

Inhibition of Differentiation in Myoblasts Deprived of the Interferon-related Protein PC4¹

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Abstract

PC4 (pheochromocytoma cell-4) is an immediate early gene related to IFN- γ , the mRNA of which is induced during the course of neuronal differentiation by nerve growth factor in the PC12 cell line. Here we report that PC4 mRNA is also expressed in the myoblast C2C12 cell line and is regulated during differentiation; its expression decreases within 6 h from the onset of differentiation, attains a minimum after 12 h, and returns to basal level within 36 h. This transient down-regulation of PC4 expression in C2C12 myoblasts is prevented by transforming growth factor β , a molecule which inhibits the differentiation of muscle. Sense and antisense PC4 cDNA transfection strategies in C2C12 cells were then used to clarify the role of PC4 in muscle differentiation. While no effect was seen by over-expression of PC4, stable transfectants underexpressing PC4 exhibited a delay in attaining the differentiated phenotype, with an impairment of myogenin and myosin expression. Myogenin was also inhibited in C2C12 cells microinjected with the anti-PC4 polyclonal antibody A451. We thus postulate a role for PC4 as a positive regulator during muscle differentiation.

Introduction

Growth factors induce cellular responses via an activation of specific patterns of gene expression. The genes induced early after stimulation are defined as IEGs³ and include the proto-oncogenes *c-fos*, *c-myc*, and *c-jun*, and zinc finger proteins such as NGFI-A. These IEGs are all induced in PC12 cells during the neuronal differentiation elicited by NGF (1–4). *c-fos*, *c-jun*, and the zinc finger proteins are transcriptional regulators, and the general idea has emerged that IEGs are activators of downstream genes, which are ultimately responsible for the growth factor-dependent cellular changes (4). Although there are indications of such a

regulatory cascade in NGF-induced PC12 cell differentiation (5), further information about the biological relevance of the above model is still required (6). Moreover, certain IEGs, which are NGF induced in PC12 cells, do not display any evident features peculiar to transcriptional regulators. For instance PC4 (pheochromocytoma cell-4), a gene with partial homology to IFN- γ (7), isolated by differential hybridization from PC12 cells (7, 8) and as a tetradecanoyl phorbol acetate-induced gene from mouse NIH3T3 cells (9), is devoid of known transcriptional motifs.

In this study we investigated the possible role of PC4 in cell differentiation by exploring its expression in the skeletal myoblast. With respect to PC12 cells, this system has the advantage that a defined set of genes with differentiation-inducing capabilities has been identified, as well as polypeptides, such as TGF β (10–13), that can block the cell differentiation and the induction of the above genes. In the myoblast the onset of differentiation is preceded by arrest of the cell cycle in G₁ (14–16). The length of time in G₁ arrest is directly proportional to the probability of entering a state of irreversible withdrawal from the cell cycle (commitment). The latter event is followed by the myoblast fusion into multinucleated fibers, the myotubes, a major component of the adult skeletal muscle morphology (15, 17). The pre-fusion state, characterized morphologically by the elongation and aligning of the cells, coincides with the activation of muscle-specific regulatory genes, such as Myogenin, *MyoD1*, *Myf-5*, and *Myf-6/herculin* (18–22). These genes, in turn, activate the expression of structural genes such as myosin heavy chain (23) and muscle creatine kinase (18, 24) responsible for the adult phenotype of the muscle. Interestingly, the muscle-specific gene induction can be reversed by inhibitory growth factors until cell fusion has occurred (17). The regulatory genes also induce the program leading to muscle differentiation in certain nonmuscle cell lines (18, 20, 24), provided that the specific cell line is permissive (25). The same genes are able to activate their own expressions as well as those of each other (18, 26), suggesting that via a positive feedback loop, the activation of a single gene is sufficient to commit the cell to the muscle phenotype. Regulatory genes are known to control transcription of the target muscle genes by binding to their control sequences with a basic region. The affinity of this interaction is affected by the property of an adjacent region, a putative helix-loop-helix structure, which induces homo- and heterodimerization between regulatory gene proteins (for review see Ref. 26).

The mouse skeletal muscle cell line we used, C2C12, was cloned from cells with regenerative potential of the skeletal muscle (satellite cells). These cells are not transformed and therefore maintain the character of a primary cell line, and have the ability to rapidly attain the differentiated phenotype (myotubes) after exposure to low serum medium (27).

Here we report that in differentiating C2C12 cells the expression of PC4 mRNA and protein shows a transient down-regulation that can be suppressed by TGF β . More-

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³ The abbreviations used are: IEG, immediate-early gene; NGF, nerve growth factor; TGF β , transforming growth factor β ; C2FM, DMEM with 2% horse serum; C2GM, DMEM with 20% FCS; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

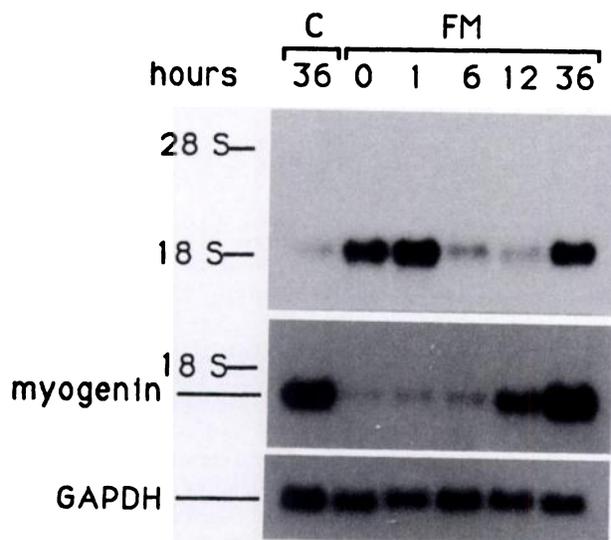


Fig. 1. PC4 mRNA levels during myoblast differentiation. A Northern blot of 10 μ g of total RNA from C2C12 cultures shifted to differentiation medium (FM) for the times indicated, or from cultures kept under confluent conditions for 36 h in growth medium (C), was hybridized to the PC4 cDNA insert 32 P-labeled (about 50×10^6 dpm total, top), to the myogenin probe (30×10^6 dpm total, middle), and to glyceraldehyde 3-phosphate dehydrogenase cDNA as a control of RNA integrity and quantitation (30×10^6 dpm total, bottom). The autoradiographs were exposed 3 days for the PC4 probe, and 24 and 4 h, respectively, for the myogenin and GAPDH probes.

over, inhibition of PC4 expression in C2C12 cells impairs the expression of myogenin and that of heavy chain myosin as well, and delays cell fusion. This suggests that the PC4 protein plays a role in the differentiation of the muscle.

Results

Regulated Expression of PC4 mRNA during Myoblast Differentiation. PC4 mRNA, which is present in skeletal muscle (7), was found by Northern analysis to be basally expressed in the satellite cell-derived C2C12 myoblasts, and transiently down-regulated during their differentiation, elicited by a shift to a low serum medium (C2FM; Fig. 1). In this medium a slight increase (30%) of expression occurred almost immediately (within 1 h), followed by a decrease. Expression was lowest (one-fourth of the basal level) at 12 h after the onset of differentiation. PC4 mRNA expression returned to basal levels within 36 h. To verify whether the transient decrease was a consequence of the removal of some serum component, we exploited the property of the myoblast of also differentiating in high serum, provided the culture density is high enough to allow cell-cell contact. As seen with the serum deprivation-mediated differentiation, more than 3-fold reduction of PC4 basal expression was observed (Fig. 1, Lane C). This suggested that the decrease of PC4 mRNA was not a direct consequence of the subtraction of the serum but more likely of the subsequent events, such as cell cycle arrest and differentiation. The expression of myogenin, a key gene in the regulation of muscle differentiation (18), was also evaluated in parallel at various times. As expected, myogenin mRNA levels increased from a nondetectable level to a maximum within 36 h, either after shifting to a low serum (45 times increase) or when differentiation occurred in high serum (38 times) (Fig. 1, Lane C; see Ref. 18).

