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Molecular cloning of PC3, a putatively secreted protein whose mRNA is induced by nerve growth factor and depolarization

(cDNA/growth factors/brain)

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ABSTRACT **PC3 is an immediate early gene induced by nerve growth factor in PC12 cells, a cell line derived from a tumor of the adrenal medulla that undergoes neuronal differentiation in the presence of nerve growth factor. This induction is independent of new protein synthesis as it can occur in the presence of cycloheximide. PC3 is also induced with similar kinetics, but at lower levels, by membrane depolarization (both *in vivo* and *in vitro*) and epidermal growth factor. It is induced at much lower levels by fibroblast growth factor and interleukin 6. *In vivo* it is found expressed in tissues, such as brain at embryonic day 13.5, placenta, amnion, and spleen, which are proliferating and/or differentiating. The deduced protein sequence from the cDNA indicates the presence of a signal peptide, suggesting that PC3 is secreted.**

Nerve growth factor (NGF), in addition to being a neurotrophic agent for sympathetic neurons and some sensory and central nervous system neurons (1, 2), also induces the differentiation of chromaffin cells of the fetal adrenal medulla (a neural crest-derived tissue) into sympathetic neurons (3). This differentiative activity is transcriptionally dependent (4), and a large number of genes induced by NGF in PC12 cells, a pheochromocytoma cell line widely used as an *in vitro* model of NGF's differentiative action (5), have been identified. These genes can be divided into two broad classes: early and late. Late genes are probably structural or contribute to the neuronal phenotype (6–9), whereas it is thought that the early genes are controllers of transcription involved in the commitment of the cell (10, 11). The finding that c-*myc* and c-*fos* (12), the glucocorticoid receptor gene NGFI-B (also known as TIS1 and nur/77) (13), and a putative transcription factor, NGFI-A (ref. 14; also known as PC1, Zif268, egr-1, Krox-24, and TIS8) are all early genes supports this idea.

In an effort toward investigating the commitment to differentiation initiated by NGF, we have isolated four early genes highly induced by NGF in PC12 cells; these were termed PC1, PC2, PC3, and PC4 (15). PC4 does not seem to correspond to the scheme of direct transcriptional control mentioned above. In fact, it encodes an interferon γ-related protein (11), which may be involved in neuronal differentiation in a fashion analogous to the differentiating effect of interferon γ on a wide variety of cells (16)—that is to say by an autocrine or paracrine mechanism.

The gene we describe here, PC3¶, encodes a putatively secreted protein that is induced by NGF with a relatively high specificity, when compared with the induction by epidermal growth factor (EGF), fibroblast growth factor (FGF), or interleukin 6 (IL-6). It is also induced by membrane depolarization both *in vitro*, caused by high KCl, and *in vivo*, by Metrazole, a convulsant agent. Membrane depolarization is a stimulus that has been shown to activate some immediate early genes induced by NGF, such as c-*fos*, NGFI-A, and NGFI-B, in the PC12 cell system as well as *in vivo* (17–19). In PC12 cells the first event involved in immediate early gene activation by depolarization appears to be the influx of Ca^{2+} through voltage-dependent channels (for review, see ref. 10). It has been reported that NGF also stimulates calcium influx (20), suggesting a possible common mechanism for immediate early gene activation.

MATERIALS AND METHODS

Cell Culture Techniques. PC12 cells (from D. Schubert, Salk Institute) were grown in Dulbecco's modified Eagle's medium with 5% supplemented calf serum (Irvine Scientific) and 5% horse serum (HyClone) in a humidified atmosphere of 12% CO_2 at 37°C. NGF (100 ng/ml, purified as described in ref. 21), EGF (5 ng/ml; Collaborative Research), N^6, $O^{2'}$-dibutyryladenosine 3',5'-cyclic monophosphate (Bt_2cAMP; 1 mg/ml; Sigma), FGF (50 ng/ml, a mixture of acidic and basic FGF from bovine brain; Boehringer Mannheim), heparin (2 μg/ml; Sigma), phorbol 12-myristate 13-acetate (PMA; 0.1 μM; Sigma), and IL-6 (1000 units/ml; purified by Giuseppe Scala, University of Naples) were added to cell cultures in the logarithmic phase of growth (≈75% confluent).

RNA Isolation and Analysis. Total cellular RNA obtained by extraction in guanidine thiocyanate (22) was used for 5' end amplification of PC3 cDNA as well as for Northern analysis. The RNA was blotted on nitrocellulose filters according to a described procedure (11). Densitometric analysis was performed where indicated.

