Stockholm, Sweden; and ‡Ludwig Institute for Cancer Research, Biomedical Center, S-751 23 Uppsala, Sweden

Abstract. L6J1 rat myoblasts and rat skeletal muscle were studied for expression of mRNAs encoding PDGF A-chain, PDGF B-chain, PDGF α-receptor, and PDGF β-receptor during in vitro and in vivo myoblast differentiation. RNA blot hybridizations demonstrated expression of the PDGF A-chain gene and the PDGF β-receptor gene in L6J1 myoblasts and in crude muscle tissue isolated from developing rats. Transcripts of the PDGF A-chain were identified at all examined stages of in vitro and in vivo myogenic differentiation. Expression of the PDGF β-receptor gene decreased in differentiated myotubes of L6J1 cells and in rat adult muscle tissue. Receptor binding assays demonstrated specific binding of PDGF-BB, but not -AA, to exponentially proliferating L6J1 myoblasts and to terminally differentiated L6J1 myotubes. The binding per cell nucleus was higher in exponentially proliferating myoblasts than in differentiated L6J1 myotubes. In serum free medium PDGF-BB was shown to increase c-*fos* protooncogene immunoreactivity in L6J1 myoblasts. In the presence of 0.5% FCS, PDGF-BB increased DNA synthesis in L6J1 myoblasts, while PDGF-AA showed no such effect. Differentiation, as monitored by myotube formation, was reduced in PDGF-BB–treated cultures. The possible role of PDGF in myoblast proliferation and differentiation is discussed.

MYOGENESIS is the differentiation of proliferating skeletal myoblasts to form contractile, multinucleated myotubes (for review, see Pearson and Epstein, 1982). This process involves cessation of DNA synthesis, cell surface changes, cell–cell recognition of G_0 myoblasts and fusion of such cells into giant, multinucleated myotubes that synthesize contractile proteins typical for skeletal muscle. In the developing rat, this process occurs mainly at the time of birth (Yaffe, 1973). Already in 10-d-old rats, the majority of myoblasts have fused into myotubes. Thereafter, muscle growth occurs mainly from enlargement of preexisting myotubes.

When myoblasts become confluent in culture, DNA synthesis is inhibited (Yaffe, 1971), and RNA synthesis is reduced (Yaffe and Fuchs, 1967). Several studies have suggested a causal relation between terminal myogenic differentiation and regulation of DNA synthesis in myoblasts. Deprivation of mitogens brings about precocious myogenic differentiation (Nadal-Ginard, 1978), and stimulation of DNA synthesis in mouse myoblasts by fibroblast growth factor delays the onset of myotube formation (Linkhart et al., 1980; Ewton and Florini, 1981). A variant mouse myoblast cell line responds by proliferation and delayed fusion to treatment with epidermal growth factor as well (Lim and Hauschka, 1984), and more recently, type β transforming growth factor was also shown to inhibit myogenic differentiation (Massagué et al., 1986). On the other hand, glucocorticoids and members of the insulin family, such as insulin, insulin-like growth factors I and II stimulate both proliferation and differentiation of rat myoblasts (Guerriero and Florini, 1980; Ewton and Florini, 1981).

To study further the role of mitogens in myoblast proliferation and differentiation, we analyzed the expression of PDGF genes and PDGF receptor genes during myogenesis. PDGF was originally observed in platelets as a cationic 30-kD protein with potent mitogenic effects on several mesenchymal cell types, such as fibroblasts, glia cells, and smooth muscle cells (Heldin et al., 1985; Ross et al., 1986). Structurally, PDGF is a dimer of two polypeptide chains linked by disulfide bonds. The PDGF polypeptides are encoded by the PDGF A-chain gene and the PDGF B-chain gene (c-*sis*). PDGF has been identified in all three possible isoforms; AA, AB, and BB (Stroobant and Waterfield, 1984; Heldin et al., 1986; Hammacher et al., 1988). The PDGF isoforms were recently shown to bind with different affinities to two distinct types of cell surface receptors (Claesson-Welsh et al., 1988; Escobedo et al., 1988; Gronwald et al., 1988; Hart et al., 1988; Heldin et al., 1988; Claesson-Welsh et al., 1989; Matsui et al., 1989). The PDGF α-receptor (also called A-type receptor) binds all three forms of PDGF with high affinity, while the PDGF β-receptor (also called B-type receptor) binds PDGF-BB with high affinity and PDGF purified from human platelets with lower affinity, but does not bind PDGF-AA. We have previously demonstrated that secondary rat

embryo myoblasts and L6J1 rat myoblasts cultured in vitro express the PDGF A-chain gene, and that L6J1 myoblasts secrete PDGF, most likely in the form of PDGF-AA (Sejersen et al., 1986*a*). We now present further results on the expression of PDGF A- and B-chains and PDGF α- and β-receptors during myogenesis in the developing rat and in L6J1 cells undergoing spontaneous in vitro myogenic differentiation. Effects of PDGF on c-*fos* protooncogene expression, DNA synthesis, cell proliferation, and myogenic differentiation are also presented.

Materials and Methods

Cell Cultures

Myoblasts of the cell line L6J1 (Yaffe, 1968; Ringertz et al., 1978) were cultured in DME, supplemented with 5% FCS, as described previously (Sejersen et al., 1986*a*). Medium was changed two or three times per week. For induction of myogenic differentiation, myoblasts were seeded at 6×10^3 cells/cm^2 in 60-cm^2 dishes and cultured for 10 d without replating. For analysis of the effect of PDGF on myoblast differentiation, cells were seeded in 20-cm^2 dishes for 24 h, before being switched to serum poor medium (DME/0.5% FCS) with or without 25 ng/ml PDGF-BB. PDGF-BB was added to the culture on a daily basis for ten consecutive days, and the medium was changed every second day.

For analysis of myoblast proliferation, cells were plated at 4×10^3 cells/cm^2 in 7-cm^2 wells in DME/5% FCS. After serum deprivation for 4 d, myoblasts were cultured in 0.5% FCS with or without 25 ng/ml of PDGF-BB. Cell numbers were counted each day in triplicate wells using an automatic cell counter (VDA 140; Analys Instrument AB, Sweden).

Determination of Myoblast Fusion

Cultures to be scored for cell fusion were washed with PBS, fixed with methanol, and stained with giemsa. The percentage of nuclei in myotubes was estimated by counting 1,200–1,500 nuclei/plate in 8–10 fields chosen at random. Only structures containing three or more nuclei were scored as representing fused cells.

