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Early gene regulation by nerve growth factor in PC12 cells: Induction of an interferon-related gene

(cDNA/brain/cAMP)

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ABSTRACT **Nerve growth factor (NGF) induces the chromaffin cell line PC12 to differentiate into cells with many of the properties of sympathetic neurons. We investigated the early differentiative phase and identified a gene, PC4, rapidly and transiently induced by NGF in PC12 cells. PC4 cDNA is homologous to the partial sequence of a putative mouse β-interferon and encodes a protein related to a lymphokine, the rat γ-interferon protein. Nonetheless, PC4 appears devoid of antiviral activity. PC4 is expressed in proliferating and differentiating tissues, such as amnion, placenta, and brain at embryonic day 13.5. The relationship of PC4 to interferons and lymphokines suggests that it could play a role in regulating gene activity in the pathways induced by NGF.**

Nerve growth factor (NGF) was the first neurotrophic factor discovered in a class of molecules responsible for nervous system development and differentiation. NGF promotes the survival and the maintenance of sympathetic and some sensory neurons, such as those of the dorsal root ganglia and the trigeminal nuclei (1–3). NGF is also able to induce the chromaffin cells of the fetal adrenal medulla, a neural crest-derived tissue, to differentiate into sympathetic neurons (4), a phenomenon well represented *in vitro* in the clonal cell line PC12 (5). Since the precursors of chromaffin cells *in vivo* become determined toward the neuronal lineage before the onset of NGF influence (6), NGF can be regarded as a specific factor of commitment toward the sympathetic phenotype. The action of NGF is transcription-dependent (7). Genes regulated by NGF have been found, such as the protooncogenes c-*fos* and c-*myc*, whose mRNA levels are rapidly increased in PC12 cells by NGF (8). It is conceivable that the genes induced rapidly after NGF exposure in PC12 cells, before a detectable neurite outgrowth occurs, are responsible for the early NGF-dependent commitment of the chromaffin cell to the neuronal phenotype. The same genes when negatively regulated, could, moreover, account for the plasticity of this cell line, which returns to the chromaffin phenotype if NGF is withdrawn from the culture medium. To explore these ideas, we attempted to identify and clone genes induced by NGF in PC12 cells within the first hour of activity. We believe that such an approach also allows the study of the proliferative activity induced by NGF (9). Although in PC12 cells proliferation can be distinguished from differentiation, they may well share features in common, such as oncogene induction and/or derepression of transcription. The temporal choice was further motivated by our desire to analyze the involvement of cyclic AMP (cAMP) in early gene regulation by NGF, since cAMP and NGF act synergistically in stimulating neurite outgrowth and cellular protein and RNA levels in PC12 cells (10, 11).

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We cloned two genes, whose RNA levels are rapidly induced by NGF in PC12 cells. One of these, PC4, shows homology to the partial sequence of a putative mouse β-interferon (β-IFN; ref. 12) and is also related to the rat γ-IFN protein (13). The other is the gene NGFI-A recently described (14), which is homologous to a transcriptional regulatory factor.*

MATERIALS AND METHODS

Cell Culture. PC12 cells were obtained from D. Schubert (Salk Institute) (5th passage) and grown in Dulbecco's modified Eagle's medium (DMEM) with 5% supplemented calf serum (Irvine Scientific) and 5% horse serum (HyClone) in a humidified atmosphere of 12% CO_2 at 37°C. COS-1 cells were grown at 5% CO_2 in DMEM and 5% fetal bovine serum (HyClone). NGF (100 ng/ml) and N^6,$O^{2'}$-dibutyryladenosine 3',5'-cyclic monophosphate (Bt_2cAMP, 1 mg/ml, from Sigma) were added to cell cultures in the logarithmic phase of growth (about 75% confluent).

RNA Isolation and Analysis. Cytoplasmic RNA was obtained from PC12 cells using the lysis method with 0.5% Nonidet P-40 (15) and was used for the construction of the cDNA libraries. Total cellular RNA was used for Northern blot analysis and was obtained by extraction in 4 M guanidine thiocyanate followed by centrifugation through a CsCl cushion (16). The RNA was separated electrophoretically on 1.2% agarose/2.2 M formaldehyde gels, transferred to nitrocellulose filters and hybridized to the PC4 probe, excised from the site *Pst* I in the pUC9 vector, and ^{32}P-labeled with the random primers procedure (17). The hybridization was in 50% formamide, 5× SSC (1× SSC = 150 mM NaCl/15 mM sodium citrate, pH 7), 5× Denhardt's solution (1× Denhardt's solution = 1% Ficoll/1% polyvinylpyrrolidone/1% bovine serum albumin), 0.1% $NaDodSO_4$, and 0.5 mg of salmon sperm DNA per ml for 20 hr at 42°C. The blots were then washed at 55°C in 0.1× SSC and 0.1% $NaDodSO_4$.

Construction and Screening of cDNA Libraries. In a first cDNA library of 8000 clones, the single-strand (ss-) and double-strand (ds-) cDNAs were synthesized from total cytoplasmic RNA of PC12 cells treated 1 hr with NGF (100 ng/ml), as described (18). After addition of homopolymer (dC) tails, the ds-cDNA was annealed to a pUC9 vector dG-tailed at the *Pst* I site and used to transform *Escherichia coli* strain HB101. Differential screening was performed as described (15). All of the 8000 transformants were individually grown in L broth with 50 μg of ampicillin per ml in 96-well microtiter plates. The collection of clones in each plate was replicated into LB agar plates using a device with 96 stainless steel rods. Filter replicas were obtained by transferring the

Abbreviations: NGF, nerve growth factor; IFN, interferon; Bt_2cAMP, N^6,$O^{2'}$-dibutyryladenosine 3',5'-cyclic monophosphate; E, embryonic day.

