Overexpression of the Nerve Growth Factor-inducible *PC3* Immediate Early Gene Is Associated with Growth Inhibition[1]

Alessia Montagnoli, Daniele Guardavaccaro, Giuseppe Starace, and Felice Tirone[2]

Istituto di Neurobiologia, Consiglio Nazionale delle Ricerche [A. M., D. G., F. T.] and Istituto di Medicina Sperimentale [G. S.], Viale Carlo Marx 15, 00156 Rome, Italy

Abstract

***PC3* (pheochromocytoma cell-3) is an immediate early gene isolated as sequence induced in the rat PC12 cell line during neuronal differentiation by nerve growth factor (NGF). *PC3*, which is expressed *in vivo* in the neuroblast when it ceases proliferating and differentiates into a neuron, has partial homology with two antiproliferative genes, *BTG1* and *Tob*. Here we report that overexpression of *PC3* in NIH3T3 and PC12 cells leads to marked inhibition of cell proliferation. In stable NIH3T3 clones expressing *PC3*, the transition from G_1 to S phase was impaired, whereas the retinoblastoma (RB) protein was detected as multiple isoforms of M_r 105,000–115,000 (indicative of a hyperphosphorylated state) only in low-density cultures. Such findings are consistent with a condition of growth inhibition. Thus, *PC3* might be a negative regulator of cell proliferation, possibly acting as a transducer of factors influencing cell growth and/or differentiation, such as NGF, by a RB-dependent pathway. This is the first evidence of a NGF-inducible immediate early gene displaying antiproliferative activity.**

Introduction

NGF[3] is able to induce the differentiation of chromaffin cells of the adrenal medulla and the PC12 cell line (derived from a rat chromaffin-cell tumor) into sympathetic neurons (1). Several genes are induced immediately after NGF stimulation, pertaining to the category of IEGs, whose transcription is induced transiently. Such IEGs are also induced by mitogenic growth factors, and some of them are proto-oncogenes, *i.e.*, c-*myc*, c-*fos*, and c-*jun* (2, 3). Gene targeting has indicated that these genes apparently are not required for the growth of the animal until the early–mid-gestation period, but they are necessary for the growth and function of some specific cell types, *i.e.*, fibroblasts for c-*jun* (4) and bone and placenta for c-*fos* (5, 6). In general, these proto-oncogenes seem to be inhibitors of cell differentiation (reviewed in Ref. 7) and promoters of G_1 progression (8, 9), behaving on the whole as proliferation activators of specific tissues with rapidly dividing cells, under the control of mitogenic growth factors.

Thus, because NGF leads PC12 cells to exit from the cell cycle to attain differentiation (10), the induction of proto-oncogenes seems contradictory. A possible explanation is that the final decision for the cell to exit or remain in the cell cycle is reached by an interplay between different NGF-induced signals, either mitogenic (such as the above proto-oncogenes) or antimitogenic, and also according to the state of the cell, as suggested by growing knowledge of the cell cycle regulatory network (11). However, thus far, the existence of any such antimitogenic IEG responsible for NGF-dependent cell cycle arrest has not been verified.

In this context, the IEG *PC3*, isolated by us between genes activated at the onset of neuronal differentiation in PC12 cells by NGF (12) and by the Herschman group as tetradecanoylphorbol acetate-induced sequence from mouse NIH3T3 fibroblast cells (TIS21; Ref. 13), presents features somewhat different from those of other IEGs. We found that *PC3* is expressed during neurogenesis with a localization peculiar among IEGs as well as neural genes. In fact, *PC3* expression is restricted in space and time to areas where the last neuroblast proliferation occurs, *i.e.*, is coincident with the gradient of neuronal birth along the germinal zone of the neural tube, both in the brain and spinal cord regions, whereas no expression is detectable in the surrounding mantle zone, where the postmitotic neuron migrates (14). This observation, together with the fact that the PC3 protein shares a partial degree of homology with two novel proteins endowed with antiproliferative activity in NIH3T3 cells, BTG1 and Tob (60 and 40% homology, respectively; see Refs. 15 and 16), led us to hypothesize that *PC3* might exert a negative control of proliferation, acting *in vivo* within a defined time-window (*i.e.*, at the moment when the cell is committed to differentiation; see also Ref. 14). In the study presented here, we show that *PC3* expression, obtained by sense PC3 cDNA transfection of NIH3T3 and PC12 cells, leads to an inhibition of proliferation in parallel with the disappearance

Received 6/27/96; accepted 7/26/96.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported by Progetto Finalizzato Ingegneria Genetica of the Italian Ministero Pubblica Istruzione and partially by Associazione Italiana per la Ricerca sul Cancro. D. G. is a recipient of a fellowship from Progetto Finalizzato Ingegneria Genetica. A. M. is a recipient of a fellowship from Specializzazione Applicazioni Biotecnologiche Università Tor Vergata.
[2] To whom requests for reprints should be addressed, at Istituto di Neurobiologia, Consiglio Nazionale delle Ricerche, Viale Carlo Marx 15, 00156 Rome, Italy. Phone: 06-86090290; Fax: 06-86090370; E-mail: tirone@biocell.irmkant.rm.cnr.it.
[3] The abbreviations used are: NGF, nerve growth factor; IEG, immediate early gene; RT-PCR, reverse transcription-PCR; EGF, epidermal growth factor; RT, reverse transcriptase.

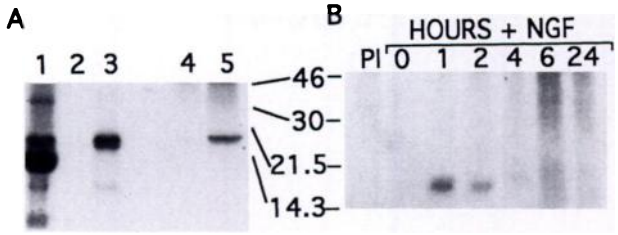

Fig. 1. NGF-dependent expression of the PC3 protein in PC12 cells. *A*, comparison of the PC3 protein [^{35}S]methionine-labeled *in vitro* and from PC12 cultures, immunoprecipitated by A3H polyclonal antibody. Rabbit reticulocyte lysates were programmed with 0.5 μg of PC3 RNA transcribed with T3 RNA polymerase. The translation product was directly loaded on the 15% SDS-polyacrylamide gel (*Lane 1*) or immunoprecipitated with pre-immune serum (*Lane 2*) and antibody A3H (*Lane 3*). *Lanes 4* and *5*, immunoprecipitates from PC12 cells (untreated or treated for 1 h with NGF). *B*, time course of the induction in PC12 cells of PC3 protein synthesis by NGF. About 2×10^6 cells in 35-mm plates preincubated in medium without methionine for 1 h were exposed to [^{35}S]methionine for 1.5 h and then harvested. Before harvesting, NGF was added to the cultures for the time indicated (h). An aliquot of the whole lysate was immunoprecipitated with the pre-immune serum (*PI*) or A3H antibody. The immune complexes were loaded on a 12% SDS-polyacrylamide gel. The molecular weight standards are indicated.

of hyperphosphorylated forms of pRB, suggesting that *PC3* might have a role in cell cycle regulation.

Results

Expression of the PC3 Protein. The PC3 cDNA predicts an open reading frame of 158 amino acid residues, with a calculated molecular mass of 17,731 kDa (12). To detect the PC3 protein, we raised a polyclonal antibody (A3H) against a bacterial fusion protein containing the PC3 protein. This was obtained by cloning the complete coding region of PC3 cDNA in frame with a polyhistidine-tagged peptide encoded by the pRSETA vector (see "Materials and Methods"). A protein of the predicted relative molecular mass was detected by immunoprecipitation with the A3H antibody of the *in vitro*-translated PC3 cDNA and the cellular PC3 protein from PC12 cells induced by NGF for 1 h (Fig. 1*A*). This would indicate that the cellular PC3 protein does not undergo post-translational modifications affecting the protein size, at least in PC12 cells. This molecular mass seems to be the same as that of the mouse homologue of PC3, TIS21 (13), as expected from a comparison of protein sequences (97.5% homologous). The expression of PC3 protein in PC12 cells, labeled for 90 min with [^{35}S]methionine and exposed to NGF for increasing lengths of time, showed a peak 1 h after NGF addition, followed by a rapid and permanent decrease within 2 h after NGF addition (Fig. 1*B*). This kinetic is coincident with that observed for mRNA induction by NGF (12).