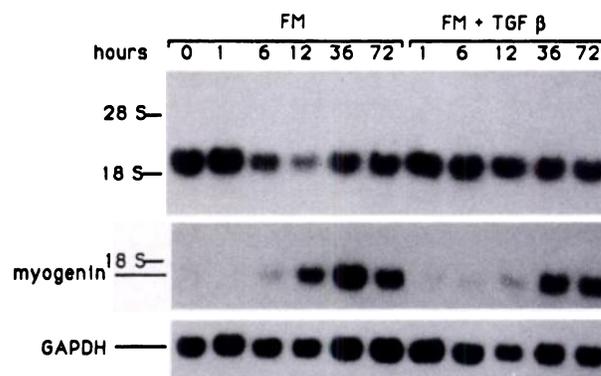


Fig. 2. Suppression of PC4 mRNA down-regulation by TGF β . An RNA blot of 10 μ g of total RNA from C2C12 cells shifted to differentiation medium (FM) for the times indicated, in the presence or absence of 5 ng/ml of TGF β , was hybridized to the PC4, myogenin, or GAPDH probes, as indicated, with specific activities and total dpm comparable to those in Fig. 1.

TGF β Counteracts PC4 mRNA Regulation during Myoblast Differentiation. To further define the relation between the changes in PC4 expression and cell differentiation, a time course study was carried out on the PC4 mRNA levels in differentiating C2C12 cells, both in the presence and absence of TGF β_1 (5 ng/ml). This molecule is able to arrest myoblast differentiation by inhibiting the muscle-specific genes (13, 28) without affecting proliferation (10, 11, 13). The presence of TGF β_1 completely inhibited the decrease of PC4 observed in C2FM after 6 h, and partially reduced the decrease after 12 h. In fact, at this latter time point, the expression of PC4 in absence of TGF β_1 was about one-fourth of the basal level of expression, while in cultures with TGF β_1 , expression of PC4 attained levels comparable to basal expression (Fig. 2). In parallel, the increase of myogenin mRNA expression observed 12 h after a shift to C2FM (about 20 times; see also Fig. 1) was also reduced by TGF β_1 (6 times), and the normal level of increased expression was regained only after 72 h (Fig. 2). At this time the cultures incubated in C2FM with TGF β_1 showed initial features of differentiated morphology, such as cell fusion. C2C12 cultures in C2FM alone, however, started to fuse within 24 h, reaching the phenotype of elongated myotubes within 48–72 h. In C2C12 cells kept 12 h in C2FM, a dose-response curve to increasing concentrations of TGF β_1 over the down-regulation of PC4 mRNA indicated an IC_{50} of about 0.2 ng/ml (Fig. 3, A and B). In the presence of growth medium with high serum (C2GM), TGF β_1 (5 ng/ml) does not stimulate the expression of PC4 (data not shown).

Immunolocalization of PC4 Protein during Myoblast Differentiation. We then sought to determine whether the PC4 protein expression was down-regulated during muscle differentiation, and to immunolocalize the protein in the muscle cell, also in the presence of NGF. In addition to inducing PC4 in PC12 cells, this neurotrophic molecule also has a trophic action on skeletal myotubes (29, 30), and its low affinity NGF receptor has been detected *in vivo* in myoblasts (31, 32). We used a polyclonal antibody raised against PC4, A-451 (see "Material and Methods" and Ref. 33). By indirect immunofluorescence, the PC4 protein was detected in the cytoplasm of myoblast cultures growing in C2GM (Fig. 4B) and in the cytoplasm of cultures incubated in low serum medium for 12, 24, and 72 h (Fig. 4, C–E), or

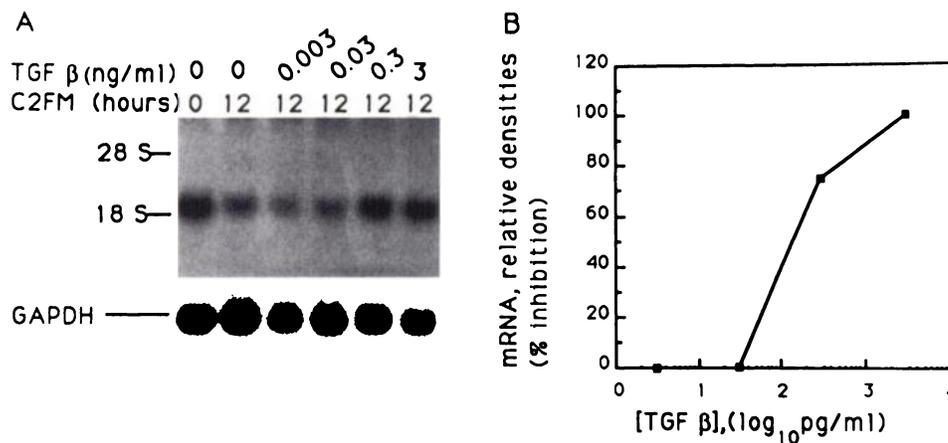


Fig. 3. Concentration dependence of TGF β inhibition on PC4 mRNA decrease. (A) An RNA blot of 10 μ g of total RNA from control C2C12 cells (C2FM, 0 h) or from cells exposed for 12 h to differentiation medium (FM) in the absence or presence of increasing concentrations of TGF β , was hybridized to the PC4 probe (40×10^6 dpm total). (B) Ordinate, relative densities of the blot in A, normalized to the GAPDH expression, as the percentage inhibition (%) of the PC4 decrease [% of relative densities = (Sample - 12-h C2FM sample without TGF β)/(0-h C2FM sample - 12-h C2FM sample without TGF β) \times 100]; abscissa, logarithm of the concentration.