Construction and Screening of the cDNA Libraries. The construction of two PC12 cell cDNA libraries, in the pUC9 and in the pcD Okayama–Berg (23) vectors, has been described, as well as the differential screening of the pUC9 library (11, 15). By using clone pUC9-PC3 (11, 15) as a probe, we isolated from the pcD library a clone that was a few nucleotides longer at the 3' end (pcD-PC3-25).

5'-End Amplification of PC3 cDNA. *Reverse transcription.* Thirty micrograms of total RNA from PC12 cells treated 1 hr with NGF in 10.9 μl of 1 mM Tris was denatured for 3 min at 65°C and added to 2 μl of 10× RVTase buffer (1× RVTase buffer = 50 mM Tris, pH 8.3 at 42°C/10 mM $MgCl_2$/70 mM KCl/1 mM dithiothreitol), 2 μl of a 20 mM solution of each dNTP, 0.5 μl (20 units) of RNasin (Promega Biotec), 1 μl (10 μCi; 1 Ci = 37 GBq) of [^{32}P]dCTP, 2.2 μl (20 pmol) of a 21-mer

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Abbreviations: NGF, nerve growth factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; IL-6, interleukin 6; Bt_2cAMP, N^6,$O^{2'}$-dibutyryladenosine 3',5'-cyclic monophosphate; PMA, phorbol 12-myristate 13-acetate; CHX, cycloheximide.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. M60921).

primer in the 5′ terminal region of pUC9-PC3 noncoding strand (5′-TCAGCTCGCTGGGCAGGAGCT-3′; called PC3-3), and 1.4 μl (20 units) of avian myeloblastosis virus reverse transcriptase (Promega Biotec) and incubated for 2 hr at 41°C. The sample was then loaded onto a Sephacryl S-200 column. The first peak of radioactivity was collected, adjusted to 23 μl, and added to 1 μl of 6 mM ATP, 6 μl of 5× tailing buffer (0.5 M potassium cacodylate, pH 7.2/10 mM $CoCl_2$/1 mM dithiothreitol), and 5 units of terminal deoxynucleotidyltransferase (Bethesda Research Laboratories), incubated 10 min at 37°C, and then heated for 15 min at 65°C.

Amplification. The volume of the cDNA reaction described above was adjusted to 200 μl, and 5 μl was taken for amplification using a modification of the RACE protocol of Frohman *et al.* (24). PCR was performed using one oligonucleotide (5′-GAGGATCCGAATTCTAGATGCAGC-TGGGGCTGGCTGAGT-3′; called PC3-2) whose sequence is complementary to a region of pUC9-PC3 found 5′ of the oligonucleotide used for cDNA synthesis and another oligonucleotide [5′-GACTCGAGTCGACATCGATTTTTT-TTTTTTTTTTT-3′, called $(dT)_{17}$ adaptor] that hybridized to the poly(A) tail added to the 5′ end of the cDNA. Both oligonucleotides contained sites for restriction enzymes used for cloning [*Xho* I, *Sal* I, and *Cla* I on the $(dT)_{17}$ adaptor and *Xba* I, *Eco*RI, and *Bam*HI on PC3-2]. Amplification was for 30 cycles (0.7 min at 94°C, 1 min at 50°C, and 1 min at 72°C) in 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris at pH 8.3, 0.01% gelatine, 250 μM dNTPs, and oligonucleotides at 1 pmol/μl. A single band was seen.

Cloning of the amplified cDNA. The amplified 5′ cDNA ends obtained from three independent procedures of amplification were purified from the gel and cloned in a Bluescript SK^+ vector (Stratagene; the constructs were called FD1, FE1, and FG1) or in a Bluescript KS^+ vector (constructs FA1, FB1, and FC1) in the *Cla* I–*Xba* I sites.

Sequence Analysis. The nucleotide sequences of FA1, FB1, FC1, FD1, FE1, and FG1 were determined with Sequenase DNA sequence kit (United States Biochemical) on the single-stranded plasmid packaged in the M13 helper phage grown in TG1 bacteria. The sequence of pUC9-PC3 was completely determined by chemical cleavage (25) and further checked with Sequenase, using oligonucleotides as primers. The nucleotide and the deduced protein sequences were then compared with those stored in GenBank, European Molecular Biology Laboratory, Swiss-Prot, and National Biomedical Research Foundation Protein Identification Resource data bases by using the programs WORDSEARCH (26) and FASTA (27).

Dissection of Embryos. The dissection of the embryo neural tube was performed as described (11) from pregnant rats (Bantin & Kingman, Fremont, CA), designated 0.5 day of gestation the morning after a midnight breeding, and included the forebrain, midbrain, and hindbrain vesicles.