Rat Skeletal Muscle Isolation

Crude muscle tissues were isolated from thighs of Sprague-Dawley rats of the following ages: 15-, 17-, 18-, and 20-d-old rat embryos; 1-, 7-, 14-, and 21-d-old rats, and from a 5-mo-old (adult) rat. Whole hind legs of 15- and 17-d-old rat embryos were used. For comparative purposes, whole 13-d-old rat embryos were used. Isolated tissues were minced by scissoring, immediately frozen and kept in liquid nitrogen until RNA extraction. Crude muscle preparations may contain a small fraction of nonmuscle cells, mainly fibroblasts.

Purification and Blot Analysis of Poly(A)$^+$ RNA

Purifications of total RNA, selections of poly(A)$^+$ RNA, gel fractionations, RNA blottings, RNA hybridizations, washing procedures, and determinations of sizes of transcripts and intensities of hybridization signals were performed essentially as described earlier (Sejersen et al., 1986*b*; Rahm et al., 1989). Concentrations of poly (A)$^+$ RNA were measured and normalized by both ethidium bromide-agarose plates and ultraviolet spectrophotometry.

DNA Probes

The following probes were used: the 1.3-kb cDNA fragment of human PDGF A-chain clone Dl (Betsholtz et al., 1986), human c-*sis* clone pSM-1 (Clarke et al., 1984), and human c-*sis* clone PDGF-B-17 (Betsholtz, C., unpublished observations), the 1.5-kb cDNA fragment of human PDGF α-receptor clone 15.3 pUC (Claesson-Welsh et al., 1989), the 1.0-kb Pst I cDNA fragment of human PDGF β-receptor (Claesson-Welsh et al., 1988), Pst I DNA fragments of mouse fast myosin heavy chain MHC 32 (Weydert et al., 1983), mouse α-actin pAM 91 (Minty et al., 1981), and chicken glyceraldehyde-3-phosphate dehydrogenase cDNA clone pGAD-28 (Dugaiczyk et al., 1983).

PDGF

PDGF-AA (short form) and PDGF-BB were recombinant human forms purified from a *Saccharomyces cerevisiae* expression system (Östman et al., 1989), and were used for studies of PDGF binding and DNA synthesis. PDGF-AA and PDGF-BB were ^{125}I-labeled using the Bolton and Hunter protocol (Bolton and Hunter, 1973) to specific activities of 13,000 (PDGF-AA) and 21,000 (PDGF-BB) cpm/ng.

Porcine PDGF-BB (Boehringer Mannheim GmBh, FRG) was used for studies of c-*fos* induction, cell proliferation, and cell differentiation.

Receptor Binding Assays

Binding experiments were performed on L6J1 myoblasts cultured in 2-cm^2 wells. Each well contained ~70,000 myoblasts or 500,000 nuclei of myotubes. Cells, with or without pretreatment of 500 μg/ml of suramin (Bayer AG, Wuppertal, FRG) for 30 min at 4°C, were rinsed with binding buffer (PBS containing 0.1% BSA, 0.9 mM $CaCl_2 \times 2H_2O$, and 0.8 mM $MgSO_4 \times 7H_2O$). Myoblast cultures were then incubated for 3 h at 4°C in 0.5 ml binding buffer containing 4 ng/ml of ^{125}I-labeled PDGF isoforms, with or without 300 times excess of the corresponding unlabeled ligand. Cultures were thereafter washed five times in ice-cold binding buffer. Myoblasts with associated ^{125}I-radioactivity were lysed by incubation in 0.5 ml of lysis buffer (1% Triton X-100/10% glycerol/20 mM Tris, pH 7.4) for 30 min at room temperature, and radioactivity was determined in a gamma spectrometer (1282 CompuGamma, LKB Wallac, Sweden). L6J1 cell numbers were determined in parallel cultures by counting giemsa-stained cell nuclei using a Zeiss light microscope.

c-fos Immunofluorescence Assay

c-*fos* immunofluorescence assay was performed as described previously (Rahm et al., 1989). xfos-3 rabbit polyclonal antibodies against amino acid residues 2–17 of human/mouse p^{55} c-*fos* (Hunt et al., 1987), provided by Dr. G. Evan, were used at 1:500 dilution in PBS/0.5% Triton X-100. For specific blocking of c-*fos* immunofluorescence xfos-3 antibodies were preincubated for 3 h at room temperature with an excess of synthetic peptides covering amino acid sequence 2–17 of human/murine c-*fos* (OP-11-3210; Cambridge Research Biochemicals, UK). The nuclear fluorescence was visualized using a Zeiss ultraviolet microscope, and the optical density of the fluorescence was scanned using a Zeiss/Kontron IBAS 2000 interactive image analysis computer.

DNA Synthesis Assay

Fractions of cells undergoing DNA synthesis were measured by studying incorporation of bromodeoxyuridine (BrdU)[1] in proliferating myoblasts. L6J1 myoblasts were seeded on coverslips and serum-deprived in DME/0% FCS for 4 d. Thereafter, 10 μM of BrdU was added, with or without various concentrations of PDGF-AA or -BB, for 24 h. After fixation in 70% ethanol for 10 d at 4°C, cells were treated with 0.07N NaOH and 0.01M $Na_2B_4O_7$ (pH 8.5) for 2 min. Thereafter, mouse monoclonal anti-BrdU antibodies (Becton Dickinson Immunocytometry Systems, Mountain View, CA) were applied for 30 min at room temperature, followed by rhodamine-conjugated anti-IgG antibodies (Dakopatts a/s, Denmark), diluted 1:10 in PBS, for 15 min at room temperature. Coverslips were mounted in PBS/glycerol (1:1), and fractions of BrdU-incorporating nuclei were determined counting 500–1,000 nuclei in randomly selected fields of vision, using a Zeiss ultraviolet microscope.

Results

PDGF A-Chain mRNA Expression in Differentiating Rat Myoblasts

PDGF A-chain gene expression has previously been reported in L6J1 myoblasts and in primary culture of rat embryo skeletal myoblasts (Sejersen et al., 1986*a*). To elucidate further the expression pattern of the PDGF A-chain gene in

1. *Abbreviations used in this paper*: BrdU, bromodeoxyuridine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MHC, myosin heavy chain.