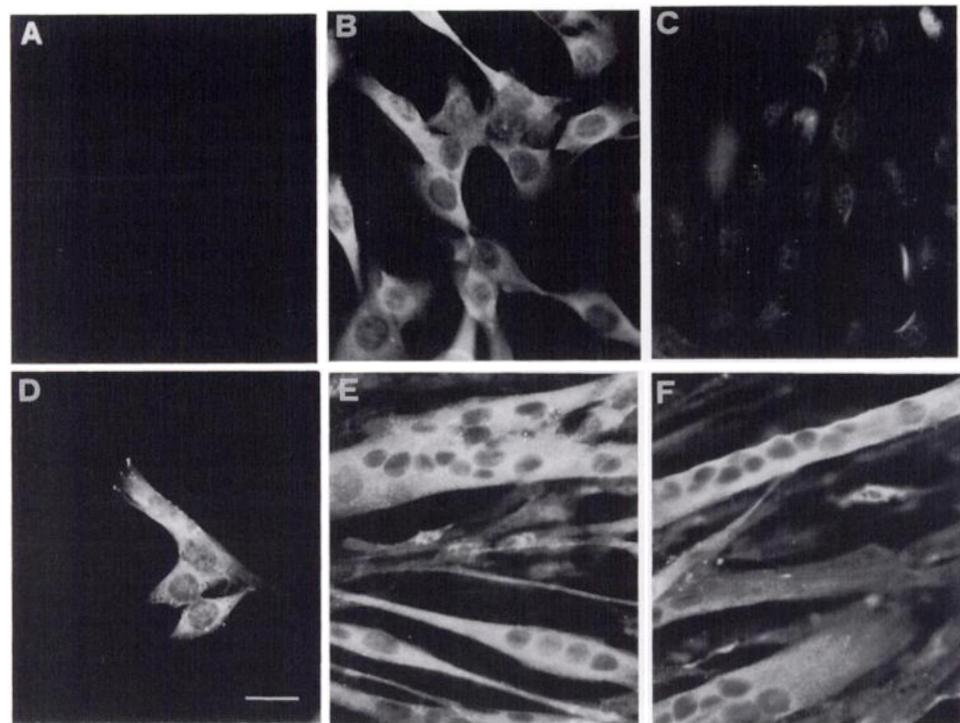


Fig. 4. Cellular localization and expression of PC4 in C2C12 skeletal myoblasts and myotubes. The antigen was revealed by FITC staining after incubation with non-immune serum as a control (A) or with affinity-purified A451 serum (B-F). B, Undifferentiated growing myoblasts; C, myoblast exposed for 12 h to FM; D, 24 h to FM; E and F, fully differentiated myotubes after 72 h in FM without or with NGF, respectively. Preabsorption of affinity-purified A451 with nonrecombinant β -galactosidase protein had no effect on PC4 staining in C2C12 cells (not shown). Bar, 40 μ m.

for 72 h in low serum medium plus NGF (200 ng/ml; Fig. 4F). We observed that in parallel with the changes in the mRNA levels of PC4 (Figs. 1-3), the protein staining decreased 12 h after the shift to C2FM, followed by a gradual return to the initial level within 72 h. At this time the cells presented the differentiated morphology of elongated and polynucleated myotubes (not apparently affected by NGF). Thus, NGF treatment did not apparently influence the expression and localization of the PC4 protein in myoblasts as judged by immunofluorescence staining with A451. (Also, in C2C12 cells allowed to differentiate in the presence of NGF [100 ng/ml] for 36 h, no change was observed in the

pattern of PC4 and myogenin mRNAs induction, data not shown).

Inhibition of Myogenin and Myosin Induction in PC4-deprived Myoblasts. To further assess the role of PC4 in muscle differentiation, we then stably transfected the full-length PC4 cDNA in the sense or antisense orientation into C2C12 cells, under the control of the β actin promoter in the vector pH β APr-1-neo [pBAP-neo-PC4 (34)]. From 6×10^5 cells transfected with each type of construct, we obtained several neomycin-resistant clones. One of them, expressing PC4 mRNA in the sense orientation (S4), and one clone expressing PC4 mRNA in the antisense orientation

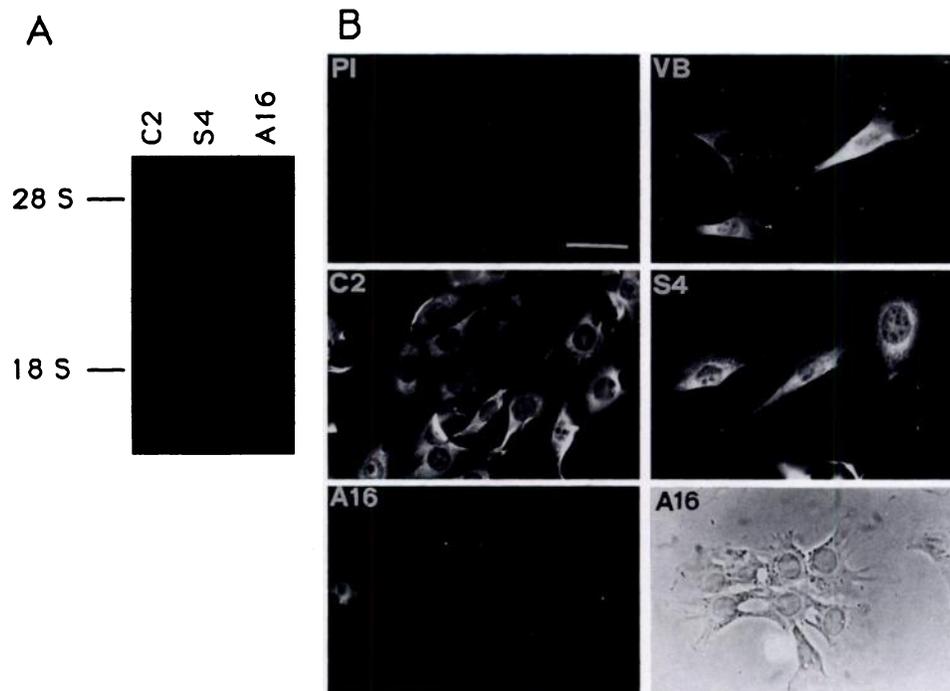


Fig. 5. Expression of the PC4 RNA and protein in the C2C12 clones stably transfected with pBAP-PC4. (A) Eight μg of total RNA from C2C12 control cells (C2) or transfected with the pBAP-neo-PC4 vector in the sense (S4) or antisense orientation (A16) were extracted, blotted on nitrocellulose filters, hybridized with the complementary RNA probe, and analyzed by autoradiography. In clone A16 the antisense RNA was revealed by sense-transcribed PC4 probe, while in S4 clone and C2C12 cells the sense RNA was detected by an antisense probe (see "Materials and Methods"). The probes had comparable specific activities (60×10^6 dpm/mg of cDNA template), and the exposure time was 24 h. (B) Immunofluorescent (FITC) staining of the PC4 protein after incubation with the A-451 antiserum in C2C12 parental cells (C2), or stably transfected with the pBAP-neo-PC4 vector in the sense (S4) or antisense orientation (A16; corresponding phase contrast field on the right), or transfected with the pBAP-neo vector (VB). FITC staining was also performed after incubation with nonimmune serum as a control (PI). Bar, 60 μm .

(A16), were chosen for further study. The PC4 sequences stably transfected in the C2C12 cell clones were detected as a transcript of about 1.9 kilobases (Fig. 5A), consistent with an expected length of 1438 nucleotides (the *Bam*I-*Hind*III fragment of PC4 cDNA containing the complete coding region), plus about 450 nucleotides of the untranslated region of the vector pBAP-neo (34). Western blot analysis indicated that the S4 clone produced several times more PC4 protein than did control cells (not shown).

In these transfectants we monitored the level of the PC4 protein by immunofluorescence (Fig. 5B) with the anti-PC4 antibody A-451. As expected, the expression of the PC4 protein was higher in the S4 clone than in a clone of cells sham transfected with the pBAP-neo vector (VB), and almost undetectable in A16 (Fig. 5B). These different levels of expression in the sense- and antisense-transfected cells were also maintained when the cultures were shifted to C2FM (data not shown; see also Ref. 34).

We then checked the effects of altered levels of PC4 protein on cell differentiation by analyzing the levels of myogenin and myosin heavy chain proteins and by observing cell fusion. Myogenin is a key regulator gene of muscle differentiation (18) also required for muscle maturation *in vivo* (35, 36), while the myosin heavy chain is a structural protein present in the developing and adult muscle, the expression of which is induced in differentiating cultured cells (23, 37) and is regulated by myogenin.