RESULTS

Isolation of Full-Length PC3. RNA from PC12 cells treated for 1 hr with NGF was used to produce a cDNA library. This was differentially screened with RNA probes from NGF-treated (1 hr) and untreated PC12 cells. As previously described (11, 15), four induced clones were isolated. One of these, pUC9-PC3, was obtained as a partial clone: its length was 2.3 kilobases (kb), whereas the size expected by Northern analysis (see below) was ≈2.6 kb. Sequence analysis revealed that both 5′ and 3′ ends were missing. Since no longer clone was found in the cDNA libraries available (see above), we used a recently published PCR technique to extend and amplify the clone (24), obtaining in this way six independent clones (called FG1, FD1, FE1, FA1, FB1, and FC1) from three separate amplifications that covered the missing 5′ region (see *Materials and Methods*). The inserts of these clones were all the same length as the ethidium bromide-stained band observed after PCR amplification of the cDNA. The missing region of the 3′ end was determined by sequencing the clone pcD-PC3-25 derived from the Okayama–Berg library described above.

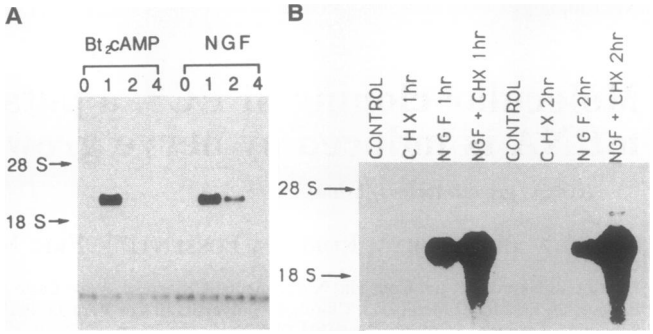


FIG. 1. Induction of PC3 RNA in PC12 cells by NGF and Bt_2cAMP (*A*) and its superinduction by NGF and CHX (*B*). (*A*) A Northern blot of 10 μg of total RNA from PC12 cell cultures treated with NGF (100 ng/ml) or Bt_2cAMP (1 mg/ml) for the indicated times was hybridized to the ^{32}P-labeled (70 × 10^6 dpm total) PC3 cDNA insert. ^{32}P-labeled mouse H4 histone cDNA (about 60 × 10^6 dpm; lower band) was added to each hybridization as a control. The filters were exposed for 6 hr at −70°C to x-ray film. (*B*) Total RNA (10 μg per lane) from PC12 cells treated with NGF (100 ng/ml) and with CHX (100 μg/ml), alone or together (in any case after a 30-min pretreatment with CHX), were hybridized to the ^{32}P-labeled PC3 probe (50 × 10^6 dpm total). The autoradiographs were exposed for 24 hr.

Regulation of PC3 mRNA by NGF, Growth Factors, and Other Agents. A labeled fragment of pUC9-PC3 that comprises most of the open reading frame (see below) was hybridized to an RNA blot from PC12 cells treated for different periods with NGF and Bt_2cAMP (Fig. 1*A*). The probe detected an RNA species of 2.6 kb, whose level was increased at least 15 times by NGF and Bt_2cAMP by 1 hr, returning to basal levels within 4 hr. This return to basal levels was quicker for Bt_2cAMP than for NGF. To find out whether this effect involves new protein synthesis, PC12 cells were treated with cycloheximide (CHX; 100 μg/ml, which is sufficient to block incorporation of >90% of [^{35}S]methionine

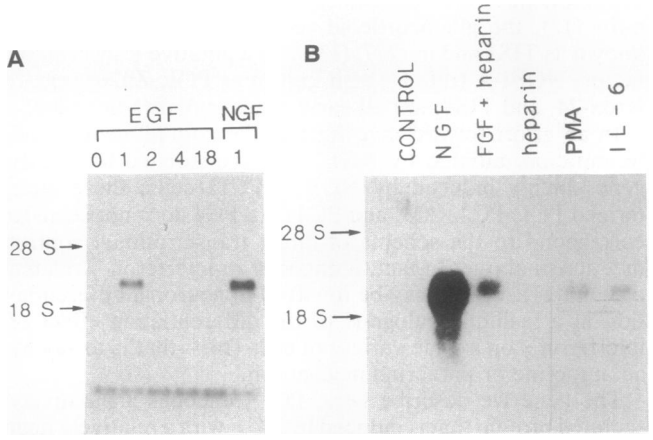


FIG. 2. Induction by EGF, FGF, PMA, and IL-6 of PC3 RNA levels in PC12 cells. Northern blots of total RNA (10 μg per lane) from PC12 cells treated for the times indicated (in hr) with EGF (5 ng/ml) and NGF (100 ng/ml) (*A*) or for 1 hr with NGF (100 ng/ml), FGF (50 ng/ml) plus heparin (2 μg/ml), heparin (2 μg/ml), PMA (0.1 μM), or human IL-6 (1000 units/ml) (*B*) were hybridized to the PC3 probe. The autoradiographs were exposed for a few hours (*A*) or overnight (*B*). The lower band in *A* represents the hybridization with the mouse H4 histone cDNA.