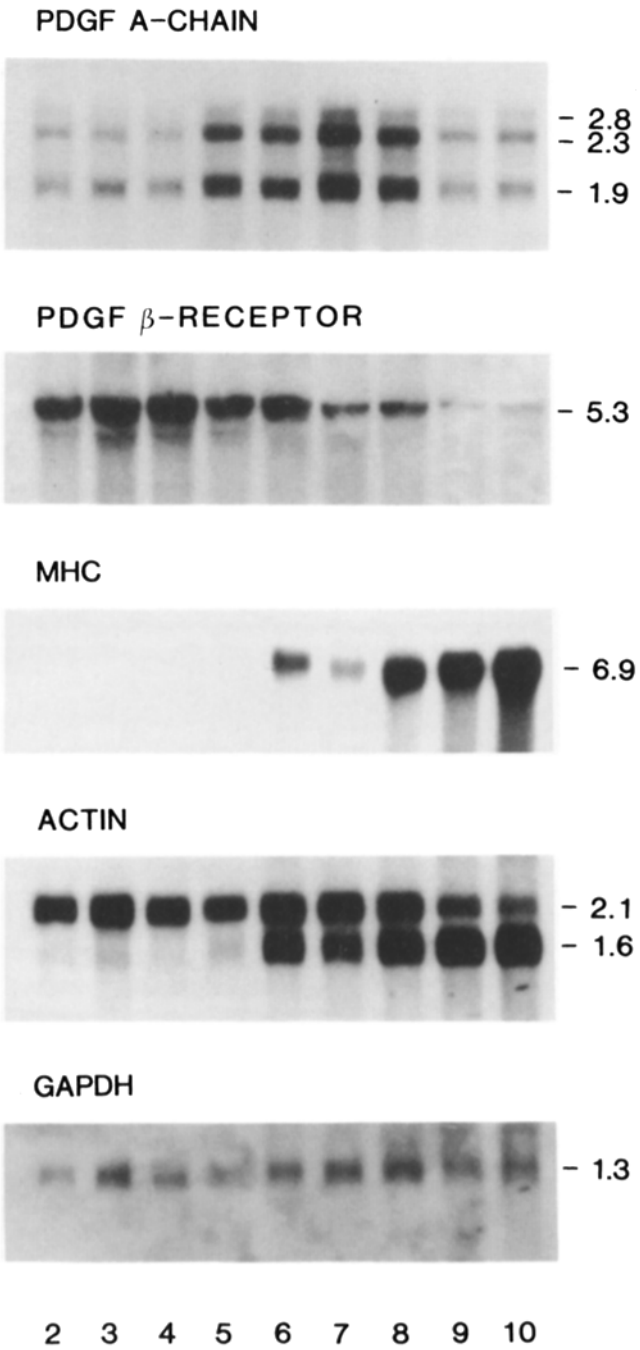

Figure 1. RNA blot illustrating PDGF A-chain, PDGF β-receptor, MHC, actin, and GAPDH transcripts in L6J1 myoblasts at day 2 to day 10 of spontaneous myogenic in vitro differentiation. 15 μg of poly A^+ selected RNA was analyzed in each lane. The same filter was sequentially hybridized to various DNA probes. Sizes of transcripts are shown in kilobase. Autoradiograph exposure times were 12 h for MHC and actin, 48 h for PDGF A-chain and PDGF β-receptor, and 96 h for GAPDH.

muscle development, we analyzed messenger RNA from L6J1 cells and from rat skeletal muscle tissue at various time points of the differentiation process.

In L6J1 myoblasts, expression of the PDGF A-chain gene was identified at all stages of the 10-d spontaneous in vitro differentiation process, although most abundantly at day 7, when the cells had undergone density-dependent arrest of DNA synthesis and fused actively to form multinucleated myotubes. Myotube formation took place in parallel with the increase of 1.6-kb α-actin and 6.9-kb myosin heavy chain (MHC) transcripts (Fig. 1). No myotubes were present at day 0, ~5–10% of the nuclei were present in myotubes at day 5, 40–50% at day 7, and 70–80% after 10 d in culture. Expression of PDGF A-chain gene 2.8-, 2.3-, and 1.9-kb transcripts increased almost six times from day 2 to 7 of the differentiation process before returning to lower levels in day 10 myotubes (Fig. 1).

Expression of the PDGF A-chain gene was also demonstrated in crude muscle tissue obtained from developing rats. Transcripts were barely detectable in 13-d-old whole embryos but appeared clearly in hind legs of 15-d-old rat embryos (Fig. 2). The abundance of the PDGF A-chain gene 2.3- and 1.9-kb transcripts then remained fairly constant throughout muscle development, including adult rat muscle (Fig. 2). Some variations were seen in the less abundantly expressed 2.8 kb transcript.

Expression of the PDGF B-chain gene (c-*sis*) was not detected in L6J1 myoblasts or in crude muscle tissue obtained from developing rats at any stage of the differentiation process, although the rat genome did contain DNA sequences that hybridized to a human c-*sis* probe (not shown), supporting previous results (Sejersen et al., 1986*a*).

PDGF β-Receptor mRNA Expression during Rat Myoblast Differentiation

Hybridizing RNA filters bearing materials from in vitro and in vivo differentiating rat myogenic cells with DNA probes specific for the PDGF α- and β-receptors, we identified a 5.3-kb PDGF β-receptor gene transcript both in L6J1 myoblasts and in rat muscle tissue. The relative abundance of 5.3-kb PDGF β-receptor transcripts in L6J1 cells was inversely related to the appearance of the myogenic markers α-actin and MHC (Fig. 1). Maximum expression was observed at day 3–4 of the spontaneous differentiation process when the growth rate of L6J1 myoblasts was exponential. The abundance rapidly declined when the cells became confluent. In day 10 L6J1 myotubes, PDGF β-receptor mRNA was barely detectable, the relative abundance being nine times lower than at day 4 (Fig. 1).

In crude muscle tissue obtained from developing rats, expression of the PDGF β-receptor gene showed kinetics comparable to that of the PDGF A-chain gene (Fig. 2). The expression was extremely low in 13-d-old whole embryos and in adult muscle tissue. Within the accuracy of the Northern technique used, a downregulation was seen around birth. Expression of PDGF α-receptor transcripts was not detected with the techniques employed, except for marginal levels in crude muscle tissue obtained from late stage rat embryos, which remains to be further confirmed (data not shown).

RNA filters were hybridized also with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene probe (Dugaiczyk et al., 1983). Expression of this gene was constant during L6J1 in vitro myoblast differentiation but appeared to be developmentally regulated in crude muscle tissue obtained from developing rats in vivo (Figs. 1 and 2).