Cell Cycle-dependent Induction of *PC3* by Serum and NGF. A common feature of NGF-inducible IEGs, such as c-*fos*, c-*myc*, c-*jun*, and Krox-24 (also known as NGFI-A, TIS8, and Zif268), is the activation of their transcription by serum during G_0 to G_1 transition (17–20). Also *BTG1*, an antiproliferative gene related to *PC3*, is induced by serum in NIH3T3 cells (15). Therefore, we sought to assess the possibility of a cell cycle-dependent expression of *PC3*. We found that PC3 mRNA expression in cells arrested in G_0 by serum starvation, either NIH3T3 (Fig. 2*A*, 0 h) or PC12 cells (Fig. 2*C*, 0 h), was equivalent to the expression observed in nonsynchronized exponentially growing cultures, *i.e.*, undetectable in NIH3T3 cells and relatively low in PC12 cells (Fig. 2, *A* and *C*, *Lane C*). When these synchronized cultures were stimulated by serum to enter and progress through the cell cycle as shown by the flow cytometry analysis (Fig. 2, *B* and *E*), PC3 mRNA levels increased considerably and transiently in NIH3T3 cells (Fig. 2*A*), whereas a barely detectable increase was seen in PC12 cells (Fig. 2*C*). In NIH3T3 cells, a rapid decrease ensued within 6 h after serum addition, returning to the undetectable level of G_0-arrested and asynchronously growing cultures (Fig. 2*A*). Clearly, serum induced the expression of PC3 mRNA in NIH3T3 cells, whereas the induction was scarcely detectable in PC12 cells, even within shorter periods after serum addition (Fig. 2*C* and data not shown).

When NGF was added to PC12 cells stimulated by serum to reenter the cell cycle, the resulting induction of PC3 mRNA was maximal in G_0 and the initial G_1 (Fig. 2*D*, 0 and 3 h after serum addition) and at the peak of the second G_1 (Fig. 2, *D* and *E*, 60 h), with similar levels. A lower level of induction of PC3 mRNA by NGF was also seen 72 h after serum addition, *i.e.*, at the onset of the second S phase, whereas it was virtually absent at the first S phase (24 h after serum addition). The most plausible explanation for this difference is a reduced homogeneity in cellular response during the second cycle progression, consequent to the reduced synchronization of the cell cultures (in this regard, in Fig. 2*E*, the G_1:S ratio of the first and second S phase, 24 and 72 h after serum addition, should be compared). As a whole, our data indicate that the induction of *PC3* expression by NGF occurs in G_0 and during the initial period of the G_1 phase, rather than during the late period.

Exogenous PC3 Overexpression Inhibits Cell Cycle Progression, with a Delay in G_1/S Transition. To verify directly if PC3 is able to inhibit cell proliferation, we overexpressed PC3 in NIH3T3 cells. To this end, we used a retroviral vector containing a neomycin resistance marker, pBABE Neo, in which the complete coding region of PC3 cDNA was cloned (pBABE Neo-PC3). NIH3T3 cultures were infected with the virus and selected by neomicin resistance. Five independent clones expressing exogenous PC3 mRNA as determined by RT-PCR were chosen for further characterization (Fig. 3, *A* and *B*). In general, positive clones were obtained with a frequency of 10–15%. The retroviral PC3 transcript expressed in these clones was translated into protein detectable by immunoprecipitation (with the expected mass of about 18 kDa; Fig. 3*C*) and immunofluorescence staining (Fig. 3*D*). The exogenous PC3 protein seems to be cytoplasmic, in agreement with data by Varnum *et al.* (21), although a nuclear localization of lesser evidence cannot be excluded (Fig. 3*D*). In control clones V1 and V2, obtained from NIH3T3 cells infected with the virus carrying no insert, the endogenous PC3 protein was almost undetectable, as expected (Fig. 3, *C* and *D*; see Refs. 13 and 21). Then, we determined the growth curve of the five PC3-expressing clones and control clones V1 and V2 as well as that of NIH3T3 parental cells by measuring the number of viable

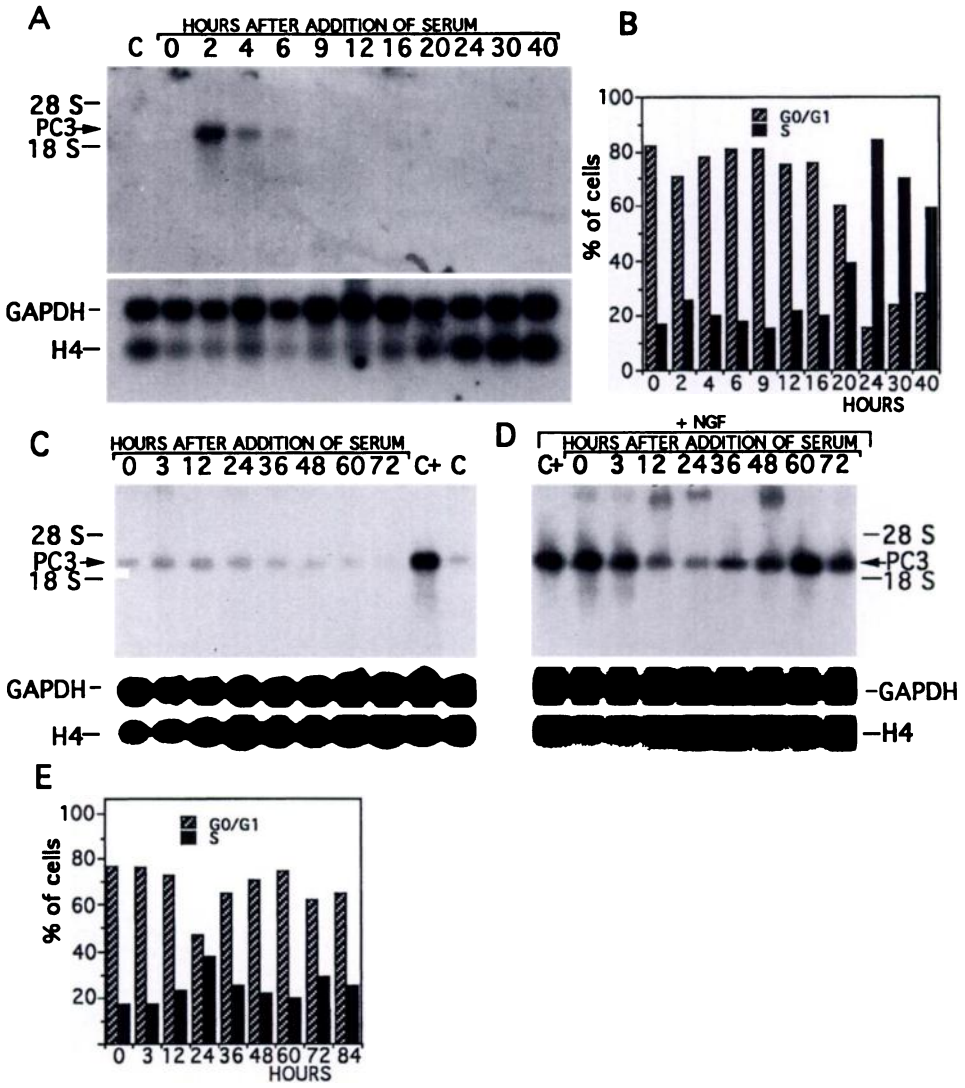

Fig. 2. Cell cycle-dependent induction of PC3 mRNA by serum and NGF. NIH3T3 cells (1×10^5/60-mm plate) were synchronized by serum starvation and collected after the addition of 10% FCS for the indicated times for parallel analysis of RNA levels by Northern blot (*A*; 5 μg RNA/sample) and DNA content by flow cytometry to determine the cell cycle profile (*B*, see "Material and Methods" for details). *Lane C* in *A* contains RNA from exponentially growing cultures. The same analysis for RNA levels (*C*) and DNA content (*E*) was performed on PC12 cell cultures (2×10^5/60-mm plate) synchronized by serum starvation and collected after the addition of 5% bovine serum and 5% horse serum for the indicated times. Cultures in *D* were as in *C* except that they were exposed to NGF (100 ng/ml) for 1 h before harvesting. *C* and *D, Lanes C* and *C*+ contained RNA from exponentially growing cultures that were untreated or treated with NGF (100 ng/ml for 1 h), respectively. Probes used had equivalent specific activity in all blots (about 1×10^6 dpm/ng for a total of about 60×10^6 dpm/7 ml), and exposure times for autoradiographs were the same (24 h for glyceraldehyde-3-phosphate dehydrogenase and 48 h for the other probes). The histone H4 expression was used as an independent observation of the S-phase period.

floating and adherent cells at different time points (Fig. 3*E*, see "Materials and Methods"). In general, clones overexpressing PC3 showed a significantly reduced proliferation rate during the exponential phase, and a plateau in growth was reached at or before confluence. The same cultures overexpressing PC3 resumed growth when replated at low density, although at a division rate lower than that of the control cells (data not shown). Furthermore, the inhibition of growth observed was not a consequence of reduced cell viability because the ratio between dead and viable cells was equivalent in control and PC3-expressing clones (data not shown). Further evidence in this direction was obtained by morphological evaluation of apoptosis using the DNA-specific stain H33258 as described (22). In fact, the nuclei of PC3-expressing cell clones were normal, with uncondensed chromatin dispersed over the whole nucleus. Fig. 4 shows a representative field from clone S7.