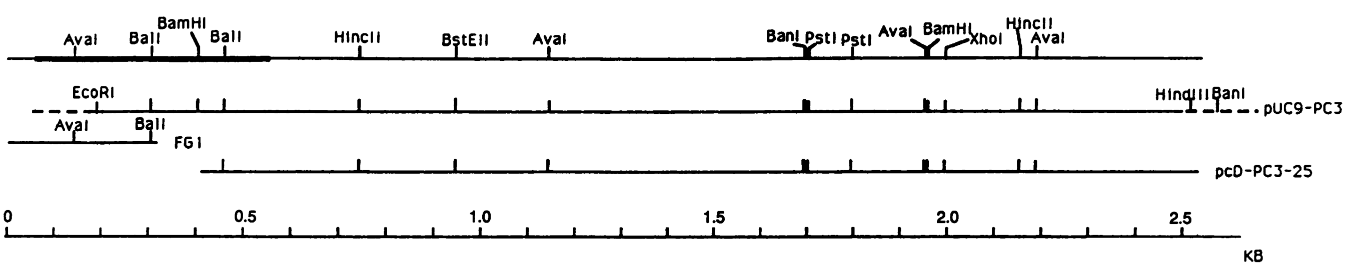
into acid-precipitable material). Instead of the 15-fold increase seen before, PC3 levels were increased in a time-dependent fashion, to a maximum of ≈100 times at 2 hr (Fig. 1*B*).

All other characterized immediate early genes induced by NGF in PC12 cells are also induced by EGF. Although the effect of this factor on PC12 cells is proliferative rather than differentiative, it displays several properties in common with NGF (28). When PC12 cells were treated with EGF or NGF at concentrations maximally effective in stimulating transcription of various NGF-induced genes (5 ng/ml and 100 ng/ml, respectively; ref. 12), PC3 was found to be induced 4-fold greater (by densitometric analysis) by NGF than by EGF, although with similar kinetics (Fig. 2*A*). Other factors that exert a phenotypic effect on PC12 cells include FGF, IL-6, and PMA. FGF (50 ng/ml), in the presence of heparin [which stabilizes and potentiates the effect of acidic FGF (29) but has no effect by itself] (Fig. 2*B*, lane 4), induced an increase of PC3 mRNA levels ≈1/15th of those induced by NGF, whereas PMA (0.1 μM) and IL-6 (1000 units) were even less effective (≈1/50th) (Fig. 2*B*).

Sequence Analysis of PC3. The complete sequence of PC3 was determined by analyzing eight overlapping clones: pUC9-PC3, pcD-PC3-25, and the six clones obtained from the PCR amplifications (Fig. 3). The full-length sequence was 2519 nucleotides long and contained two major open reading frames, one of 474 nucleotides (nucleotides 65–538, Fig. 3*B*) and a shorter one of 372 nucleotides (nucleotides 1838–2197), in addition to several much smaller ones (not shown). The first open reading frame, in addition to being the longest and bearing the first ATG start codon of the gene, is also, according to Gribskow *et al.* (30), the only portion of the molecule with a significant probability of encoding a protein. The protein encoded by this open reading frame is 158 residues long with a molecular weight of 17,731. The 3′ untranslated region is 1981 nucleotides long and occupies most of the mRNA. Within it can be found three A+T-rich sequences, containing the motif ATTTA (nucleotides 645, 1826, and 1849), which has been associated with the rapid degradation of mRNA and appears in several lymphokines and protooncogenes (31), as well as in PC4 (11). The lack of a polyadenylylation signal in pUC9-PC3 suggested that the 3′ end was incomplete. This was confirmed when the 3′ end of the clone pcD-PC3-25 was sequenced. This contained 20 additional bases, within which was also found a consensus polyadenylylation signal (nucleotide 2501) (32).