L6J1 Myoblasts Bind PDGF-BB

^{125}I-labeled recombinant PDGF-AA and -BB were used to

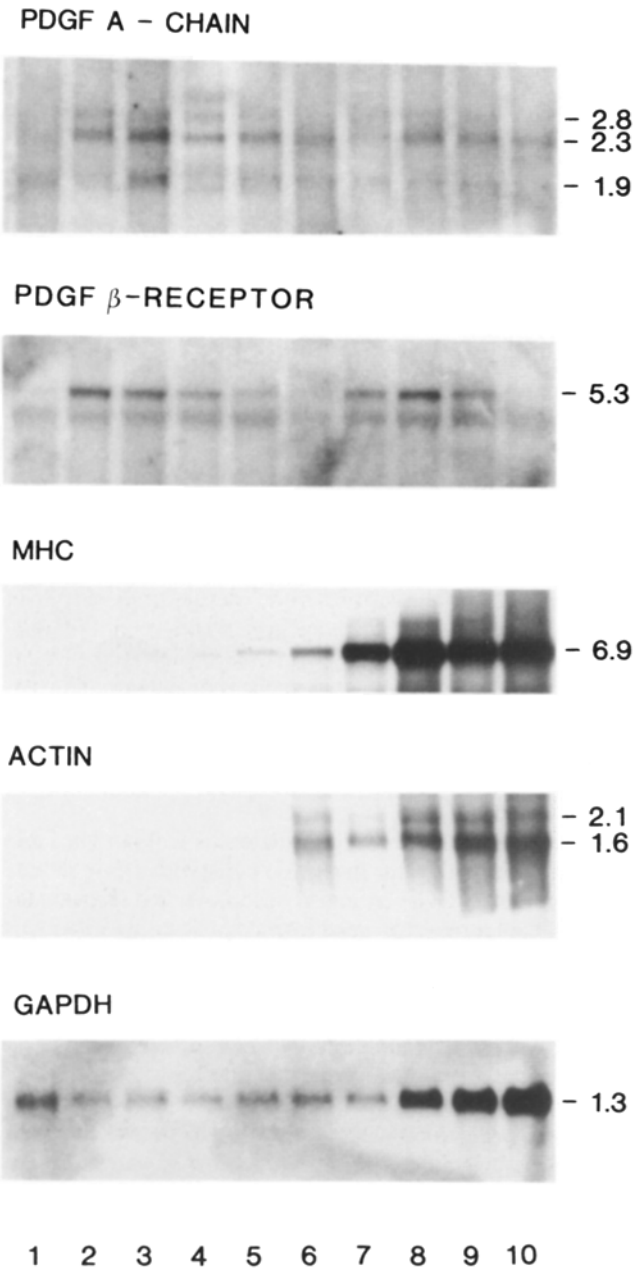

Figure 2. RNA blot illustrating PDGF A-chain, PDGF β-receptor, MHC, actin, and GAPDH transcripts in total embryo and crude muscle tissues isolated from thighs of Sprague-Dawley rats of different ages. 9 μg of poly A^+ selected RNA was analyzed in each lane. The same filter was sequentially hybridized to various DNA probes. Sizes of transcripts are shown in kilobase. Autoradiograph exposure times were 12 h for MHC, actin and GAPDH, and 48 h for PDGF A-chain and PDGF β-receptor. (Lane *1*) Whole 13-d-old embryos; (lanes *2–5*) crude muscle from 15-, 17-, 18-, and 20-d-old embryos; (lanes *6–9*) crude muscle from 1-, 7-, 14-, and 21-d-old rats; (lane *10*) crude muscle from a 5-mo-old (adult) rat.

detect binding of PDGF to exponentially proliferating and terminally differentiated L6J1 myoblasts. Specificity of the binding was determined in the absence and presence of 300 times excess of the corresponding unlabeled ligand. Preincubation with suramin, which previously has been shown to dissociate PDGF from its receptors (Williams et al., 1984; Hosang, 1985), was used in parallel experiments to dissociate binding of endogenously produced PDGF. PDGF-BB specifically bound to L6J1 cells (Fig. 3). Exponentially proliferating myoblasts showed higher binding of PDGF-BB than did the differentiated myotubes, indicating a higher number of PDGF β-receptors per nucleus on proliferating myoblasts than on myotubes. Suramin pretreatment did not influence the binding of PDGF-AA or -BB (Fig. 3). Separate binding studies of PDGF purified from human platelets (Heldin et al., 1987) showed results similar to that of PDGF-BB (not shown). PDGF purified from human platelets contains ~70% PDGF-AB and 30% PDGF-BB. Specific binding of PDGF-AA to L6J1 myoblasts could not be demonstrated, indicating no or very low amounts of PDGF α-receptors on these cells. Results similar to the above described were obtained in three separate experiments.

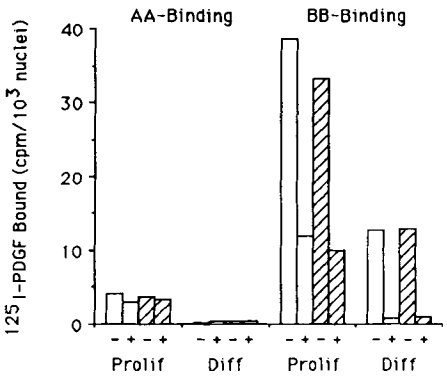

Figure 3. Binding of ^{125}I-labeled recombinant human PDGF-AA and -BB to exponentially proliferating (*Prolif*) L6J1 myoblasts and differentiated (*Diff*) myotubes, respectively. Cells with (*stippled bars*) or without (*open bars*) pretreatment of suramin were rinsed with binding buffer, and then incubated for 3 h at 4°C in binding buffer supplemented with 4 ng/ml of ^{125}I-labeled PDGF-AA or -BB with (+) or without (−) 300 times excess of the corresponding unlabeled ligand. Cell densities were determined in parallel cultures by counting Giemsa-stained cell nuclei using a Zeiss light microscope. The results are shown as mean values of triplicate cultures.

PDGF-BB Transiently Induces c-fos Immunoreactivity

c-*fos* is an early response gene to PDGF treatment in various cell types (Kruijer et al., 1984; Müller et al., 1984). To see if this was the case also in L6J1 myoblasts, L6J1 cells, deprived of serum for 4 d, were treated with 25 ng/ml of PDGF-BB, and fixed after different times. c-*fos* proteinlike staining could be detected 1 h after PDGF treatment (not shown), and maximal fluorescence was observed at 2 h (Fig. 4). The nuclear fluorescence induced by PDGF could be blocked by preincubation of the c-*fos* antiserum with an excess of c-*fos* synthetic peptides (not shown), indicating specific c-*fos* antibody immunoreactivity. The intensity of the nuclear immunofluorescence was scanned using a Zeiss/Kontron IBAS 2000 interactive image analysis computer. Mean values and standard deviations of the optical densities, based on 250–300 nuclei for each sample, are shown in Fig. 4 *G*. The experiment was repeated twice with similar results.