Immunoblot analysis of the myogenin protein levels at various times after shifting the cultures to C2FM showed

that after 48 h myogenin was maximally increased in the sense (S4) and control cultures [control C2C12 cells and the pBAP sham-transfected clones (see Fig. 6; Table 1)] and almost undetectable in the A16 clone (Fig. 6; Table 1). Similarly, an increase of myosin protein levels was observed after 48 h in C2FM, only in the sense and control clones (Fig. 6; Table 1).

The stable expression of antisense PC4 RNA and the inhibition of myogenin protein induction during differentiation was correlated in three other C2C12 clones too, called A7, A1, and A3 (see Fig. 7; Table 1), transfected with vectors constitutively transcribing the PC4 insert *Bam*I-*Hind*III in the antisense direction under the control of other promoters, respectively SV40 (vectors pCDL-SR α and pSV2; Refs. 38 and 39) or the cytomegalovirus promoter (vector pRc-CMV). These clones presented, in common with A16, an impairment of myosin protein induction, measured after 48 h of exposure to C2FM. Sham-transfected clones showed normal increases in myogenin or myosin expression (Fig. 7; Table 1).

Thus, these results indicate that the inhibition of myogenin expression, as well as that of myosin, is genuinely dependent on the expression of antisense PC4 RNA, irrespective of the type of vector used.

Morphological analysis by phase contrast microscopy indicated that cultures of clones transfected with antisense construct kept 48 h in C2FM presented less cell fusion than did cultures transfected with the vector or the sense construct. In Fig. 8A are shown clones A16, VB, and S4. However, all the clones attained the fully differentiated phenotype when

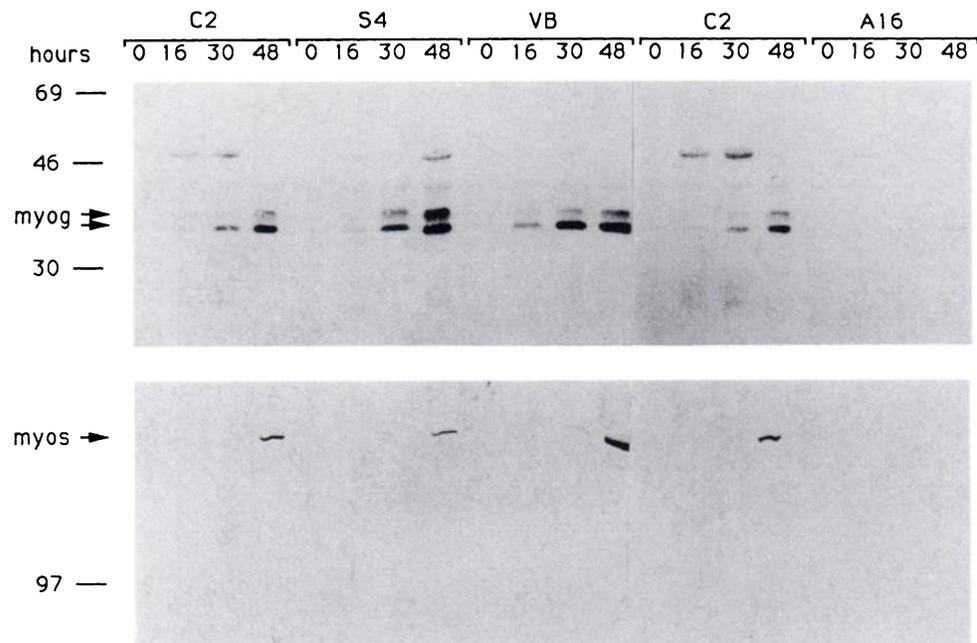


Fig. 6. Immunoblot analysis of the myogenin and myosin proteins in the C2C12 clones stably transfected with pBAP-PC4 and exposed to C2FM for the times indicated. Equal amounts of proteins were loaded onto 12 (*myog*) or 6% (*myo*) SDS-polyacrylamide gel, blotted on nitrocellulose filters, and incubated with the mouse mAbs against myogenin or myosin. The signal was detected by incubation with alkaline phosphatase-conjugated goat anti-mouse, followed by colorimetric development. The clones are indicated as in Fig. 5B. Ordinate, protein molecular weight standards in thousands.

Table 1 Analysis of the effects of under- or overexpressing PC4 on myogenin and myosin levels in C2C12 cells

Clone name	Vector transfected	Orientation of PC4 insert	PC4 RNA ^a (sense or antisense)	PC4 protein ^b	Myogenin induction (%) ^c	Myosin induction (%) ^c
S4	pBAP-neo-PC4	Sense	++++ ^d	++++	90	29
VB	pBAP-neo	Vector alone	N.D.	+++	100	100
VB-2	pBAP-neo	Vector alone	N.D.	+++	91	95
A16	pBAP-neo-PC4	Antisense	+++	---	2	1
VR	SR α	Vector alone	N.D.	+++	100	100
A7	SR α -PC4	Antisense	++	-	35	10
V2	pSV2	Vector alone	N.D.	+++	100	100
A1	pSV2-PC4	Antisense	+(+)	-	40	12
VM	pRc-CMV	Vector alone	N.D.	+++	100	100
A3	pRc-CMV-PC4	Antisense	+	+	76	14

^a Relative values of the vector's promoter-driven expression of PC4 RNA, measured by Northern analysis with riboprobes complementary to the sense or antisense sequence of PC4.

^b Relative PC4 protein expression, revealed by immunofluorescence staining.

^c Myogenin and myosin protein levels of the transfected cultures exposed 48 hours to C2FM, as detected by immunoblot analysis of the lysates. The amount of the latter two proteins was measured for each clone by densitometric scanning of the immunoblots and calculated as the percentage of the signal observed in the corresponding control clone (transfected with the same vector without PC4 insert). The clone pBAP was considered as control of pBAP-neo-PC4 transfected clones. The clones analyzed represent those detected by screening about 30 neomycin resistant clones for each type of vector transfected (about 6×10^5 cells per transfection).

^d +++++, 100–200%; +++, 75–100%; +(), 25–35%; ++, 35–50%; -, 5–10%; +, 10–25%; ---, 0%; ND, not detectable.

exposed for longer periods to C2FM (Fig. 8B, cultures maintained 144 h in C2FM). In these latter conditions in clone A16, the expression of myogenin, myosin, and PC4 proteins (virtually absent after 48 h in C2FM) was detectable (Fig. 8, C and D). This further indicates a correlation between the expression of PC4 and that of myogenin and myosin.

We then attempted to verify these findings with an independent approach, inhibiting the activity of the endogenous PC4 protein in C2C12 cells by microinjecting anti-PC4 affinity-purified antibody. C2C12 cells were injected in the cytoplasm, refed after 4 h with C2FM, and allowed to differentiate by exposure to C2FM for 48 h. The cells injected with A451 antibody expressed myogenin with significantly lower frequency than did cells injected with control antibodies (Fig. 9; Table 2).

This technique permits unequivocal identification of the microinjected cells and thus directly correlates the microinjection of antibodies to the cellular effects with no uncertainty. Therefore, we conclude that the activity of the PC4 protein in some way plays a part in the induction of myogenin protein during myoblast differentiation. This suggests that PC4 might be necessary for normal muscle development.