A computer-assisted search (Dec. 1990) for similarities of the deduced protein sequence with other proteins (see *Materials and Methods*) did not reveal any significant homologies. The search for active sites using the program PROSITE (33) showed two potential phosphorylation sites by protein kinase C [Ser-82 (Ser-Tyr-Arg) and Thr-108 (Thr-Cys-Lys)]. The hydropathic profile of PC3 protein obtained by the Kyte and Doolittle algorithm (34) showed a unique region of

A



B

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                                                                                  -28                             -21
                                                                                  Met Ser His Gly Lys Arg Thr Asp
   1            AGAGCCCGTGAGGCGGCCGGACCATCATCGTCCTGCTAATACAGCTACTTCCTCAACCACCGGT ATG AGC CAC GGG AAG AGA ACC GAC
                                       -11                                 -1  +1                                 10
     Met Leu Pro Glu Ile Ala Ala Ala Val Gly Phe Leu Thr Ser Leu Leu Arg Thr Arg Gly Cys Val Ser Glu Gln Arg Leu Lys Val Phe
  89 ATG CTC CCG GAG ATC GCC GCC GCC GTA GGT TTC CTC ACC AGT CTC CTG AGG ACT CGG GGC TGC GTG AGC GAG CAG AGA CTC AAG GTT TTC
                                       20                                  30                                     40
     Ser Arg Ala Leu Gln Asp Ala Leu Thr Asp His Tyr Lys His His Trp Phe Pro Glu Lys Pro Ser Lys Gly Ser Gly Tyr Arg Cys Ile
 179 AGT AGG GCG CTC CAG GAC GCA CTG ACC GAT CAT TAC AAA CAC CAC TGG TTT CCA GAA AAG CCA TCC AAG GGC TCC GGC TAT CGC TGT ATC
                                       50                                  60                                     70
     Arg Ile Asn His Lys Met Asp Pro Ile Ile Ser Lys Val Ala Ser Gln Ile Gly Leu Ser Gln Pro Gln Leu His Gln Leu Leu Pro Ser
 269 CGC ATC AAC CAC AAG ATG GAC CCC ATC ATC AGC AAG GTG GCC AGC CAG ATC GGA CTC AGC CAG CCC CAG CTG CAC CAG CTC CTG CCC AGC
                                       80                                  90                                     100
     Glu Leu Thr Leu Trp Val Asp Pro Tyr Glu Val Ser Tyr Arg Ile Gly Glu Asp Gly Ser Ile Cys Val Leu Tyr Glu Glu Ala Pro Val
 359 GAG CTG ACC CTG TGG GTC GAT CCC TAC GAA GTG TCC TAC CGC ATC GGG GAA GAT GGA TCC ATC TGC GTG CTG TAT GAG GAG GCG CCG GTG
                                       110                                 120                                    130
     Ala Thr Ser Tyr Gly Leu Leu Thr Cys Lys Asn Gln Met Met Leu Gly Arg Ser Ser Pro Ser Lys Asn Tyr Val Met Thr Val Ser Ser
 449 GCC ACC TCC TAC GGG CTC CTC ACC TGC AAG AAC CAG ATG ATG CTG GGC AGG AGC AGT CCA TCG AAG AAC TAC GTG ATG ACT GTC TCC AGC