DNA Synthesis and Differentiation in PDGF-Treated L6J1 Myoblasts

Having demonstrated the expression of the PDGF β-receptor

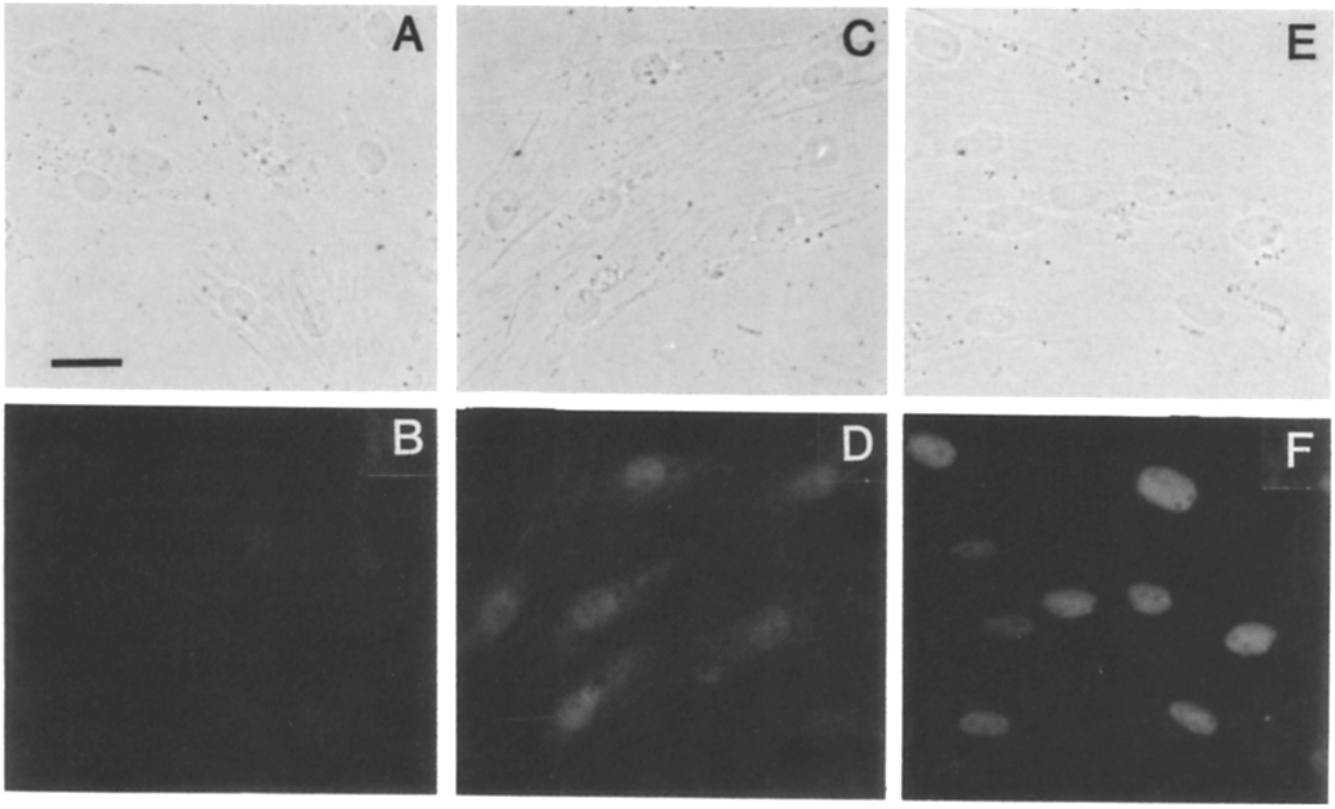

Figure 4. Expression of c-*fos* immunoreactivity in L6J1 myoblasts deprived of serum for 4 d (*B*), and in serum-deprived L6J1 myoblasts treated either with 25 ng/ml of porcine PDGF-BB (*D*), or 5% FCS (*F*) for 2 h. Corresponding phase-contrast photographs are shown in *A*, *C*, and *E*. Cells were fixed with 4% paraformaldehyde and incubated with a rabbit anti-*fos* antibody (xfos-3) followed by a TRITC-conjugated swine antirabbit IgG antibody. Nuclear fluorescence was scanned using a Zeiss/Kontron IBAS 2000 interactive image analysis computer, setting background fluorescence to 1. Bar, 30 μm. (*G*) Mean values and standard deviations of the immunofluorescence intensities based on 250–300 nuclei for each sample. Significance level for comparison between 0% FCS and 0% FCS/PDGF-BB is indicated; $***P < 0.01$.

gene, the specific membrane binding ability, and the induction of c-*fos* protooncogene, it was of interest to study further the effects of PDGF on DNA synthesis, cell proliferation, and myogenic differentiation of L6J1 rat myoblasts. As shown in Fig. 5, low concentrations of PDGF-BB increased the percentage of BrdU-incorporating nuclei in the presence of 0.5% FCS. Half maximal stimulation of DNA synthesis was obtained with ~1 ng/ml of PDGF-BB. At 10 ng/ml of PDGF-BB, the mitogenic activity reached a maximum. No further increase could be observed with higher concentrations of PDGF-BB. PDGF-AA did not affect DNA synthesis, except for a marginal increase observed at 45 ng/ml of PDGF-AA, the highest concentration used (Fig. 5). The functional activity of the PDGF-AA preparation used has previously been shown (Heldin et al., 1988). PDGF purified from human platelets increased L6J1 myoblast DNA synthesis by 25% in the presence of 0.5% FCS (data not shown). PDGF-BB, PDGF-AA, and PDGF purified from human platelets were not mitogenic for rat myoblasts in the absence of FCS (data not shown). Essentially similar results were obtained in three consecutive experiments.

To analyze possible effects of PDGF on L6J1 myoblast proliferation, we studied proliferation of cells, deprived of serum for 4 d, in the absence or presence of 25 ng/ml of PDGF-BB in 0.5% FCS. As shown in Fig. 6, no stimulation of cell proliferation was seen during 7 d in the PDGF-BB containing medium, as compared to proliferation in 0.5% FCS. The experiment was repeated once with similar results. A possible explanation of the above described findings could be that PDGF exerts its effects only during the first cell cycle after stimulation of serum-deprived myoblasts. To investigate this possibility, we analyzed the increase in fractions of BrdU-incorporating nuclei during 0–24 h and 24–48 h after addition of 25 ng/ml of PDGF-BB to the culture medium. As demonstrated in Fig. 7, the increase of cells present in the cell cycle after PDGF-BB treatment was markedly reduced during the 24–48-h time period as compared to the 0–24-h time period.