BrdU incorporation, measured in cultures of clones S1 and V2 (plated as duplicate samples of the growth curve of Fig. 3*E*), indicated a significant inhibition of DNA synthesis in clone S1 (*e.g.*, at day 3, mean ± SEM percent values of incorporation in clone S1 were 40.3 ± 1.1, whereas those for clone V2 were 97.4 ± 0.5; data not shown).

As a complementary approach to assess the inhibition of proliferation by PC3, we employed the colony formation assay, a technique widely used to evaluate the inhibition of proliferation by genes such as *RB* (23). The assay was performed in NIH3T3 and PC12 cells. The resulting number of G418-resistant colonies was significantly lower in NIH3T3 and PC12 cells transfected with the pBABE Neo-PC3 expression construct, compared to cultures transfected with the pBABE Neo vector without the insert or containing PC3 oriented in antisense direction (Table 1, experiments 1–3; Fig. 5; see "Materials and Methods"). Furthermore, we sought to rule out the possibility of differences in proliferation as a consequence of aspecific actions (*e.g.*, toxic) by the exogenous PC3 mRNA. Therefore, we transfected NIH3T3 cells with a pBABE Neo-PC3 expression construct bearing a nonsense mutation in the ATG codon (pBABE Neo-PC3/P–; see "Materials and

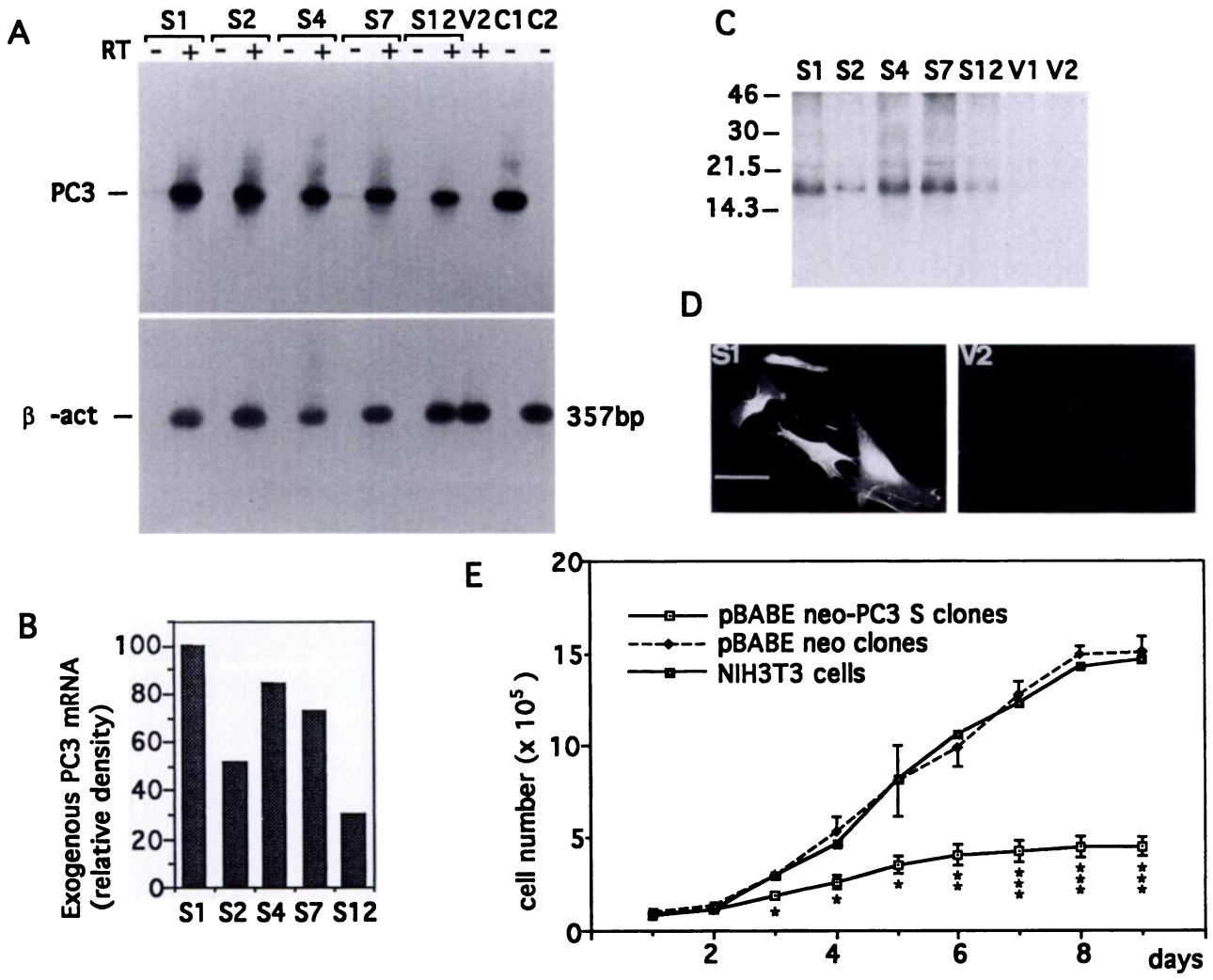

Fig. 3. Inhibition of proliferation in NIH3T3 cell clones stably overexpressing PC3. *A*, equal amounts of RT-PCR products amplified from NIH3T3 clones stably infected by a virus carrying PC3 (*S1, S2, S4, S7* and *S12*) or without PC3 (*V2*) were electrophoresed, blotted on filter, and hybridized to PC3 and β-actin probes. *RT+* and *RT−*, products of coamplification performed in parallel on two aliquots of each RNA starting sample preincubated with RT or not preincubated with RT, respectively, to verify the absence of DNA contaminations. *C1* and *C2*, control amplifications with pBABE Neo-PC3 or β-actin cDNA as templates. *B*, relative levels of exogenous PC3 RNA calculated from densities of Southern blot (*A*) as ratios of PC3/β-actin PCR products, assuming the ratio of sample S1 = 100 [relative density = sample density × actin density sample S1 × 100/actin density sample × sample S1 density]. *C*, immunoprecipitates with A3H antibody from whole lysates of cell clones labeled with 0.1 mCi/ml [^{35}S]methionine (in 35-mm plates) for 90 min. An additional control clone (*V1*) obtained by infection with the virus carrying no insert was analyzed. *D*, immunofluorescence staining of cells from S1 and V2 clones. The antigen was revealed by FITC staining after incubation with A3H antibody. *Bar*, 60 μm. *E*, proliferation rates of cell clones overexpressing PC3 (*S1, S2, S4, S7* and *S12*) or without expression (*V1* and *V2*) and of parental NIH3T3 cells. Equal numbers of cells were plated at day 0 (6 × 10^4 cells in duplicate 35-mm plates). Shown at each time point is the number of viable cells (means ± SEM) of the two control or five PC3-expressing independent clones. Starting data for analysis were the mean values of each duplicate determination. Parental NIH3T3 cell values represent the means of duplicate determinations (see also "Material and Methods"). * ($P < 0.05$), ** ($P < 0.01$), or *** ($P < 0.001$) *versus* the corresponding value of vector-infected clones (Student's *t* test).

Methods"), producing an RNA differing essentially only for the inability of being translated, and found that the number of resulting G418-resistant colonies was not lower than in NIH3T3 cell cultures transfected in parallel with the same amount of pBABE Neo construct (Table 1, experiments 4–6). This indicates that the effect on proliferation (seen as inhibition of colony formation) is consequent only to expression of the PC3 protein.