     End
 539 TAG AGAGGAGCCGCCCCGCCCTGGCACTCTACTGTTCTCATGCTGCCCTGACAACAGGCCACCGTATACCTCAACCTGGGGAACTGTATTTTTAAAGTGAAGAGCTATTTATACATGT
 657 TATTTTTTTTTTTAAGAAAAGAGGAGGAAAAAAACCAAAAGTTTTTTTTAAAAAAACAAAAAAGAAAAAACAATTCGTTAACGGGAGCTGCTTGGAAGTGGTCTCCCCAGGTGCCTTT
 776 GGAGAGAACTGTTCTTGATTGAGTCTATGAGCCAGTGTTTGCCTAGGGGAGTGGGTTGGGGATTGGCCTAGCCAAGGTAAAAGGGGATTCTTGGCTGATCCCCCAGGAGGTGGTGGAAG
 895 GGAGCAAGGTTAGCAACTGTGAACGAGAGGGGTCAGGGTCTGCTCTGGGTTACCGTTCCCGCTGGGATGCCTGTATTCCTGGTCCCTCTCTTACTCAGGGGCATTCAAGCCTGGTCTCA
1014 AATAATACTACATTGCCTAATCTTCTCTTTTGTTTTTCTGCTGAGATCCTGGGCACACGGAAAGGCCTCTCCTGTCCCTTCCGTCTGAGCAGAGTTTCTTGAAACTGTGTCTCGTTTCT
1133 GATCCTACCCTCGGGGTCCTGAAGAGGTGGTTTCCCGGCCTAGAATCTATCTAAACGTTTTTGGAGGGTGGGCTATAAGGCAGATATAATGGAGGGGAACCGCACAAACCCTTTGCTTT
1252 GCTCTGTGCTGCTTTGTATGGATGGATGGTTAATAACTTAGGGATGATTTGCAATGGAATTTTGGGACCCAAAGAGTATCCAATGGGGGTGGGTGTTTTGGACCTAAGCCCTCCTTTTG
1371 GGAACCACGTGACAGTCTGAATGCTGCTACCATTATTCCTTTGAGAGGTGGCTCAAAGCTCCAGGGAACTCCAGGTCCTTTCTTACTGCCTTCTCTTCAAGAGCAACCTCCCCCATTTC
1490 TTTTCCCTCTTTCCTGCGGTTGGGTCCTGGAGGGCCCCATTTCCTAGGACAAGAGTTCTCAATCACTGTGCAATAGTCCCAGGAAGCTCTGGAACTGGGCCTCCCAGCCCCTCCTGATT
1609 CCTGGTGGGTTTTAGGACCCCGCCTTCCCCGTTCTTCTGACTGGCTGGTGGGCCTTGAGGAGATCTCCCTCGGCCGCAGGGAGGGCACCTGTGCACTGCAGGACTACCTGGTACTCCTG
1728 TGGGGCTGCCACGGAGAGCCAAACCTTAGGCATAGCTTTGTCTCCTCGGTGCTCAGAGCACCTGCAGGGGGAGGTTGCCCCCCTCAGTAAAAATCCAAATTTATTTGTAGATGTGTGCA
1847 ATATTTACTGTTCTGGGTTGGAGAAAATCGGGAAACACTGGGAAGAAGTGGCCTTCCTTCAGGTTCAGTGACACTGATGAGGGCTTCTCAGAAGGCCTCGAGTCTCTCAAACCAAAGGA
1966 CAGAGCTAGAGCCAGCCAGTCACCCTTAGTGAGGATCCCCTTCCCCATGTCTCTCCACTGCCGTGGCATCCCATGTCCTGGATTTCTCAATTCCTCAGTTTCTACTCAAAGGTGCTACT
2085 TACCAAACACTCTGCCCGTCCCGCTCTCCCCAGCTTCGCACAGCCGTCCCAGGTGGCTTCGTCTCTCCTGCTTTAAAGTTAACTTTGGGCCCACAGACCCGAGAGCTGTGGGTTGAAGC
2204 AAAGCTGTGAATCGCTCCAGATGGTCCCTGTGTTCTGTCCACACACAGGTCCCCGCCTTTTTAGAAGCAGCCTCTCGGTCTCATGCTTAAATCTGTTCCTCACTGCCCGTGTTCACTTT
2323 AGAAATGGCAGAACCACAGAGCTGGACTGTTGAGCAGGCCTGTCTCTCTCATTAAATAGAAATAAGTAAGTTTGTAAGCTATTCCGACAGAAGAGACAAAGGTTACTGATTGTACAATA
2442 GCGCTTTTATATGGAAGACTGTACAGCTTTATGGACAAATGTAAAACTTTTTTGTTTTTAATAAAAATGTAGCAGACC
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FIG. 3. Restriction map of full-length PC3 (*A*) and its complete nucleotide and predicted protein sequence (*B*). (*A*) Thick line, translated region; thick dashed line, plasmid pUC9; thin line, untranslated region. (*B*) The nucleotide sequence of PC3 is presented in the 5′-to-3′ orientation. Numbers above the translated sequence indicate amino acid residues of the translational open reading frame, assuming that the predicted signal peptide is cleaved as expected. Nucleotide numbers are on the left of the sequence. The polyadenylation signal and the ATTTA motifs are underlined. The dashed line corresponds to the putative signal peptide cleavage region.