To study possible effects of PDGF on L6J1 myoblast spontaneous in vitro differentiation, myoblasts were cultured in DME/0.5% FCS in the absence or presence of 25 ng/ml of PDGF-BB supplied daily. The percentage of nuclei present in myotubes was scored at different time points of the spontaneous differentiation. In the PDGF-BB containing medium 7% of the nuclei were found in myotubes at day 4, corresponding to 13% in the control medium. At day 7, 24%, and

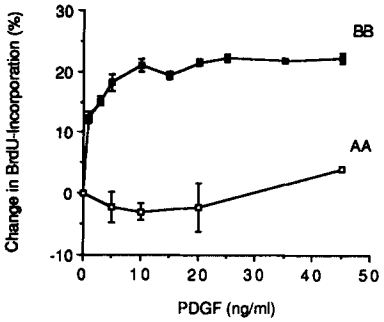

Figure 5. Increase in DNA synthesis in L6J1 myoblasts treated with recombinant human PDGF-AA or PDGF-BB. Myoblasts were seeded in DME/5% FCS on coverslips at 6 × 10^3 cells/cm^2, and cultured in serum free DME for 4 d. The cells were then switched to DME/0.5% FCS supplemented with 10 μM BrdU and various concentrations of either PDGF-AA or -BB for 24 h. Cell fixations and antibody reactions were carried out as described in Materials and Methods. Fractions of BrdU-incorporating nuclei were determined by counting 500–1,000 cells in randomly selected fields of vision, using a Zeiss ultraviolet microscope. Mean values and standard deviations of triplicate assays are shown. Levels of BrdU-incorporation in nuclei without PDGF treatment have been adjusted to zero.

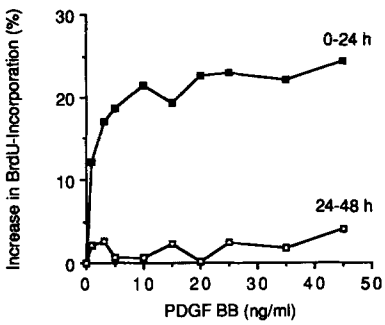

Figure 7. Increase in DNA synthesis in L6J1 myoblasts treated with porcine PDGF-BB. Myoblasts were seeded in DME/5% FCS on coverslips at 6 × 10^3 cells/cm^2, and cultured in serum free DME for 4 d. After serum deprivation, cells were switched to DME/0.5% FCS supplemented with various concentrations of PDGF-BB. 10 μM of BrdU was added to the medium either 0–24 h or 24–48 h thereafter. Cell fixations and antibody reactions were carried out as described in Materials and Methods. Increase in fractions of BrdU-incorporating nuclei were determined by counting 500–1,000 cells in randomly selected fields of vision, using a Zeiss ultraviolet microscope. Levels of BrdU-incorporation in nuclei without PDGF treatment have been adjusted to zero.

at day 10 41% of the nuclei were present in myotubes in PDGF-BB–treated cultures. These contrasted with 36% and 61% in the control cultures at day 7 and day 10, respectively. Thus, the formation of myotubes in PDGF-BB–treated L6J1 myoblasts was reduced by ~30% at day 10. This assay was repeated once with essentially similar results.

Discussion

The results presented here show that the genes coding for the PDGF A-chain and the PDGF β-receptor are expressed in rat myoblasts, and that PDGF-BB is a partial mitogen for L6J1 rat myoblasts. Several polypeptide growth factors have in previous studies been shown to be mitogenic for established or primary rat myoblasts. These include insulin, insulin-like growth factors I and II, fibroblast growth factor, transferrin, and growth hormone (Florini et al., 1977; Ewton and Florini, 1981; Hayachi and Kobylecki, 1982; Nissley et al., 1984; Massagué et al., 1986). Insulin-like growth factors I and II have been shown to be produced by rat myoblasts and to stimulate cell growth in an autocrine manner (Nissley et al., 1984).

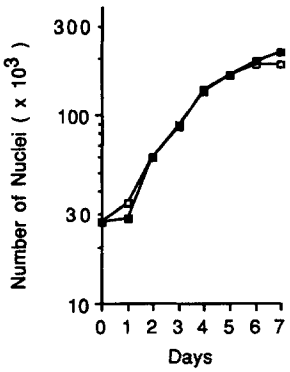

Figure 6. Proliferation of L6J1 myoblasts. Cells were seeded in DME/5% FCS at 4 × 10^3/cm^2 in 7-cm^2 wells, and cultured in serum free DME for 4 d. Myoblasts were then maintained in DME/0.5% FCS supplemented with (—□—) or without (—■—) 25 ng/ml of porcine PDGF-BB. At the indicated time points (days), myoblasts were trypsinized, and cell numbers were determined in triplicate wells using an automatic cell counter.

At the RNA level, we found that the PDGF A-chain gene was expressed at all examined stages of rat myogenesis, in vitro and in vivo. However, the function of endogenously produced PDGF-AA on rat myoblasts is not clear, and expression of its receptor remains to be examined using more sensitive methods. Our experiments showed that L6J1 myoblasts did not bind PDGF-AA, and only a marginal increase in DNA synthesis was observed with very high concentrations of PDGF-AA.

Transcripts for the PDGF β-receptor were present in undifferentiated L6J1 rat myoblasts in vitro and in crude rat muscle tissue in vivo. PDGF β-receptor mRNA was down-regulated in differentiated L6J1 myotubes and mature adult rat muscle in a manner different from the expression pattern of PDGF A-chain transcripts. This was reflected also at the protein level as decreased capacity of L6J1 myotubes to bind PDGF-BB, compared to the binding capacity of proliferating L6J1 myoblasts. It should further be commented on the PDGF binding experiments that the background binding, per nucleus, was consistently found to be much higher for proliferating than for differentiated myoblasts. This may partly be due to the large difference in cell density and free plastic surface area between the two states of differentiation analyzed, but other factors (e.g., altered membrane composition) are also likely to have influenced the observed difference in background binding.