We further investigated whether the impairment of proliferation by PC3 was specific to a defined stage of cell cycle progression. To this end, we analyzed by flow cytometry the kinetics of cell cycle reentry in cultures of S1, S4, and V2 NIH3T3 clones, made quiescent by serum starvation and stimulated to reenter the cell cycle by serum addition. We observed that clones expressing PC3, with respect to the control clone, presented a higher percentage of cells in G_0/G_1 at all times analyzed, with a significant difference 24 h after serum addition (Fig. 6). This latter time corresponded to the entry of the synchronized cultures into S phase (Figs. 6 and 2, *A* and *B*). Thus, overexpression of PC3 significantly impairs cell cycle progression from G_0/G_1 to S phase. Cell division was inhibited but not totally arrested, consistent with

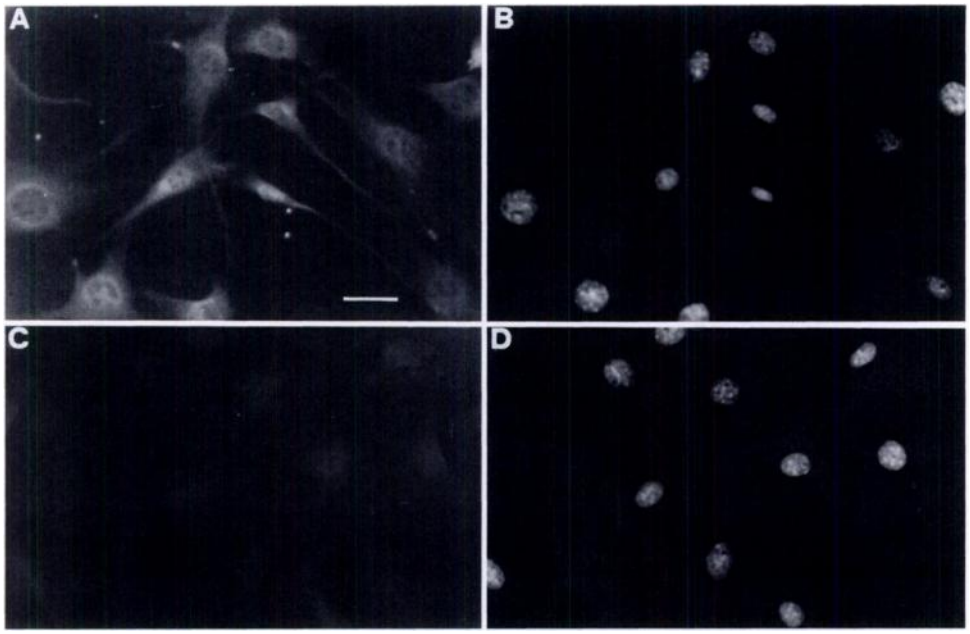

Fig. 4. Nuclear morphology of NIH3T3 cells stably expressing PC3. Immunofluorescence staining of cells from S7 (*A*) and V1 control clone (*C*); *right*, the corresponding field with fluorescent H33258-stained nuclei (*B* and *D*). *Bar*, 35 μm.

Table 1 Analysis by colony formation assay of the effects of *PC3* expression on NIH3T3 and PC12 cell growth

Transfected cells	Transfected DNA	Growth suppression by *PC3*[a] No. of G418-resistant colonies (experiment no.)					
		1	2	3	4	5	6
NIH3T3	pBABE Neo-PC3	13	1	10	15	12	12
	pBABE Neo	58	25	82	71	103	91
	pBABE Neo-PC3AS	52	53	95	–	–	–
	pBABE Neo-PC3/P–	–	–	–	74	101	107
PC12	pBABE Neo-PC3	192	4	18	–	–	–
	pBABE Neo	1155	251	83	–	–	–

[a] Assays were carried out as described in Fig. 5 and in "Materials and Methods."

the previous observation that clones expressing PC3 cultured in conditions stimulating cell growth (*i.e.*, plating at low density) do not cease to divide. As a whole, these experiments indicate that PC3 negatively affects the proliferation rate of both NIH3T3 and PC12 cells.

PC3 Overexpression Is Associated Preferentially to the Hypophosphorylated pRB Isoform. pRB is a key growth-suppressor protein whose activity is regulated by phosphorylation (for a review, see Ref. 24). In cultures growing exponentially and asynchronously, pRB is present mainly in a hyperphosphorylated state, whereas it is hypophosphorylated in conditions limiting growth (*i.e.*, leading to predominance of G_0/G_1 phase), such as serum deprivation or high cell density (25–27). Hyperphosphorylated pRB can typically be detected as multiplets of M_r 105,000–115,000, whereas the hypophosphorylated species can typically be detected as a single band of about M_r 105,000 (25, 26). Thus, we sought to assess whether pRB expression or its isoforms were modified by overexpressing PC3. To this end, we analyzed the expression pattern of pRB in control and PC3-expressing clones at different times after plating, in correlation with the cell density (Fig. 7). We observed that the pattern of multiple bands, consistent with pRB being in the hyperphosphorylated state, was detectable in clones S1 and S4 only at an early time point (day 1, corresponding to a cell density of about 8×10^3 cells/cm^2; see Fig. 7 legend), unlike control clones V1 and V2, in which multiplets could be detected clearly until day 3 (corresponding to a density of about 20×10^3 cells/cm^2; see Fig. 7 legend). At day 3, both V1 and V2 control clones and S1 and S4 clones were not yet confluent, having a density comparable to that seen at the corresponding time point of the growth curve (Fig. 3*E*). At day 5, control clones reached confluence at a density of about 70×10^3 cells/cm^2, and this was associated with the disappearance of forms corresponding to hyperphosphorylated pRB.

Thus, in PC3-expressing cells, the timing of the appearance of growth inhibition (day 3, 14×10^3 cells/cm^2 *versus* 20×10^3 cells/cm^2 in control clones) was correlated with the detection of the unphosphorylated pRB singlet species.

Discussion

Induction of PC3 by Serum and NGF. We have shown in this report that overexpression of the PC3 protein inhibits cell

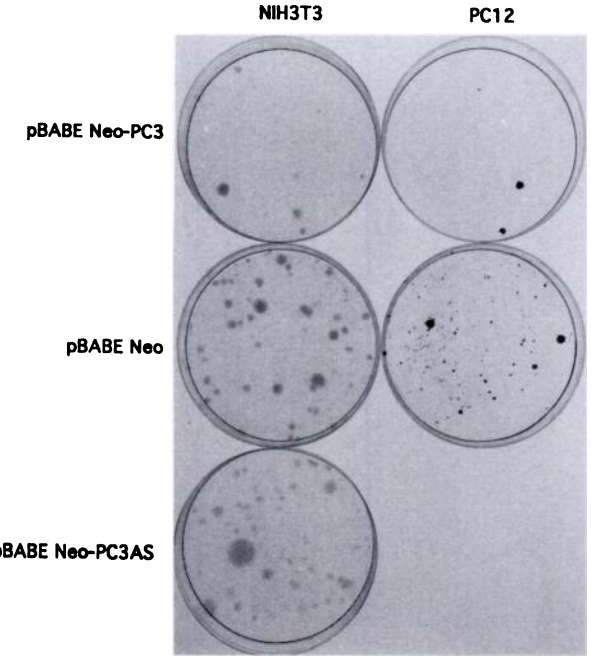

Fig. 5. Inhibition of proliferation by PC3 in NIH3T3 and PC12 cells observed by colony formation assay. Cell cultures (in 60-mm plates) were transfected with the indicated plasmids. After 48 h, cell cultures were trypsinized, diluted into 90-mm plates (1:150 for NIH3T3 cells and 1:40 for PC12 cells), and cultured in medium containing G418. The cultures were fixed and stained with Giemsa solution. The experiment shown is one of three reported in Table 1.

proliferation. The expression of PC3 mRNA is induced by serum when quiescent cells reenter the cell cycle and by NGF in the G_0 and G_1 phases. The strong induction by serum in NIH3T3 cells, as well as that observed previously in NIH3T3 cells by 12-*O*-tetradecanoylphorbol-13-acetate and in PC12 cells by the proliferative EGF (12, 13), seems somewhat contradictory for a gene induced by a differentiative and antiproliferative factor such as NGF (10). Activation by serum and 12-*O*-tetradecanoylphorbol-13-acetate in NIH3T3 cells and EGF in PC12 cells is common to all NGF-inducible IEGs; nonetheless, the conflict seems more relevant in the case of PC3, in consideration of its antiproliferative effects. However, recent data indicate that this conflict might be only apparent. It has been suggested that in PC12 cells, the difference in the effects of NGF and EGF can be ascribed to a quantitative rather than qualitative difference in the induction of the mitogen-activated protein (MAP) kinase cascade (whose activation is in itself sufficient to induce differentiation; Refs. 28–30). Indeed, quantitative as well as qualitative modifications might influence the effect of the PC3 protein. In fact, we have observed in NIH3T3 and PC12 cells that the inhibition of proliferation correlates with the level of expression of the PC3 protein and that the state of phosphorylation of PC3 changes in response to NGF.[4] Thus, additional studies aimed at clarifying the role of the PC3 protein should analyze its antiproliferative action as a function of the length of time of its expression, the cell cycle phase at which it occurs, and specific posttranslational modifications. Such analysis should also be performed for the induction of PC3 protein expression by EGF in PC12 cells, which is about one-fourth of that by NGF[5] (12).