Discussion

Functional Significance of PC4 in the Differentiating Myoblast. We have shown here that expression of the PC4 gene, which is NGF inducible in the PC12 cell line, decreases transiently in differentiating myoblasts, and that the

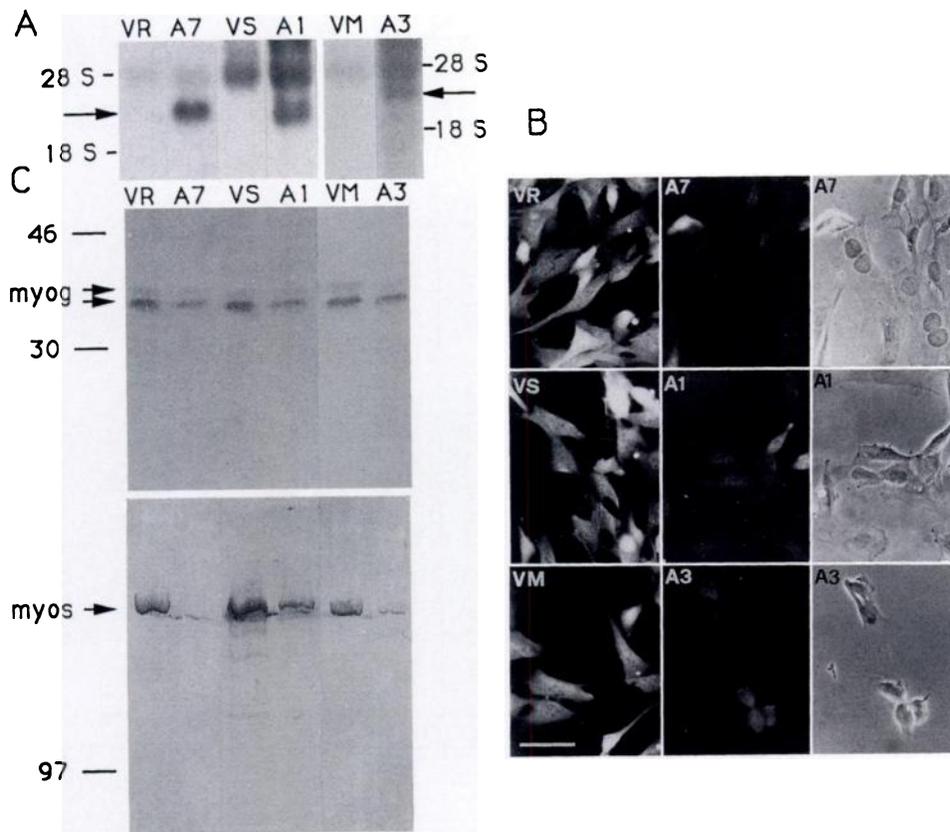


Fig. 7. Expression of PC4 antisense RNA, PC4 protein, and myogenin and myosin proteins in C2C12 clones stably transfected with pCDL-SR α , pSV2, and pRc-CMV vectors bearing antisense PC4 cDNA. PC4 antisense RNA (by Northern analysis; A), PC4 protein (by immunofluorescence staining; B), and myogenin and myosin proteins (by immunoblot; C), were detected as described in Figs. 5 and 6. Clones VR, V2, and VM were transfected with vectors (pCDL-SR α , pSV2, and pRc-CMV, respectively) without insert; clones A7, A1, and A3 were transfected with the corresponding vectors bearing PC4 antisense-oriented cDNA. (A) Arrow, PC4 antisense RNA; exposure time of the autoradiographs was 24 h. (B) Bar, 70 μ m. Myogenin and myosin protein were analyzed in cultures kept 48 h in C2FM (C).

inhibition of its expression impairs the induction of myogenin and myosin gene expression. Levels of *PC4* expression were similar in growing and fully differentiated cultures, and in cultures the differentiation of which was impaired by TGF β . On the other hand, TGF β counteracted the transient down-regulation of *PC4* expression. Since TGF β prevents myoblasts incubated in low serum medium from arresting the cell cycle (11), these latter observations point to an association between the transient down-regulation and either the proliferative arrest that precedes differentiation or the initial phase of the differentiation itself. However, we should consider that in our hands TGF β treatment delayed but did not prevent myogenin expression (which attained high levels within 36 h) and, thus, myoblast differentiation. Therefore a transient decrease of *PC4*, which was completely inhibited by TGF β treatment, does not appear to be required for differentiation. Furthermore, despite the absence in both the sense and antisense *PC4*-transfected clones of a transient down-regulation of the *PC4* protein (expressed respectively at a steadily high or low level), only the clones with a low level of *PC4* protein had impaired myogenin regulation. Such impairment was also observed in cells deprived of *PC4* protein by microinjection of anti-*PC4* antibodies shortly before inducing differentiation. Thus, the presence of *PC4*, rather than its transient decrease, might be a requirement for myogenin induction.

In clones with reduced expression of *PC4* and myogenin, myosin induction was impaired as well. Since heavy chain myosin is a structural protein marker for myoblast differentiation, this indicates that the inhibition of *PC4* expression negatively affects not only myogenin induction but also the

process of differentiation. Indeed, we detected morphologically a reduced extent of differentiation. Such effect is possibly in relation to the inhibition of myogenin. However, the antisense-transfected clones, which after 48 h in C2FM presented reduced myogenin and myosin levels, had not lost the ability to fuse and, eventually, to fully differentiate after long exposure to C2FM. In fact, in clone A16 we observed that after 144 h in C2FM the cellular fusion and the induction of myogenin and myosin, as well as the expression of *PC4*, did not significantly differ from the control clone. While attainment of differentiation can be explained by the restoration of myogenin levels, this in turn might be consequent to the recovered expression of *PC4*. We speculate that this latter event can be due to a feedback up-regulation of the endogenous *PC4* promoter activity. Alternatively, it could be supposed that cells expressing *PC4* antisense are prevented from differentiating only until the ongoing proliferation has diluted the antisense vector molecules in the cellular progeny to a number insufficient to inhibit endogenous *PC4* mRNA. Such a hypothesis would assume that the vector remains episomic.

In our view, in functional terms, adequate levels of *PC4* might be necessary at the moment when the cell receives the signal to differentiate, and myogenin induction is delayed until such levels are reached. On the contrary, the decrease of *PC4* expression which rapidly follows the onset of differentiation could be a marker of the arrest of proliferation or differentiation. Furthermore, the ensuing restoration of the basal levels of *PC4* appears to be unnecessary for myogenin induction, which begins before this restoration occurs (see Fig. 1).