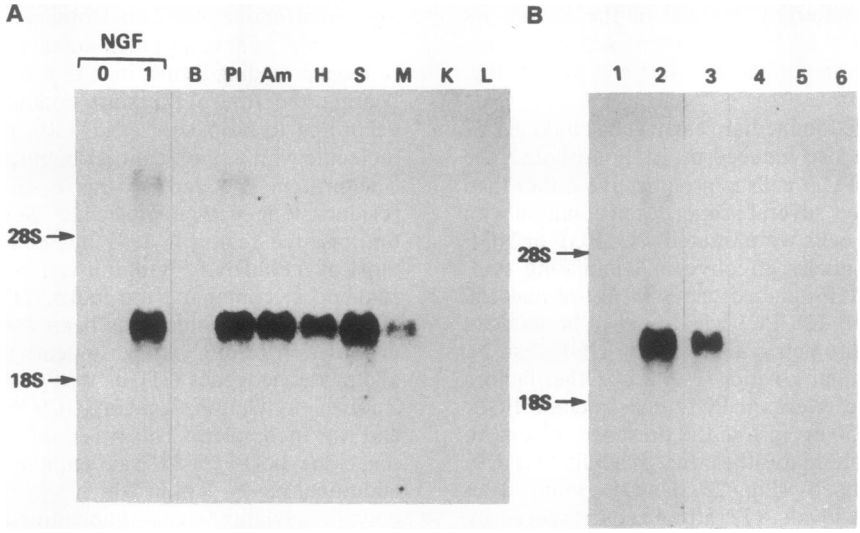

FIG. 4. Expression of PC3 in adult rat tissues (*A*) and in embryonic rat brain (*B*). (*A*) Total RNA (8 μg per lane) obtained from PC12 cells [control or treated 1 hr with NGF (100 ng/ml)], adult rat brain (B), placenta (Pl), amnion (Am), heart (H), spleen (S), muscle (M), kidney (K), and liver (L) were hybridized with the ^{32}P-labeled PC3 cDNA. Amnion and placenta were from 14-day pregnant animals. The lanes "0 hr NGF" and "1 hr NGF" were exposed for about one-seventh of the time of the other lanes. (*B*) Total RNA (8 μg per lane) isolated from the brains of embryos or of postnatal animals was hybridized to the PC3 cDNA. Lane 1, RNA from PC12 cells; lane 2, RNA from PC12 cells treated 1 hr with NGF (100 ng/ml); lane 3, RNA from the neural tube of 13.5-day embryos; lane 4, RNA from the brain of 15.5-day embryos; lane 5, RNA from the brain of 1-day postnatal animals; lane 6, RNA from the adult brain. Lanes 1 and 2 were exposed for a shorter time (as in *A*). The presence of equivalent amounts of RNA in *A* and *B* was assessed by ethidium bromide staining and by hybridization to a mouse β-actin probe.

hydrophobicity at the N terminus (amino acids 13–24 of the full length protein, or amino acids −16 to −5 if a signal sequence is removed). That this could be a signal peptide was confirmed by applying Von Heijne's algorithm (35, 36). PC3 is unlikely to be a membrane protein as no further hydrophobic regions, which could span the membrane, are found, suggesting that it is secreted.

Expression of PC3 in Tissues. The expression of PC3 was assessed in several tissues of the adult rat. PC3 mRNA is absent in the brain, liver, and kidney, whereas it is strongly expressed in placenta and amnion [tissues that are actively proliferating and differentiating (37)], heart, spleen, and, to a lower extent, in skeletal muscle (Fig. 4*A*). Although absent from adult rat brain, PC3 mRNA is present in the neural tube of 13.5-day embryos (a time when neuroblast proliferation and differentiation occurs) and disappears 2 days later (Fig. 4*B*).

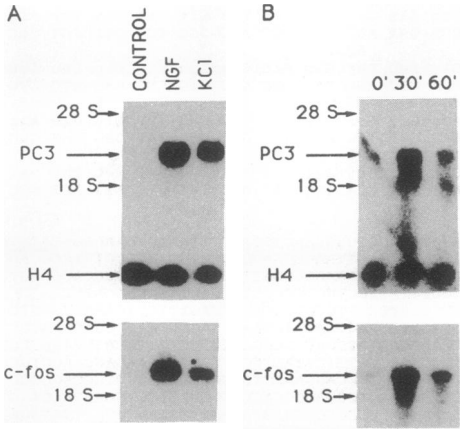

FIG. 5. Regulation of PC3 RNA after cell depolarization. Northern blots of 10 μg of total RNA from PC12 cells exposed to NGF (100 ng/ml) or to 75 mM KCl for 1 hr (*A*) and from the brains of rats sacrificed at the indicated times after i.v. treatment with Metrazol (40 mg/ml) (*B*) were hybridized to ^{32}P-labeled PC3 (≈50 × 10^6 dpm) together with the mouse H4 histone cDNA, as indicated (≈40 × 10^6 dpm). The same filter was hybridized to a rat c-*fos* probe (38) (about 60 × 10^6 dpm). The filters were exposed overnight at −70°C to x-ray film.