Antiproliferative Action of PC3. The demonstration of an antiproliferative action of PC3 raises a compelling question about its physiological role. Cell growth inhibition by PC3 could be a signal for a temporary or definitive exit from the cell cycle, possibly acting as a prerequisite for other cellular states (*e.g.,* in PC12 cells, the NGF-dependent neuronal differentiation). Of note, the recently identified protein Tob (M_r about 45,000) presents homology in its amino-terminal half to PC3 and displays antiproliferative activity in NIH3T3 cells. The carboxyl-terminal half of Tob directly interacts with $p185^{erbB-2}$ (the product of the gene *c-erb-2*, a member of the EGF receptor family), and it has been proposed that Tob can act as a negative modulator of the proliferative signal triggered by $p185^{erbB-2}$ (16). We have not yet identified the PC3 target molecules. However, that observation, together with our data, raises the possibility of a general effect of PC3 on cell cycle linked to the signal transduction cascade of factors negatively or positively regulating cellular proliferation. This would be in line with the fact mentioned above that PC3 is induced not only by the proliferation inhibitor NGF but also by proliferative stimuli. In PC12 cells, exogenous overexpression of PC3 inhibits proliferation, as seen by the colony formation assay, but does not elicit differentiation in the absence of NGF.[6] This would indicate that PC3 is not sufficient to induce differentiation and that additional cellular signals are required to effect differentiation. Furthermore, the transient induction of PC3 by NGF in PC12 cells closely resembles the transient pattern of induction seen *in vivo* during neurogenesis, in which the expression of PC3 is limited to the moment of transition from the proliferating neuroblast to the postmitotic neuron (14). The present data are compatible with our previous hypothesis that PC3 might be a physiological effector of the growth arrest induced by developmentally regulated cellular cues in the neuroblast (14). However, neither NIH3T3 cells growth-arrested by serum starvation, the postmitotic neuron in the mantle zone of the neural tube, nor the neuronally differentiated chromaffin cell express PC3 (Ref. 14; data not shown). This indicates that in physiological conditions, PC3 is not associated with permanent growth arrest. Thus, if *in vivo* PC3 leads the cell to growth arrest, such a role, being effective only within a very short period, is preliminary to other signals downstream locking the cell in a permanent G_0 state.

Possible Mechanisms of PC3 Action. One of these signals might be pRB, a key growth-suppressor protein (reviewed in Refs. 31, 32, and 24). This protein regulates the cycle in most cell types (including the neuron cell, Ref. 33). Overexpression or hypophosphorylation of pRB inhibits progression into S phase; conversely, all the conditions deter-

[4] Unpublished observations.

[5] Unpublished observations.

[6] D. Guardavaccaro and F. Tirone, unpublished observations.

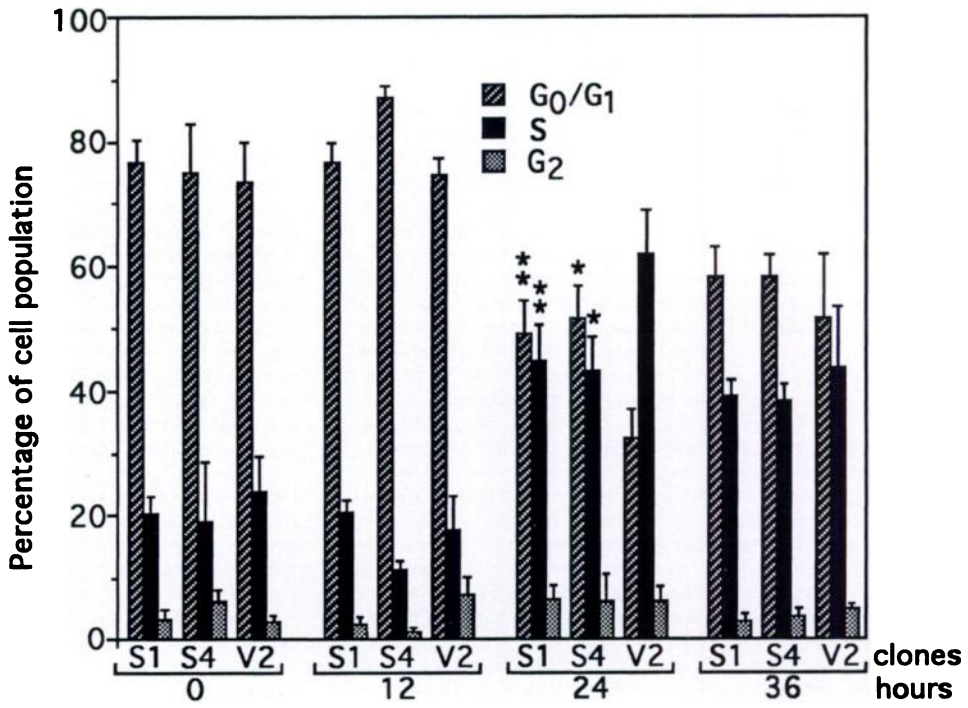

Fig. 6. Cell cycle progression after serum stimulation of G_0-arrested NIH3T3 clones overexpressing PC3. Clones S1, S4, and V2 were rendered quiescent by serum starvation and then stimulated to reenter the cell cycle. The percentages of cells in G_0/G_1, S, and G_2-M phase for each cell line at various times after serum stimulation were calculated from flow cytometric measurements of DNA contents during the ensuing cycle. Values are the means ± SEM of three experiments. *, $P < 0.05$ *versus* the corresponding value of the V2 clone; **, $P < 0.01$ *versus* the corresponding value of the V2 clone (Student's paired *t* test). Parental cells did not behave differently than the V2 clone infected with the virus without a PC3 insert.

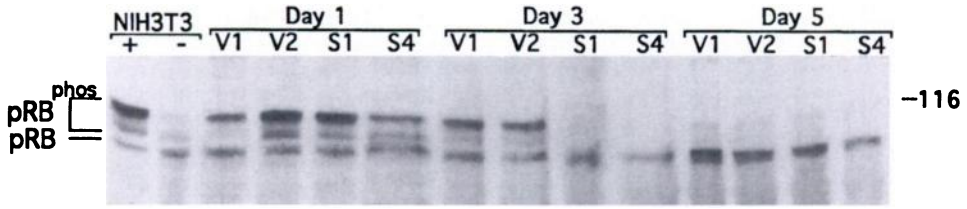

Fig. 7. Immunoblot analysis of the relative abundance of pRB isoforms in NIH3T3 clones overexpressing PC3. Clones stably infected with the virus either carrying PC3 (S1 and S4) or not (V1 and V2) were grown in 10% FCS. Parental cells were either grown in 10% FCS (*NIH3T3+*) or subsequently shifted to 0.5% FCS (*NIH3T3−*). Equal numbers of cells (4×10^5) were seeded into 90-mm culture plates. Clones were harvested at the indicated time points after plating, while parental cells were harvested after 2 days (*NIH3T3+*) or after 3 days plus an additional 72 h of exposure to 0.5% FCS, *i.e.*, when confluence had been attained (*NIH3T3−*). Fifty μg of whole cell extract protein were loaded on an SDS/7% polyacrylamide gel and blotted and incubated with G3-245 anti-pRB monoclonal antibody (0.5 μg/ml; PharMingen), followed by chemiluminescent assay. Multiplets (slower migrating bands) represent the hyperphosphorylated forms of pRB (*phos*). Cell densities at the different time points, calculated from cultures grown in parallel, were as follows (indicated as mean value for each pair of clones, cells/cm^2). Day 1: V1/V2, 7.8×10^3; S1/S4, 7.7×10^3; day 3: V1/V2, 20×10^3; S1/S4, 14×10^3; day 5: V1/V2, 70×10^3; S1/S4, 29×10^3.

mining pRB phosphorylation lead to cell proliferation (see Ref. 31). Thus, the control exerted by pRB on G_1 progression, by regulating the activity of genes responsible for the advancement of the cell through the initial phase of the cycle, is a function of the state of phosphorylation of pRB. In PC3-expressing NIH3T3 cultures, the pRB singlet species of M_r 105,000 corresponding to the hypophosphorylated (thus active) isoform becomes predominant at a cell density largely permissive for growth, whereas in control clones, the species corresponding to hyperphosphorylated pRB are still detected at an even higher density. This is consistent with the condition of growth inhibition observed in PC3-expressing cells. Certainly, based on these data, it is not yet possible to define whether in NIH3T3 cells expressing PC3 the earlier appearance of the active form of pRB, responsible for growth inhibition, is directly regulated by PC3 or not. Of note, it has been recently found that NGF represses pRB phosphorylation in PC12 cells, possibly by inhibition of the specific cyclin-dependent kinases responsible for pRB phosphorylation, indicating that these events are of central importance in the NGF-mediated block of cell cycling (34, 35). Thus, further work is necessary to ascertain the molecular mechanism by which PC3 influences pRB in NIH3T3 cells and whether these findings can be extended to the NGF-activated machinery in PC12 cells.