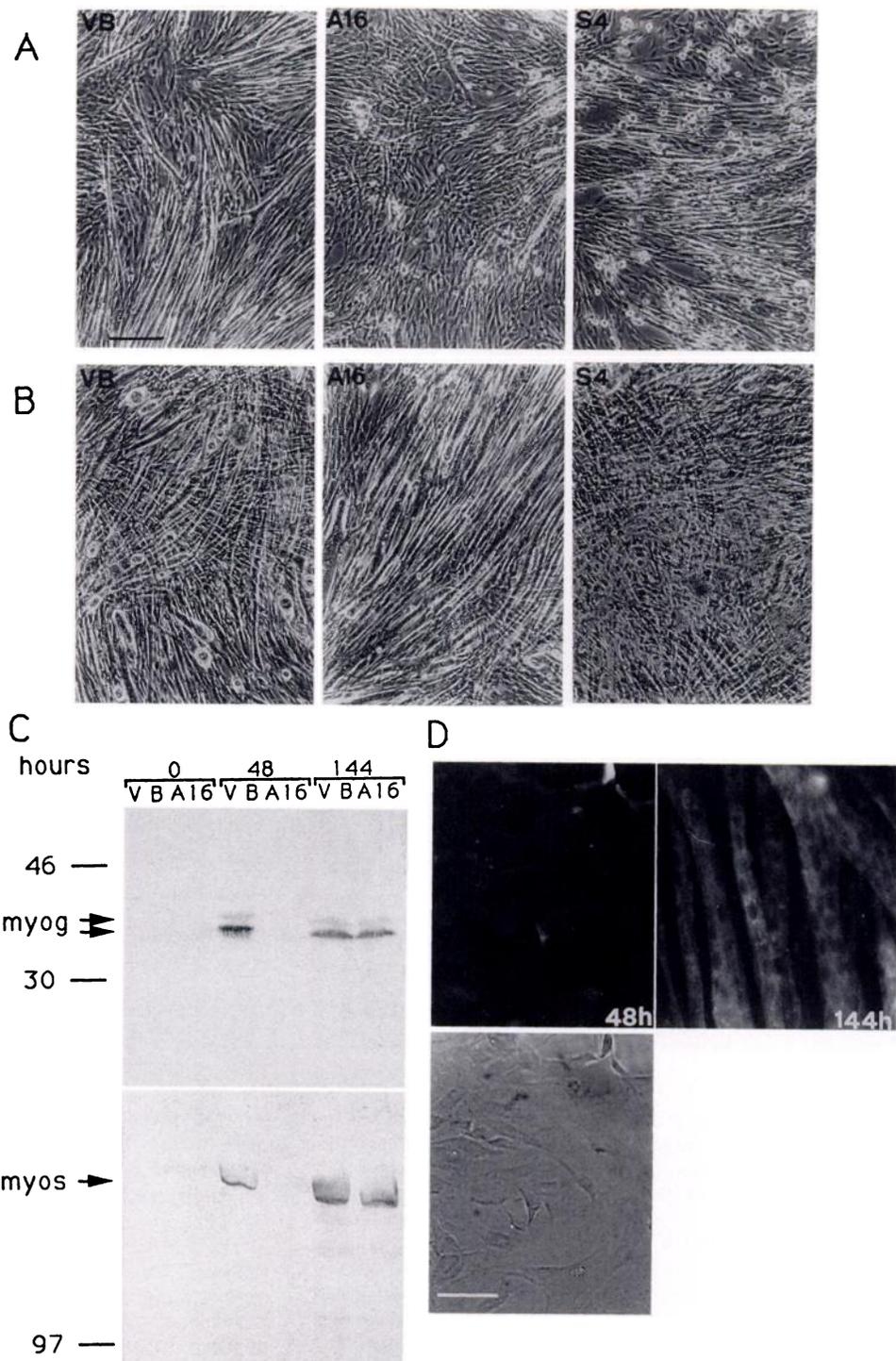


Fig. 8. Morphology of C2C12 cells transfected with antisense PC4 cDNA. Phase contrast micrographs of pBAP (VB), A16, and S4 clones cultured 48 h in C2FM (A) and 144 h in C2FM (B). Bar, 250 μ m. (C) Levels of myogenin and myosin proteins (by immunoblot, in clones pBAP and A16 cultured in C2FM for the times indicated); (D) Levels of PC4 protein in clone A16 [by immunofluorescence staining using the A-451 anti-serum in cultures kept 48 h (corresponding phase contrast field below) and 144 h in C2FM]. (D) Bar, 100 μ m. The procedures were as described above (Figs. 5 and 6).

Cellular Localization and Function of PC4 in Muscle. In C2C12 cells the PC4 protein appears to be localized in the cytoplasm. In undifferentiated PC12 cells PC4 is also clearly restricted to the cytosol. However, as the PC12 cultures reach a state of advanced neuronal differentiation elicited by NGF, the PC4 protein becomes localized in the nucleus (33). Several examples are known of proteins imported from the cytoplasm to the nucleus depending on extracellular

signals or biological cues [e.g., the IFN-stimulated gene factor 3a translocates to the nucleus on activation by IFN- α (ref. 40; for review see Refs. 41 and 42)]. The absence of nuclear translocation of PC4 in C2C12 cells, either differentiating or treated with NGF, might indicate that the translocation in PC12 cells is cell type specific. We cannot however completely rule out the possibility of a hormone-dependent mechanism common to both cell types; in fact,