Induction of PC3 mRNA by Membrane Depolarization. The depolarization of PC12 cells by high K^+ was accompanied by a rapid increase of PC3 mRNA levels within 1 hr, comparable to that induced by NGF (Fig. 5*A*). Interestingly this effect was also seen *in vivo* when rats were given the convulsant Metrazole, with a peak of 30 min (Fig. 5*B*). A second band of ≈1.8 kb is also seen *in vivo*, whose nature is not known. The expected c-*fos* induction (see ref. 19) was similar in kinetics and level.

DISCUSSION

We have described the sequence of an NGF-inducible immediate early gene that has no homologue in the European Molecular Biology Laboratory, National Biomedical Research Foundation Protein Identification Resource, or Swiss-Prot data bases.

In common with the other immediate early genes, including PC4 (F.T., unpublished data), PC3 is superinduced by NGF in the presence of CHX, indicating that the levels of PC3 mRNA are directly regulated by NGF without the need for the synthesis of new proteins after the NGF receptor has been stimulated. The accumulation of PC3 mRNA also suggests the existence of a protein with fast turnover whose function is either to inhibit transcription or to degrade the transcript once formed. The existence of a signal peptide consensus in the PC3-deduced protein sequence has no precedent in the other known immediate early genes and suggests that it is secreted or retained as a free protein in one of the many vesicular compartments of the cell. The first possibility appears more probable, since no consensus sequence for retention in cell compartments was found. If so, and considering that PC3 mRNA is very rapidly and transiently produced, the question arises about the need for a cell stimulated by NGF to produce, and secrete, this protein so soon after stimulation.

The expression of PC3 in the neural tube observed at embryonic day 13.5 suggests that PC3 could be involved in either proliferative or differentiative processes, since neuro-

blast proliferation is associated, between embryonic days 11 and 14, with the differentiation of postmitotic neurons (39). Proliferation and differentiation are also present in spleen, amnion, and placenta, whereas in heart and skeletal muscle both proliferation and differentiation have stopped. *In vivo*, therefore, although PC3 is expressed in some tissues that are proliferating and differentiating, it can also be expressed in those that are terminally differentiated.

Given that PC3 is induced in PC12 cells by NGF *in vitro*, it is interesting that the pattern of PC3 mRNA expression *in vivo* generally mirrors that of NGF mRNA (with the exception of adult brain) and its receptor (40–43). This is consistent with the possibility that NGF is responsible for PC3 induction *in vivo*. The decrease of PC3 mRNA in the adult brain, irrespective of the high expression of NGF mRNA, could be the *in vivo* counterpart of the time window observed in the *in vitro* response of PC3 to NGF.

In these considerations, the specificity of induction is also relevant: PC3 is also induced in PC12 cells by EGF but considerably less so than by NGF. This is unlike PC4 and NGFI-A/PC1, which are induced to similar levels by NGF and EGF under the same conditions (data not shown). Bt_2cAMP is the only agent tested able to elicit an increase of PC3 mRNA comparable to that of NGF. Although PC3 is the immediate early gene with the most NGF-specific profile of induction described so far, no truly NGF-specific early gene (including PC3) has been described. This is in contrast to the specificity seen for some of the late genes and begs the question of whether such a degree of early relative specificity is sufficient by itself to explain the cellular changes peculiar to NGF treatment, or whether we should be searching for more NGF-specific genes. It is conceivable that it is the effect of patterns of early genes induced by different stimuli, which is more important than that of individual genes. The different transcriptional activities, some stimulatory and some inhibitory, of different dimers of the leucine zipper family provide an example of a mechanism by which this could occur (44). In this way it is easier to understand how stimuli inducing genes at such similar levels can result in such vastly different phenotypic effects, either proliferative or differentiative. Such a scheme need not be limited to transcriptional control of genes but may also be operative at a cellular level.

How does this relate to the induction of PC3 mRNA by depolarization in PC12 cells (caused by high K^+) and *in vivo* (caused by Metrazole)? Many other immediate early genes are induced by depolarization, and of these, some (c-*fos* and NGFI-A) have been associated with physiological depolarization found *in vivo* (18). Although NGFI-A is induced during long-term potentiation (LTP), it can also be induced when LTP inhibitory pathways are activated, suggesting that no simple correlation exists between the activation of this gene and LTP. Although it would be attractive to hypothesize a role for PC3 in this context it is clear that the results obtained thus far can do no more than suggest that an investigation in this field may be fruitful.

Although still to be shown, it is significant that PC3 is probably a secreted protein. What could the function of such a protein be? Its size is similar to that of most molecules responsible for communication between cells, and certainly one could imagine a role for a protein secreted in response to either NGF or depolarization. Such a protein could act as a messenger between different neuronal subsets or even between the nervous system and non-neuronal cells, but further work will be necessary to clarify this point.

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