Materials and Methods

Cell Culture. NIH3T3 cells were cultured in DMEM containing 10% FCS (Hyclone Laboratories, Logan, UT) in a humidified atmosphere of 5% CO_2 at 37°C. PC12 cells were grown in DMEM containing 5% bovine serum and 5% horse serum (Hyclone Laboratories) in 10% CO_2. Synchronization was obtained by serum starvation for 72 h, *i.e.*, for NIH3T3 cells in DMEM

with 0.5% FCS and PC12 cells in DMEM without serum, according to procedures described by Rudkin *et al.* (10) and Roualt *et al.* (15). Medium exchange was preceded by three washes of cell cultures in serum-free DMEM.

Production of Anti-PC3 Polyclonal Antibody. A male New Zealand White rabbit (Gennari, Rome, Italy) was immunized with the entire PC3 protein (158 amino acids long; Ref. 12) fused at the NH_2-terminal with a Histidine-tagged peptide. The fusion protein was produced by expressing in *Escherichia coli* the vector pRSETA (Invitrogen, San Diego, CA) in which had been cloned the coding region of PC3 cDNA (nucleotides 65–541, with the stop codon), amplified by PCR using primers that incorporated 5′ *Xho*I and 3′ *Eco*RI sites and confirmed by sequencing. Bacteria were induced to express the fusion protein by the addition of 2 mM isopropyl β-thiogalactopyranoside. The protein was affinity-purified from inclusion bodies under denaturing conditions using a column of Ni^{2+}-charged Sepharose resin according to the manufacturer's instructions (Invitrogen), refolded from 8 M urea by dialysis against PBS, and concentrated with a Centricon 3 ultrafilter (Amicon, Danvers, MA). SDS-PAGE visualized a single band. Purified fusion protein (0.5 mg) was emulsified with an equal volume of complete Freund's adjuvant for the first injection and incomplete Freund's adjuvant for booster injections for a total of three injections every three weeks followed by monthly booster injections. The immune serum was then purified by affinity chromatography, the first time through a column of cyanogen bromide-activated Sepharose 4B resin (Pharmacia, Uppsala, Sweden) conjugated to a fusion protein of polyhistidine with the extracellular domain of the TrkB receptor protein (to subtract IgGs directed against the polyhistidine peptide); thereafter, the eluted volume was loaded through another column of cyanogen bromide-activated Sepharose 4B resin conjugated to the PC3 fusion protein. The buffer of the flow through was exchanged with PBS, and the antibody was concentrated with a Centricon 3 ultrafilter. This affinity-purified antibody, called A3H, was used in all the procedures for PC3 protein detection. The specificity of A3H was verified by size comparison of immunoprecipitates of the PC3 protein either *in vivo* from whole cell lysates of NGF-treated PC12 cells or *in vitro* synthesized in a rabbit reticulocyte lysate system (Promega, Madison, WI).

Detection of PC3 Protein *in Vitro* and *in Vivo*. The full-length PC3 protein was produced *in vitro* using a capped mRNA template synthesized by T3 RNA polymerase from a pBluescript KS vector in which the complete cDNA sequence of PC3 (see Ref. 12) had been cloned by us in the *Cla*I-*Xba*I sites.[7] The synthetic RNA was translated using a nuclease-treated rabbit reticulocyte lysate (Promega) in the presence of Pro-mix^{35}S (Amersham, Little Chalfont, United Kingdom) to a final concentration of 0.8 mCi/ml [^{35}S]methionine. Immunoprecipitation of the *in vitro* PC3 protein was performed using a described procedure (36). Briefly, the protein was denatured in 4% v/v (final concentration) SDS followed by heating in boiling water. The sample was precleared with pre-immune rabbit serum and immunoprecipitated with immune serum in the presence of 150 mM NaCl, 5 mM EDTA, 40 mM Tris-HCl (pH 7.4), 0.4% SDS, 1% Triton X-100, and Sepharose-protein A CL-4B (Pharmacia) as immunoadsorbent at 4°C for 3 h. The immune complexes were pelleted by centrifugation (5 min at 200 × *g* at 4°C), washed three times in immunoprecipitation buffer with 140 mM NaCl, and washed once in immunoprecipitation buffer without NaCl.

In PC12 and NIH3T3 cells, the PC3 protein was immunoprecipitated from cultures (about 1 × 10^6 cells onto 35-mm plates) preincubated in medium without methionine for an hour, then labeled by incubation in medium containing Pro-mix^{35}S (0.1 mCi/ml [^{35}S]methionine) added 90 min before harvesting. In PC12 cells, exposure to NGF (100 ng/ml) before harvesting was for the time lengths indicated. Lysis and immunoprecipitation were essentially as described (37). Cells were incubated for 30 min at 4°C in ice-cold lysis buffer [50 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 150 mM NaCl] containing 1% NP40, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. The sample was then precleared for 1 h at 4°C and immunoprecipitated for 2 h at 4°C in lysis buffer, and the immunocomplexes were washed three times in lysis buffer. In the immunoprecipitation procedures, no appreciable difference was observed between A3H anti-PC3 antibody and crude immune serum. Immunofluorescence staining of the PC3 protein in NIH3T3 cells was performed after the procedure described previously (38). Briefly, cells were fixed with 3.75% paraformaldehyde in PBS for 10 min at room temperature, permeabilized with 0.2% Triton in 0.1 M Tris-HCl (pH 7.5) for 5 min at room temperature, and incubated with A3H and then with the secondary antibody.

Infection of NIH3T3 Cells. The retroviral vector used was pBABE Neo, a Moloney murine leukemia virus-based vector carrying the neomycin resistance gene *neo* (39). In the *Bam*HI site of the pBABE Neo polylinker, a *Hin*cII-*Dra*I fragment of PC3 cDNA containing the entire coding region was cloned by addition of nonphosphorylated *Bam*HI linkers. The pBABE Neo-PC3 construct as well as the pBABE Neo vector without insert was transfected by conventional calcium phosphate procedures (40) into the helper-free ecotropic packaging cell line ψ*2* (grown in DMEM with 10% FCS; Ref. 41). Stably infected virus-producing ψ*2* cells were then obtained by selecting the transfected ψ*2* cultures in G418 (0.5 mg/ml). The virus was obtained by allowing the resistant clones to reach confluence, replacing the medium, and harvesting the virus-containing conditioned medium after 24 h. The latter medium was then filtered with a 0.45-μm filter and frozen in liquid N_2. The virus titer was >10^5/ml. This virus-containing medium, diluted to different extents (1:10 or 1:100) in medium and supplemented with polybrene (8 mg/ml), was added to NIH3T3 cultures (2 ml/100-mm plate) for 2 h. Fresh medium was then added, and after 48 h, the cultures were split 1:20 into selection medium (with 0.5 mg/ml G418). Several resistant clones were isolated from cultures infected with the virus either carrying or not carrying the PC3 sequence. The former were analyzed for PC3 expression by RT-PCR assay.

Cell Counts. The number of viable cells was determined as described by Soto and Sonneschein (42). The nuclei of viable cells are clearly visualized as phase-bright circles surrounded by a dark ring, whereas those of dying cells are opaque and smaller (see Refs. 43 and 44). Cell counts of samples analyzed in parallel by this method and by trypan blue dye exclusion presented equivalent values, within the experimental error. Briefly, to measure floating cells, the culture medium was removed, centrifuged, and decanted, and the resulting pellet was lysed with 0.5% ethylhexadecyldimethylammonium bromide, 0.28% acetic acid, 0.5% Triton X-100, 3 mM NaCl, and 2 mM $MgCl_2$ in PBS (pH 7.4) diluted 1:10. The same lysate was then applied to the corresponding cell plate, and after 2 min, the suspension was collected, and intact nuclei were counted. This procedure allowed us to count viable adherent and floating cells.

Morphological Evaluation of Apoptosis. Apoptosis was evaluated by analysis of nuclear morphology, as described (22). Immediately after immunofluorescence staining, samples were incubated with Hoechst dye 33258 (Sigma Chemical Co.; 8 mg/ml) for 5 min, washed, and mounted with PBS:glycerol (3:1).

BrdU Incorporation. DNA synthesis assay was performed as described (45). Cells were incubated for 18 h in culture medium containing 50 mM BrdU and fixed thereafter in 3.7% paraformaldehyde in PBS. DNA was partially denatured by a 10-s incubation in 50 mM NaOH followed by three washes in PBS. The incorporated BrdU was then revealed by an anti-BrdU mouse monoclonal antibody (Amersham) and a FITC-conjugated goat anti-mouse IgG (Sigma).