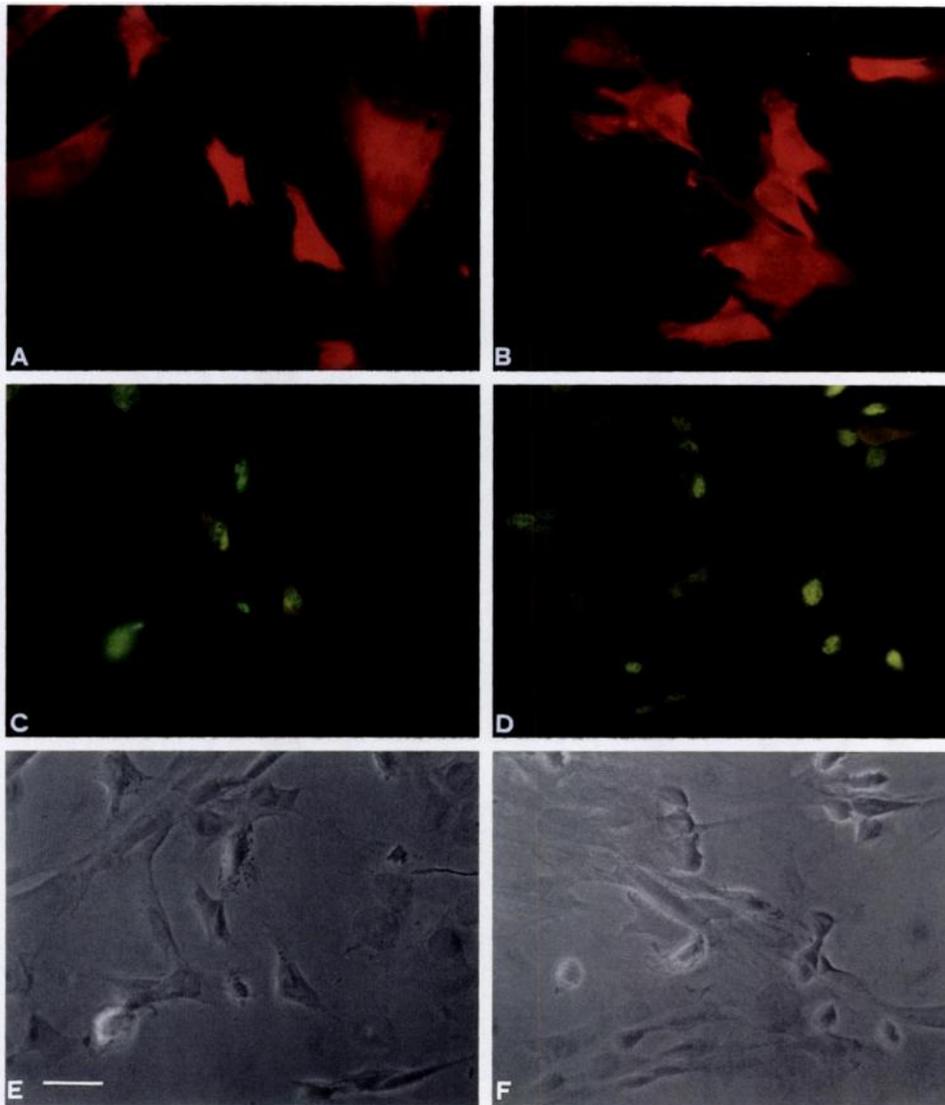


Fig. 9. Analysis of myogenin expression in C2C12 cells microinjected with anti-PC4 antibody. C2C12 cells growing on coverslips in C2GM were microinjected in the cytoplasm with control IgG (A, C, and E) or with A451 (B, D, and F). Four h later C2FM medium was added and left for 48 h (with an intermediate refeeding after 24 h). Thereafter, cells were fixed as described and processed for immunofluorescence. Each coverslip was double stained with goat anti-rabbit tetramethylrhodamine isothiocyanate-conjugated antibodies to visualize cells successfully microinjected (A and B), and with anti-myogenin [mouse monoclonal anti-myogenin/goat anti-mouse FITC-conjugated antibodies (C and D)]. The corresponding phase contrast images are presented (E and F). The fields shown are representative of experimental data (see Table 2). Bar, 40 μ m.

the lack of nuclear translocation in C2C12 cells might be dependent on the absence of appropriate signal transduction pathways for NGF. This is suggested by the inability of NGF to influence PC4 (and myogenin) mRNA expression in this cell system.

In speculating about the mechanisms underlying the regulation of PC4 in myogenesis, the localization of PC4 in the cytoplasm argues against direct effects on gene regulation in the nucleus; indeed, sequence analysis of the PC4 protein does not indicate the presence of any motif involved in control of transcription such as the helix-loop-helix motif of the muscle-specific regulatory proteins (26). However, in consideration of the effects on myogenin expression, it is possible that PC4 exerts a transcriptional regulation through unknown motifs or by indirect mechanisms, *e.g.*, by influencing the activity of transcription regulators. The possibility that the PC4 protein is transiently associated to the myoblast plasma membrane, as observed in PC12 cells by electron microscopy (33), should be investigated. In such a case, the function of PC4 could be related to the transduction of signals across the plasma membrane, at the onset of

the differentiative process. However, at this point it seems unlikely that PC4 could exert its biological actions by means of an autocrine mechanism, as is the case for interferons (7), considering that thus far we have been unable to detect any secretion of the protein in the cell medium (33). Interestingly, we observed that myogenin induction is impaired by microinjecting antibodies directed not only against the amino-terminal region of PC4, such as A451, but also against the carboxy-terminal region, such as the P1 antibody (see ref. 33).⁴ This suggests that the correlation observed between PC4 levels and myogenin induction might depend on molecular events requiring the integrity of the entire PC4 protein molecule (*e.g.*, protein conformation).

Functional Differences in Myogenesis between PC4 and Other NGF-inducible Genes. Interestingly, the expression of other genes induced by NGF in PC12 cells is down-

⁴ D. Guardavaccaro and F. Tirone, unpublished results.

Table 2 Effect of anti-PC4 antibody injection on myogenin induction

Experiment ^a	Control immunoglobulin			Anti-PC4 immunoglobulin		
	No. of injected cells analyzed ^b	No. of myogenin-positive cells	Myogenin induction (%) ^c	No. of injected cells analyzed ^b	No. of myogenin-positive cells	Myogenin induction (%) ^d
I	69	35	51	72	26	36
II	33	9	27	55	8	15
III	37	16	43	98	18	18

^a Performed as described in Fig. 9.

^b As visualized by detection with goat anti-rabbit tetramethylrhodamine isothiocyanate-conjugated antibodies.

^c Mean \pm SE = 40.3 \pm 7.0.

^d Mean \pm SE = 23.0 \pm 6.5; $P < 0.05$ vs. control immunoglobulin (Student's paired t test).

regulated in the differentiating skeletal myoblast, namely the proto-oncogenes *c-jun*, *c-myc*, and *c-fos* (3, 43–46), which all have the ability to inhibit the differentiation of the muscle cell (and, in the case of *c-fos*, probably also of the PC12 cells; see Ref. 47), albeit with different mechanisms. *c-myc* inhibits differentiation probably by stimulating cell proliferation and without directly affecting the regulation of *MyoD* (48), while *c-jun* and *c-fos* act by blocking the expression and function of *MyoD1* and myogenin through a direct interaction between the leucine zipper domain (at least in the case of *c-jun*) and the helix-loop-helix region of *MyoD* (43, 49, 50). Unlike *PC4*, the mRNA levels of the proto-oncogenes are permanently down-regulated in differentiated muscle cells (43–45), with the *c-fos* and *c-myc* proteins undergoing nucleus-cytoplasm exchange in all the cell types tested (51), including the muscle cell.⁵ It appears, therefore, that *PC4* has a peculiar profile, different from the other NGF-inducible genes regulated during muscle differentiation. In fact, *PC4* does not inhibit differentiation but appears to be necessary for the correct expression of the differentiated phenotype of the muscle cell.

Definition of the mechanisms through which *PC4* acts promises an insight into the process of muscle differentiation. It will also be important to assess whether the involvement of *PC4* in differentiation is muscle specific or also occurs in neuronal differentiation, as it has been suggested (7).

Materials and Methods

Cell Culture. C2C12 cells from the 16th passage were obtained from H. Blau (Stanford University, Stanford, CA) and propagated in C2GM (see above) in a humidified atmosphere of 12% CO₂ at 37°C. Differentiation was obtained by shifting the cultures to C2FM (see above), with a change of medium every 24 h. FCS and horse serum were from Hyclone Laboratories (Logan, UT). NGF (100 ng/ml) and porcine TGF β ₁ (British Biotechnology, Oxon, UK) were added to the cell cultures in the logarithmic phase of growth (about 40% confluent).

RNA Isolation and Analysis. Cytoplasmic RNA for Northern analysis was obtained from C2C12 cell cultures by homogenization in 4 M guanidine thiocyanate, followed by extraction with phenol-chloroform (52). The RNA was separated electrophoretically on 1.2% agarose/2.2 M formaldehyde gel, transferred to nitrocellulose filters, hybridized to a *PstI*-*PstI* fragment 0.8 kilobase long, and excised from the pCD-PC4 vector (7) and to a rat myogenin probe of 1.6

kilobases obtained by *EcoRI* digestion of the vector pEMS-myogenin (19). The probes were recovered after gel separation by GeneClean II (BIO 101, La Jolla, CA); ³²P-labeled with the hexamer primers procedure (53); and hybridized to filters in 50% formamide, 5 \times SSC (150 mM NaCl and 15 mM Na citrate, pH 7), 5 \times Denhardt's (1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumine), 0.1% SDS, and 0.5 mg/ml salmon sperm DNA for 20 h at 42°C. Then probes were washed at 55°C in 0.1 \times SSC and 0.1% SDS for 1 h. The integrity and the amounts of the RNAs were checked either by hybridizing the filters to the cDNA GAPDH or by ethidium staining of the gel. The intensity of the hybridization bands of the autoradiographs was quantified by an EPA 3000 densitometer (Sanwatsusho, Tokyo, Japan) in the linear range of the film. The intensity values of the sample, where indicated, were normalized to the corresponding values of GAPDH or ethidium bromide. The RNA probe for the analysis of the transfected clones was synthesized from a pGEM-3 vector (Promega, Madison, WI) bearing a *BamI*-*HindIII* fragment of the PC4 cDNA (pGEM-PC4), either in the sense or antisense orientation, linearized by *BamHI*, and transcribed by SP6 polymerase in the presence of [α -³²P]rCTP, with a transcription kit from Promega. Filters were hybridized in a 50 mM sodium phosphate buffer at pH 6.5 with 50% formamide, 5 \times SSC, 8 \times Denhardt's, 0.1% SDS, 0.25 mg/ml salmon sperm DNA, and 0.5 mg/ml of yeast RNA, for 20 h at 60°C. They were washed at 65°C in 0.1 \times SSC and 0.1% SDS for 1 h.