The presence of functional PDGF β-receptors on L6J1 myoblasts was further supported by the PDGF-BB induced increase of c-*fos* immunoreactivity and the DNA synthesis induced by PDGF-BB; this form of PDGF was previously shown to bind to and act through the PDGF β-receptor (Hart et al., 1988; Heldin et al., 1988). PDGF-BB is also likely to induce DNA synthesis in developing rat myoblasts in vivo, since they express PDGF β-receptor mRNA. Surprisingly, PDGF-BB did not increase the total cell number of L6J1 myoblasts in proliferation assays, although it induced DNA synthesis. The lack of long term effect of growth factor on

L6J1 myoblasts may, at least partially, be explained by our finding that DNA synthesis was stimulated in serum-deprived myoblasts during the first 24 h after PDGF addition, but not during the next 24 h. The reason for this decreased responsiveness to PDGF remains to be further analyzed. One factor likely to contribute to the lack of proliferative effect is deficiency of other growth factors required by L6J1 myoblasts.

Myoblast differentiation has in previous studies been shown to be inhibited or stimulated by various growth factors (Yaffe, 1971; Linkhart et al., 1980; Ewton and Florini, 1981; Lim and Hauschka, 1984; Massagué et al., 1986). PDGF-BB partially inhibited myogenic differentiation in vitro, decreasing myotube formation by ~30%.

In conclusion, we have found that rat myoblasts express the PDGF A-chain gene and functional PDGF β-receptors. PDGF-BB induces c-*fos* immunoreactivity and DNA synthesis, but not cell proliferation in short term assays. Myogenic differentiation of L6J1 myoblasts was inhibited by the presence of PDGF-BB in the culture medium.

We thank Mrs. M.-L. Alun, G. Dombos, and U. Krondahl, and Mr. W.-Q. Jiang for excellent technical assistance. Suggestions and comments on the manuscript by Drs. N. R. Ringertz and J. Thyberg are gratefully acknowledged. We are indebted to the following colleagues for providing us with cloned genes and antibodies: Drs. C. Betsholtz (PDGF A-chain and c-*sis*), M. Buckingham (actin pAM 91 and MHC 32), M. Groudine (pGAD-28), F. Wong-Staal (c-*sis*), and G. Evan (xfos-3 antibodies).

Financial support was obtained from the Swedish Medical Research Council (grant 6832), the Swedish Cancer Society (grant 2280), and the funds of Karolinska Institutet.

Received for publication 20 July 1989 and in revised form 16 January 1990.

References

Betsholtz, C., A. Johnsson, C.-H. Heldin, B. Westermark, P. Lind, M. S. Urdea, R. Eddy, T. B. Shows, K. Philpott, A. L. Mellor, T. J. Knott, and J. Scott. 1986. cDNA sequence and chromosomal localization of human platelet-derived growth factor A-chain and its expression in tumour cell lines. *Nature (Lond.).* 320:695–699.

Bolton, A. E., and W. M. Hunter. 1973. The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. *Biochem. J.* 133:529–539.

Claesson-Welsh, L., A. Eriksson, A. Moren, L. Severinsson, B. Ek, A. Östman, C. Betsholtz, and C.-H. Heldin. 1988. cDNA cloning and expression of a human platelet-derived growth factor (PDGF) receptor specific for B-chain-containing PDGF molecules. *Mol. Cell. Biol.* 8:3476–3486.

Claesson-Welsh, L., A. Eriksson, B. Westermark, and C.-H. Heldin. 1989. cDNA cloning and expression of the human A type PDGF receptor establishes structural similarity to the B type PDGF receptor. *Proc. Natl. Acad. Sci. USA.* 86:4917–4922.

Clarke, M. F., E. Westin, D. Schmidt, S. F. Josephs, L. Ratner, F. Wong-Staal, R. C. Gallo, and M. S. Reitz, Jr. 1984. Transformation of NIH 3T3 cells by a human c-*sis* cDNA clone. *Nature (Lond.).* 308:464–467.

Dugaiczyk, A., J. A. Haron, E. M. Stone, O. E. Dennison, K. N. Rothblum, and R. J. Schwartz. 1983. Cloning and sequencing of a deoxyribonucleic acid copy of glyceraldehyde-3-phosphate dehydrogenase messenger ribonucleic acid isolated from chicken muscle. *Biochemistry.* 22:1605–1613.

Escobedo, J. A., S. Navankasatussas, L. S. Cousens, S. R. Coughlin, G. I. Bell, and L. T. Williams. 1988. A common PDGF receptor is activated by homodimeric A and B forms of PDGF. *Science (Wash. DC).* 240:1532–1534.

Ewton, D. Z., and J. R. Florini. 1981. Effects of the somatomedins and insulin on myoblast differentiation *in vitro*. *Dev. Biol.* 86:31–39.

Florini, J. R., M. L. Nicholson, and N. C. Dulak. 1977. Effects of peptide anabolic hormones on growth of myoblasts in culture. *Endocrinology.* 101: 32–41.

Gronwald, R. G. K., F. J. Grant, B. A. Haldeman, C. E. Hart, P. J. O'Hara, F. S. Hagen, R. Ross, D. F. Bowen-Pope, and M. J. Murray. 1988. Cloning and expression of a cDNA coding for the human platelet-derived growth factor receptor: evidence for more than one receptor class. *Proc. Natl. Acad. Sci. USA.* 85:3435–3439.

Guerriero, V., Jr., and J. R. Florini. 1980. Dexamethasone effects on myoblast proliferation and differentiation. *Endocrinology.* 106:1198–1202.

Hammacher, A., M. Nistér, B. Westermark, and C.-H. Heldin. 1988. A human glioma cell line secretes three structurally and functionally different dimeric forms of platelet-derived growth factor. *Eur. J. Biochem.* 176:179–186.

Hart, C. E., J. W. Forstrom, J. D. Kelly, R. A. Seifert, R. A. Smith, R. Ross, M. J. Murray, and D. F. Bowen-Pope. 1988. Two classes of PDGF receptors recognize different isoforms of PDGF. *Science (Wash. DC).* 240: 1529–1531.

Hayachi, I., and J. Kobylecki. 1982. Growth of myoblasts in hormone-supplemented, serum-free medium. *In* Cold Spring Harbor Conferences on Cell Proliferation. G. H. Sato, editor. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 857–865.

Heldin, C.-H., Å. Wasteson, and B. Westermark. 1985. Platelet-derived growth factor. *Mol. Cell. Endocrinol.* 39:169–187.

Heldin, C.-H., A. Johnsson, S. Wennergren, C. Wernstedt, C. Betsholtz, and B. Westermark. 1986. A human osteosarcoma cell line secretes a growth factor structurally related to a homodimer of PDGF A-chains. *Nature (Lond.).* 319:511–514.