Colony Formation Assay. pBABE Neo vector carrying the full-length PC3 coding region in both sense (pBABE Neo-PC3; see above) and antisense (pBABE Neo-PC3AS) orientations or without the insert was transfected into NIH3T3 and PC12 cultures as described (23). Additionally, a pBABE Neo vector carrying a PC3 insert with a nonsense mutation in the ATG codon (pBABE Neo-PC3/P−) was transfected in NIH3T3 cells. pBABE Neo-PC3/P− was produced by cloning the coding region of PC3 cDNA, corresponding to the *Hin*cII-*Dra*I fragment of PC3 cDNA amplified by PCR using primers incorporating 5′ *Eco*RI and 3′ *Sal*I sites and carrying a mutated ATG codon (TAG), into pBABE Neo (*Eco*RI-*Sal*I sites). An equal number of cells were plated into 60-mm plates 16–20 h before transfection (performed at 25–60% confluence). Transfections of the plasmids pBABE Neo-PC3, pBABE Neo, and pBABE Neo-PC3AS were performed by either liposomes for PC12 cells (Lipofectamine Reagent; Life Technologies, Inc., Gaithersburg, MD; 3 μg DNA/plate), after the procedure described by the producer, or calcium phosphate (5 μg DNA/plate; Ref. 40) for NIH3T3 cells. After 48 h, the cultures were split into a 90-mm plate at serial dilutions and grown in medium containing G418 (0.5 mg/ml for NIH3T3 and 0.8 mg/ml for PC12 cells) to allow resistant cells to form

[7] A. Montagnoli and F. Tirone, unpublished data.

colonies. These were usually obtained in 2–3 weeks for NIH3T3 cells or 7 weeks for PC12 cells. The cutoff point for colony size was >20 cells/colony.

Flow Cytometry Analysis. For DNA content measurements, cells were trypsinized, pelleted by centrifugation, resuspended in PBS, further dissociated by 10 passages through a syringe with a 22-gauge needle, and fixed immediately thereafter in ethanol (70% final concentration). Fixed cells were then stained with propidium iodide (50 μg/ml) in PBS containing RNase A (Sigma Chemical Co., 100 kilounits/ml). The fluorescence was measured by an EPICS 541 flow cytometer (Coulter Electronics Inc.), exciting the fluorescence with the 488-nm line of an Argon-ion laser Innova 90 (Coherent) emitting a power of 50 mW and collecting the fluorescence after a 590-nm long-pass filter. DNA histograms were analyzed by a suitable mathematical model (46).

Preparation of RNA. Total cellular RNA used for Northern analysis and RT-PCR assay was obtained from cell cultures by homogenization in 4 M guanidine thiocyanate followed by extraction with phenol-chloroform (47). RNA for Northern analysis was separated electrophoretically, transferred to nitrocellulose filters, and hybridized as described (38) to a PC3 *PstI-PstI* 1.5-kb fragment excised from the pUC9-PC3 vector (12) and a mouse histone H4 cDNA fragment (48) labeled with the hexamer primers procedure (49). RNA was checked by hybridizing the filters to the glyceraldehyde-3-phosphate dehydrogenase cDNA. RNA for RT-PCR assay was digested before cDNA synthesis in RNase-free DNase (Promega; 1000 units/ml) for 30 min at 37°C.

RT-PCR Assay. Total cellular RNA (10 μg) was denatured at 75°C for 5 min and added to a total reaction volume of 50 μl containing 1 × RT buffer [10 mM Tris-HCl (pH 8.8), 50 mM KCl, and 0.1% Triton X-100], 5 mM $MgCl_2$, 0.5 mM of each deoxynucleotide triphosphate, 1 unit of RNAsin (Promega), and 600 pmol of random hexamer primers. Moloney murine leukemia virus RT (200 units; Promega) was added to half the reaction volume (25 μl) and incubated for 2 h at 37°C (the remaining reaction volume without RT was kept for use in PCR amplifications as a control for possible contamination of the sample with genomic DNA). RT reactions were stored at −20°C and then used for PCR amplification of specific samples. Two μl of each RT reaction were then amplified in a 100-μl PCR reaction containing 1 × PCR buffer [10 mM Tris-HCl (pH 9 at 25°C), 50 mM KCl, and 0.1% Triton X-100], 0.2 mM of each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, 20 pmol of each primer, and 2 units of *Taq* polymerase (Promega) after covering with 50 μl of mineral oil. Amplifications were carried out in an automated DNA thermal cycler (M. J. Research, Inc.) with the following profile: denaturation at 94°C for 60 s, primer annealing at 48°C for 60 s, and primer extension at 72°C for 90 s. The number of cycles was designed to maintain the reactions of amplification in exponential phase. This was determined to correspond to 30 cycles for the reactions with primer pairs specifying the PC3 sequence and to 20 cycles for the reaction with β-actin primer pairs, which were added after 10 cycles to each sample. Coamplification of β-actin mRNA gave a measure of the efficiency of the reaction and the starting RNA amount in each sample because β-actin is constitutively expressed in the cell lines used. One-tenth of the PCR sample was electrophoresed on 1.2% agarose gel, blotted on nylon filter, and hybridized to specific ^{32}P-labeled probes. These were the 1.5-kb *PstI-PstI* fragment of PC3 excised from the pUC9-PC3 vector (12) and a mouse β-actin cDNA fragment. The relative product amounts were quantitated by analysis with a Molecular Dynamics 400A PhosphorImager system. The PCR primers used were: (*a*) PC3, 5′ [5′-ATGAGCCACGGGAAGAGA-3′] and 3′ [5′-GTCGACCACTGTGCTGG-3′] (the latter primer is reverse complementary to the pBABE poly-linker in the 3′ region of the cloning site of PC3, thus the PC3-amplified product derives only from the PC3 exogenous viral transcript); and (*b*) cytoplasmic β-actin, [5′-TTGAGACCTTCAACACCC-3′] and 3′ [5′-GCAGCTCAT-AGCTCTTCT-3′] (50).

Acknowledgments

We thank Delio Mercanti for the gift of NGF, Maurizia Caruso for suggestions throughout the experimental work and critical reading, Patrizia Piccioli for critical reading, Giulio Draetta for comments on the manuscript, Tadashi Yamamoto for sharing data with us in the course of publication, Roberto D'Amico for statistical analysis, and Mariano Scippacercola for his contribution to our ideas and experiments. The mouse histone cDNA was a gift of M. L. Birnstiel.

References

1. Greene, L. A., and Tischler, A. S. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. USA, *73:* 2424–2428, 1976.

2. Greenberg, M. E., Greene, L. A., and Ziff, E. B. Nerve growth factor and epidermal growth factor induce rapid transient changes in proto-oncogene transcription in PC12 cells. J. Biol. Chem., *260:* 14101–14110, 1985.

3. Wu, B-Y., Fodor, E. J. B., Edwards, R. H., and Rutter, W. J. Nerve growth factor induces the proto-oncogene c-*jun* in PC12 cells. J. Biol. Chem., *264:* 9000–9003, 1988.

4. Johnson, R. S., van Lingen, B., Papaioannu, V. E., and Spiegelmann, B. M. A null mutation at the c-*jun* locus causes embryonic lethality and retarded cell growth in culture. Genes & Dev., *7:* 1309–1317, 1993.

5. Johnson, R. S., Spiegelmann, B. M., and Papaioannu, V. E. Pleiotropic effects of a null mutation in the c-*fos* proto-oncogene. Cell, *71:* 577–586, 1992.

6. Wang, Z. Q., Ovitt, C., Grigoriadis, A. E., Mohle-Steinlein, U., Ruther, U., and Wagner, E. F. Bone and hematopoietic defects in mice lacking c-*fos*. Nature (Lond.), *360:* 741–745, 1992.

7. Gonos, E. S., and Spandidos, D. A. Oncogenes in cellular immortalisation and differentiation. Anticancer Res., *13:* 1117–1122, 1993.

8. Goodrich, D. W., and Lee, W-H. Abrogation by c-*myc* of G_1 phase arrest induced by RB protein but not by p53. Nature (Lond.), *360:* 177–179, 1992.

9. Cosenza, S. C., Yumet, G., Soprano, D. R., and Soprano, K. J. Induction of c-*fos* and c-*jun* mRNA at the M/G_1 border is required for cell cycle progression. J. Cell. Biochem., *55:* 503–512, 1994.

10. Rudkin, B. B., Lazarovici, P., Levi, B-Z., Abe, Y., Fujita, K., and Guroff, G. Cell cycle-specific action of nerve growth factor in PC12 cells: differentiation without proliferation. EMBO J., *8:* 3319–3325, 1989.