Expression of PC4 in *Escherichia coli* and Generation of Anti-PC4 Polyclonal Antibody. The antibody used, A451, had already been produced and characterized (33). Briefly, a fusion protein of β -galactosidase with part of the PC4 protein (amino acids 52–144) was obtained by subcloning the *PstI*-*PstI* fragment (nucleotides 299–577) from the coding region of PC4 cDNA in the *PstI* site of the vector pUEX-3 (Amersham, Little Chalfont, England). The resulting protein was expressed in *E. coli*, purified by elution from an SDS polyacrylamide gel onto which the bacteria lysate had been loaded, and used as immunogen in rabbits. The immune serum was then precipitated by addition of saturated ammonium sulfate (40% final concentration), dialyzed against PBS, and affinity purified through a column of cyanogen bromide-activated-Sepharose 4B resin conjugated to β -galactosidase-PC4 protein according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden; see Ref. 33). The buffer was exchanged with PBS and the antibody was concentrated with a Centricon 3 ultrafilter (Amicon, Danvers, MA). The antibody used for microinjection before the passage through Sepharose 4B resin conjugated to β -galactosidase-PC4 protein was additionally passed over a similar column conjugated to purified β -galactosidase protein.

⁵ S. Alemá, personal communication.

Microinjection was performed with the antibody at a final concentration of 4 mg/ml in 0.5× PBS. The specificity of the affinity-purified A451 antibody was analyzed by comparing the size and the tryptic digestion pattern of immunoprecipitates from whole cell lysates and from *in vitro* translated PC4 protein (33).

Control antibodies used for microinjection were immunoglobulins purified from nonimmune rabbit serum by affinity chromatography with protein A-agarose CL 4B resin, as per manufacturer's instructions (Pharmacia). The buffer was exchanged with PBS (0.5 ×) and the antibody was concentrated with Centricon 3, to 4 mg/ml.

Immunofluorescence Microscopy. Cells grown on polylysine-coated coverslips were washed 3 times with PBS, fixed for 10 min at room temperature in PBS containing 4% paraformaldehyde, and then permeabilized with 0.2% Triton in 0.1 M Tris-HCl (pH 7.5). After a PBS wash, the cells were incubated for 60 min at room temperature with the primary affinity-purified antibody, A451, diluted in PBS. After 2 washes in PBS the cells were incubated for 30 min at room temperature with FITC-conjugated (Myles-Yeda, Rehovot, Israel) goat anti-rabbit. Immunofluorescence was performed on a Leitz Dialux 22 microscope.

Calcium Phosphate Transfection. Cultures of C2C12 cells in 100-mm dishes containing approximately 6×10^5 cells were transfected by calcium phosphate precipitation (54). Vectors used for transfections were the β -actin promoter-driven vector pBAP-neo (34), the SV40 promoter-driven vectors pCDL-SR α (38) and pSV2 (39), and the human cytomegalovirus promoter-driven vector pRc-CMV (Invitrogen, San Diego, CA). These vectors were transfected either alone or bearing the *Bam*I-*Hind*III fragment of PC4 cDNA in the sense (only in the case of pBAP-neo) or antisense orientation. This fragment was obtained by excision from the pcD-PC4 cDNA (7) and subcloned in *Sal*I site of each of these vectors, with the exception of pRc-CMV. In this latter vector the fragment *Bam*I-*Hind*III of PC4, obtained from the vector pGEM-3-PC4 (as *Hind*III-*Sma*I fragment within the poly-linker; see Ref. 33), was subcloned in the *Hind*III-*Xba*I sites after blunt ending the *Xba*I site. Forty-eight h after transfection the cultures were transferred for selection into C2GM containing 800 mg/ml of G418 (GIBCO, Grand Island, NY), the resistance of which was provided by the neomycin resistance gene present (under control of the SV40 early promoter) in the vectors pBAP-neo, pRc-CMV, and pSV2 neo (this latter cotransfected with either pCDL-SR α or with pSV2 vectors; see Ref. 39). After 10 days, individual colonies were isolated and propagated into stable cell lines (about 30 per transfection of 6×10^5 cells with each sense or antisense construct) and analyzed for the expression of antisense PC4 RNA and PC4 protein.

The clones studied represent all those positive for the expression of antisense (or sense) PC4 mRNA.

Immunoblot Analysis. Transfected and control clones (*i.e.*, sham transfected with the vector alone) were plated to the same density of 1×10^6 cells in 60-mm plates and utilized after 24 h for the differentiation experiments by exposure to C2FM for the time indicated. Then transfected C2C12 cell cultures were harvested, centrifuged, and resuspended in loading buffer (50 mM Tris-HCl, pH 6.8-2% SDS-10 mM DTT-1 mM phenylmethylsulfonyl fluoride-1 mM leupeptin) and heated for 5 min at 100°C. Aliquots containing equal amounts of protein were analyzed by SDS/12 (for myogenin detection) or 6% (for myosin detection) PAGE. After electrophoresis, proteins were electrophoretically

transferred to nitrocellulose (4 h for the myogenin blot and overnight for myosin) at 120 mA in 24 mM Tris-HCl, pH 8.0-166 mM glycine-20% methanol. The filters were then incubated for 3 h with either a mouse monoclonal anti-myogenin (55) or anti-heavy chain myosin antibody (MF-20; see Ref. 43). As a secondary antibody, alkaline phosphatase-conjugated goat anti-mouse IgG (Pierce, Rockford, IL) was used following the manufacturer's instructions.

Microinjection. For microinjection, C2C12 myoblasts were grown on circular coverslips (10 mm diameter) in 35-mm Petri dishes containing 10^5 cells in C2GM. The cells were microinjected by an Eppendorf automatic microinjector (Model 5242; Eppendorf, Hamburg, Germany) as described (56) with the use of glass micropipettes (femtotips; Eppendorf), with a plastic tip for back filling (microloaders; Eppendorf). Each cell localized within a circular mark on the underside of the coverslip was injected in the cytoplasm for 0.5 s at a constant pressure of 100 hPa. With these conditions we estimate that the injection volume was about 10% of the volume of the cell. Four h after injection each culture was washed with PBS, shifted to C2FM, and analyzed after 48 h by double immunofluorescence labeling for the presence of the antibody injected and of myogenin. The protocol was as described above for PC4 detection, except that the antibody injected (either A451 or control) was visualized by goat anti-rabbit tetramethylrhodamine isothiocyanate-conjugated antibodies (Caltag Laboratories, S. Francisco), while myogenin was revealed by a mouse monoclonal anti-myogenin (55), followed by goat anti-mouse FITC-conjugated antibodies (Sigma Chemical Co., St. Louis, MO).

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