Heldin, C.-H., A. Johnsson, B. Ek, S. Wennergren, L. Rönnstrand, A. Hammacher, B. Faulders, Å. Wasteson, and B. Westermark. 1987. Purification of human platelet-derived growth factor. *Methods. Enzymol.* 147:3–13.

Heldin, C.-H., G. Bäckström, A. Östman, A. Hammacher, L. Rönnstrand, K. Rubin, M. Nister, and B. Westermark. 1988. Binding of different dimeric forms of PDGF to human fibroblasts: evidence for two separate receptor types. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:1387–1393.

Hosang, M. 1985. Suramin binds to platelet-derived growth factor and inhibits its biological activity. *J. Cell Biochem.* 29:265–273.

Hunt, S. P., A. Pini, and G. Evan. 1987. Induction of c-*fos*-like protein in spinal cord neurons following sensory stimulation. *Nature (Lond.).* 328:632–634.

Kruijer, W., J. A. Cooper, T. Hunter, and I. M. Verma. 1984. Platelet-derived growth factor induces rapid but transient expression of the c-*fos* gene and protein. *Nature (Lond.).* 312:711–716.

Lim, R. W., and S. D. Hauschka. 1984. A rapid decrease in epidermal growth factor-binding capacity accompanies the terminal differentiation of mouse myoblasts in vitro. *J. Cell Biol.* 98:739–747.

Linkhart, T. A., C. H. Clegg, and S. D. Hauschka. 1980. Control of mouse myoblast commitment to terminal differentiation by mitogens. *J. Supramol. Struct.* 14:483–498.

Massagué, J., S. Cheifetz, T. Endo, and B. Nadal-Ginard. 1986. Type β transforming growth factor is an inhibitor of myogenic differentiation. *Proc. Natl. Acad. Sci. USA.* 83:8206–8210.

Matsui, T., M. Heidaran, T. Miki, N. Popescu, W. La Rochelle, M. Kraus, J. Pierce, and S. Aaronson. 1989. Isolation of a novel receptor cDNA establishes the existence of two PDGF receptor genes. *Science (Wash. DC).* 243:800–803.

Minty, A. J., M. Caravatti, B. Robert, A. Cohen, P. Daubas, A. Weydert, F. Gros, and M. Buckingham. 1981. Mouse actin messenger RNAs. *J. Biol. Chem.* 256:1008–1014.

Müller, R., R. Bravo, J. Burckhardt, and T. Curran. 1984. Induction of c-*fos* gene and protein by growth factors precedes activation of c-*myc*. *Nature (Lond.).* 312:716–720.

Nadal-Ginard, B. 1978. Commitment, fusion and biochemical differentiation of a myogenic cell line in the absence of DNA synthesis. *Cell.* 15:855–864.

Nissley, P., S. Adams, L. Gaynes, W. Anderson, L. Nagarajan, D. Hill, Y. Yang, and M. Rechler. 1984. Growth of cells in defined environments: the role of endogenous production of insulin-like growth factors. *In* Proceedings of the International Symposium on Growth and Differentiation of Cells in Defined Environment. H. Murakami, I. Yamane, D. W. Barnes, J. P. Mather, I. Hayashi, and G. H. Sato, editors. Kodansha Ltd., Tokyo. 337–344.

Östman, A., G. Bäckström, N. Fong, C. Betsholtz, C. Wernstedt, U. Hellman, B. Westermark, P. Valenzuela, and C.-H. Heldin. 1989. Expression of three recombinant homodimeric isoforms of PDGF in *Saccharomyces cerevisiae*. Evidence for differences in receptor binding and functional activities. *Growth Factors.* 1:271–281.

Pearson, M. L., and H. F. Epstein. 1982. Muscle Development: Molecular and Cellular Control. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 559–568.

Rahm, M., P. Jin, J. Sümegi, and T. Sejersen. 1989. Elevated c-*fos* expression inhibits differentiation of L6 rat myoblasts. *J. Cell. Physiol.* 139:237–244.

Ringertz, N. R., U. Krondahl, and J. R. Coleman. 1978. Reconstitution of cells by fusion of cell fragments. *Exp. Cell Res.* 113:233–246.

Ross, R., E. W. Raines, and D. F. Bowen-Pope. 1986. The biology of platelet-derived growth factor. *Cell.* 46:155–169.

Sejersen, T., C. Betsholtz, M. Sjölund, C.-H. Heldin, B. Westermark, and J. Thyberg. 1986*a*. Rat skeletal myoblasts and arterial smooth muscle cells express the gene for the A-chain but not the gene for the β-chain (c-*sis*) of PDGF and produce a PDGF-like protein. *Proc. Natl. Acad. Sci. USA.* 83:6844–6848.

Sejersen, T., H. Björklund, J. Sümegi, and N. R. Ringertz. 1986*b*. N-*myc* and c-*src* genes are differentially regulated in PCC7 embryonal carcinoma cells undergoing neuronal differentiation. *J. Cell. Physiol.* 127:274–280.

Stroobant, P., and M. D. Waterfield. 1984. Purification and properties of porcine platelet-derived growth factor. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:2963–2967.

Weydert, A., P. Daubas, M. Caravatti, A. Minty, G. Bugaisky, A. Cohen, B.

Robert, and M. Buckingham. 1983. Sequential accumulation of mRNAs encoding different myosin heavy chain isoforms during skeletal muscle development in vivo detected with a recombinant plasmid identified as coding for an adult fast myosin heavy chain from mouse skeletal muscle. *J. Biol. Chem.* 258:13867–13874.

Williams, L. T., P. M. Tremble, M. F. Lavin, and M. E. Sunday. 1984. Platelet-derived growth factor receptors form a high affinity state in membrane preparations. Kinetics and affinity cross-linking studies. *J. Biol. Chem.* 259:5287–5294.

Yaffe, D. 1968. Retention and differentiation potentialities during prolonged cultivation of myogenic cells. *Proc. Natl. Acad. Sci. USA.* 61:477–483.

Yaffe, D. 1971. Developmental changes preceding cell fusion during muscle differentiation in vitro. *Exp. Cell Res.* 66:33–48.

Yaffe, D. 1973. Rat skeletal muscle cells. *In* Tissue Culture Methods and Applications. P. F. Kruse and M. K. Patterson, editors. Academic Press, New York. 106–114.

Yaffe, D., and S. Fuchs. 1967. Autoradiographic study of the incorporation of uridine-^{3}H during myogenesis in tissue culture. *Dev. Biol.* 15:33–50.