11. Graña, X., and Reddy, E. P. Cell cycle control in mammalian cells: role of cyclins, cyclin-dependent kinases (CDKs), growth suppressor genes, and cyclin-dependent kinase inhibitors (CKIs). Oncogene, *11:* 211–219, 1995.

12. Bradbury, A., Possenti, R., Shooter, E. M., and Tirone, F. Molecular cloning of PC3, a putatively secreted protein whose mRNA is induced by nerve growth factor and depolarization. Proc. Natl. Acad. Sci. USA, *88:* 3353–3357, 1991.

13. Fletcher, B. S., Lim, R. W., Varnum, B. C., Kujubu, D. A., Koski, R. A., and Herschman, H. R. Structure and expression of TIS21, a primary response gene induced by growth factors and tumor promoters. J. Biol. Chem., *266:* 14511–14518, 1991.

14. Iacopetti, P., Barsacchi, G., Tirone, F., Maffei, L., and Cremisi, F. Developmental expression of the *PC3* gene is correlated with neuronal cell birthday. Mech. Dev., *47:* 127–137, 1994.

15. Rouault, J-P., Rimokh, R., Tessa, C., Paranhos, G., Ffrench, M., Duret, L., Garoccio, M., Germain, D., Samarut, J., and Magaud, J-P. *BTG1*, a member of a new family of antiproliferative genes. EMBO J., *11:* 1663–1670, 1992.

16. Matsuda, S., Kawamura-Tsuzuku, J., Oshugi, M., Yoshida, M., Emi, M., Nakamura, Y., Onda, M., Yoshida, Y., Nishiyama, A., and Yamamoto, T. Tob, a novel protein that interacts with $p185^{erbB2}$, is associated with antiproliferative activity. Oncogene, *12:* 705–713, 1996.

17. Ryseck, R-P., Hirai, S. I., Yaniv, M., and Bravo, R. Transcriptional activation of c-*jun* during the G_0/G_1 transition in mouse fibroblasts. Nature (Lond.), *334:* 535–537, 1988.

18. Morgan, C. J., and Pledger, W. J. Cell cycle-dependent growth factor regulation of gene expression. J. Cell. Physiol., *141:* 535–542, 1989.

19. Lin, D., Shields, M. T., Ullrich, S. J., Appella, E., and Mercer, W. E. Growth arrest induced by wild-type p53 protein blocks cells prior to or near the restriction point in late G_1 phase. Proc. Natl. Acad. Sci. USA, *89:* 9210–9214, 1992.

20. Lemaire, P., Revelant, O., Bravo, R., and Charnay, P. Two mouse genes encoding potential transcription factors with identical DNA-binding domains are activated by growth factors in cultured cells. Proc. Natl. Acad. Sci. USA, *85:* 4691–4695, 1988.

21. Varnum, B. C., Reddy, S. T., Koski, R. A., and Herschman, H. R. Synthesis, degradation, and subcellular localization of proteins encoded by the primary response genes *TIS7/PC4* and *TIS21/PC3*. J. Cell. Physiol., *158:* 205–213, 1994.

22. Oberhammer, F. A., Pavelka, M., Sharma, S., Tiefenbacher, R., Purchio, A. F., and Schulte-Hermann, R. Induction of apoptosis in cultured hepatocytes and in regressing liver by transforming growth factor β1. Proc. Natl. Acad. Sci. USA, *89:* 5408–5412, 1992.

23. Qin, X-Q., Chittenden, T., Livingston, D., and Kaelin, W. G. Identification of a growth suppression domain within the retinoblastoma gene product. Genes & Dev., *6:* 953–964, 1992.

24. Weinberg, R. A. The retinoblastoma protein and cell cycle control. Cell, *81:* 323–330, 1995.

25. Chen, P-L., Scully, P., Shew, J-Y., Wang, J. Y. J., and Lee, W-H. Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. Cell, *58:* 1193–1198, 1989.

26. Buchkovich, K., Duffy, L. A., and Harlow, E. The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. Cell, *58:* 1097–1105, 1989.

27. Polyak, K., Lee, M-H., Erdjument-Bromage, H., Koff, A., Roberts, J. M., Tempst, P., and Massagué, J. Cloning of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell, *78:* 59–66, 1994.

28. Cowley, S., Paterson, H., Kemp, P., and Marshall, C. J. Activation of MAP kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH3T3 cells. Cell, *77:* 841–852, 1994.

29. Traverse, S., Gomez, N., Paterson, H., Marshall, C., and Cohen, P. Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Biochem. J., *288:* 351–355, 1992.

30. Traverse, S., Seedorf, K., Paterson, H., Marshall, C., Cohen, P., and Ullrich, A. EGF triggers neuronal differentiation of PC12 cells that overexpress the EGF receptor. Curr. Biol., *4:* 694–701, 1994.

31. Cobrinik, D., Dowdy, S. F., Hinds, P. W., Mittnacht, S., and Weinberg, R. A. The retinoblastoma protein and the regulation of cell cycling. Trends Biochem. Sci., *17:* 312–315, 1992.

32. Goodrich, D. W., and Lee, W-H. Molecular characterization of the retinoblastoma susceptibility gene. Biochim. Biophys. Acta, *1155:* 43–61, 1993.

33. Lee, E. Y-H. P., Hu, N., Yuan, S-S. F., Cox, L. A., Bradley, A., Lee, W-H., and Herrup, K. Dual roles of the retinoblastoma protein in cell cycle regulation and neuron differentiation. Genes & Dev., *8:* 2008–2021, 1994.

34. Buchkovich, K. J., and Ziff, E. B. Nerve growth factor regulates the expression and activity of $p33^{cdk2}$ and $p34^{cdc2}$ kinases in PC12 pheochromocytoma cells. Mol. Biol. Cell, *5:* 1225–1241, 1995.

35. Yan, G. Z., and Ziff, E. B. NGF regulates the PC12 cell machinery through specific inhibition of the Cdk kinase and induction of cyclin D1. J. Neurosci., *15:* 6200–6212, 1995.

36. Guardavaccaro, D., Montagnoli, A., Ciotti, M. T., Lotti, L., Di Lazzaro, C., Torrisi, M-R., Gatti, A., and Tirone, F. NGF regulates the sub-cellular localization of the NGF-inducible protein PC4 in PC12 cells. J. Neurosci. Res., *37:* 660–674, 1994.

37. Xiong, Y., Zhang, H., and Beach, D. Subunit rearrangement of the cyclin-dependent kinases is associated with cellular transformation. Genes & Dev., *7:* 1572–1583, 1993.

38. Guardavaccaro, D., Ciotti, M. T., Schäfer, B. W., Montagnoli, A., and Tirone, F. Inhibition of differentiation in myoblasts deprived of the interferon-related protein PC4. Cell Growth & Differ., *6:* 159–169, 1995.

39. Morgenstern, J. P., and Land, H. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res., *18:* 3587–3596, 1990.

40. Graham, F. L., and Vander Eb, A. J. A new technique for the assay of infectivity of human adenovirus DNA. Virology, *52:* 456–467, 1973.

41. Mann, R., Mulligan, R. C., and Baltimore, D. Construction of a retrovirus-packaging mutant and its use to produce helper-free defective retrovirus. Cell, *33:* 153–159, 1983.

42. Soto, A. M., and Sonneschein, C. The role of estrogen on the proliferation of human breast tumor cells (MCF-7). J. Steroid Biochem., *23:* 87–94, 1985.

43. Rukenstein, A., Rydel, R. E., and Greene, L. A. Multiple agents rescue PC12 cells from serum-free cell death by translation- and transcription-independent mechanisms. J. Neurosci., *11:* 2552–2563, 1991.

44. Volonté, C., Ciotti, M. T., and Battistini, L. Development of a method for measuring cell number: application to CNS primary neuronal cultures. Cytometry, *17:* 274–276, 1994.

45. Del Sal, G., Ruaro, M. E., Philipson, L., and Schneider, C. The growth arrest-specific gene, *gas 1*, is involved in growth suppression. Cell, *70:* 595–607, 1992.

46. Bertuzzi, A., Gandolfi, A., Germani, A., and Vitelli, R. A general expression for sequential DNA-fluorescence histograms. J. Theor. Biol., *102:* 55–62, 1984.

47. Chomczynski, P., and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem., *162:* 156–159, 1987.

48. Seiler-Tuyns, A., and Birnstiel, M. L. Structure and expression in L-cells of a cloned H4 histone gene of the mouse. J. Mol. Biol., *151:* 607–625, 1981.

49. Feinberg, A. P., and Vogelstein, B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem., *132:* 6–13, 1983.

50. Nudel, U., Zakut, R., Shani, M., Neuman, S., Levy, Z., and Yaffe, D. The nucleotide sequence of the rat cytoplasmic β-actin gene. Nucleic Acids Res., *11:* 1759–1771, 1983.