*The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04511).

clones into Whatman 541 filter paper and, after a denaturation step in 0.5 M NaOH, were hybridized to ^{32}P-labeled ss-cDNA probes generated by reverse transcriptase from the RNA of PC12 cell cultures, either naive or treated 1 hr with NGF (100 ng/ml). A second cDNA library was constructed from the same total RNA (40 μg) using the simian virus 40 early promoter-based expression vector pcD as described (19). Briefly, the first strand was synthesized by reverse transcriptase using the oligo(dT)-tailed plasmid pcDV-1 as primer and, after addition of homopolymer (dC) tails, annealed to the (dG)-tailed pL1 linker. The second strand was synthesized by using *E. coli* DNA polymerase and RNase H. Competent *E. coli* (DH5α) cells were transformed with the recombinant plasmid. Approximately 60,000 transformants were plated.

Sequence Analysis. The nucleotide sequences were all obtained by chemical cleavage (20) and compared to those stored in GenBank, EMBL, and National Biomedical Research Foundation (NBRF) nucleic files, using the program Wordsearch (21). The amino acid sequences were then compared to those stored in the NBRF protein database using the program FASTP (22).

Transfection Experiments. Fresh monolayers of COS-1 cells in 9-cm plates were transfected with 10 μg of uncleaved pcD-PC4 cDNA by the calcium phosphate precipitation technique (23). After 48 hr, the culture medium and the cell extract [obtained by sonication in 10 mM Hepes (pH 7.5) with 5 mM dithiothreitol] were assayed for antiviral activity.

Antiviral Assay. The antiviral activity of PC4 was determined by measurement of the protective effect against cytocidal infection in the rat kidney fibroblast cell line RtKII (Lee Biomolecular Research, San Diego, CA) by the vesicular stomatitis virus (VSV). RtKII cells seeded in 96-well microplates in DMEM/2% fetal bovine serum were incubated for 24 hr at 37°C with serial dilution of the medium or the cell extract from COS-1 cultures transfected with pcD-PC4. Then VSV (3×10^4 plaque-forming units) was added. After 90 min at 37°C the cells were washed, incubated 24 hr in DMEM/2% fetal bovine serum, fixed in absolute methanol, and stained in 0.5% aqueous crystal violet. The IFN standards were from Lee Biomolecular Research.

Dissection of Embryos. Pregnant rats (Bantin & Kingman, Fremont, CA) were designated 0.5 day of gestation the morning after a midnight breeding. The neural tube from 11.5-, 12.5-, and 13.-5-day-old embryos was dissected and cleared from epithelial cells in Hanks' balanced salt solution, with the aid of a dissecting microscope (Wild, Heerbrugg, Switzerland), using electrolytically sharpened 0.5-mm tungsten wires. This dissection included the forebrain, midbrain, and hindbrain vesicles.

RESULTS

Isolation of cDNA Clones Induced by NGF. Total mRNA from PC12 cells treated for 1 hr with NGF (100 ng/ml) was used to generate a cDNA library of about 8000 independent clones with an average length of 1.5 kilobases (kb). Differential screening of all the transformants (see *Materials and Methods*) gave eight colonies that hybridized to probes from NGF-treated PC12 cells but not to probes from naive cell cultures. The cDNA inserts of these clones were further analyzed by Northern blot analysis. Four clones represented RNAs induced by NGF. One of them, designated PC4, is described here.

Induction of PC4 by NGF and Bt$_2$cAMP in PC12 Cells. The patterns of hybridization of PC4 cDNA to the RNA from PC12 cells treated for different times with NGF or Bt$_2$cAMP are shown in Fig. 1. PC4 hybridized to an RNA species whose levels were maximally induced by NGF after 2 hr (about 11 times, as measured by densitometry scanning) and then decreased to basal level within 18 hr. The size of the transcript hybridizing to PC4 was about 1.95 kb. We also tested the inducibility of the RNA levels of PC4 by Bt$_2$cAMP, as an indication of the involvement of cAMP in the early regulation of gene expression by NGF. No appreciable induction was detected (Fig. 1).

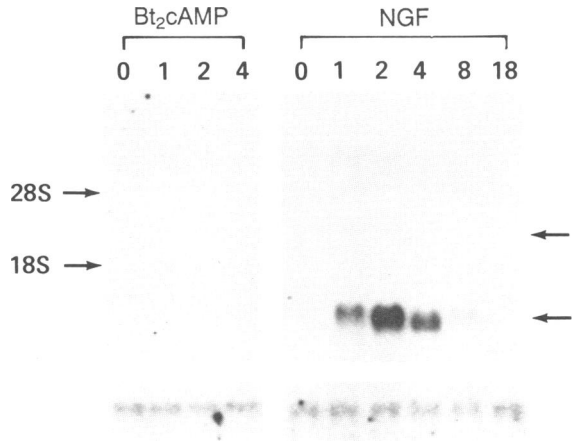

FIG. 1. Induction by NGF and Bt$_2$cAMP of the RNA hybridizing to the cDNA clone PC4. Ten micrograms of electrophoretically separated RNA, extracted from PC12 cell cultures treated with NGF (100 ng/ml) or Bt$_2$cAMP (1 mg/ml) for the indicated times (hours), was blotted on nitrocellulose filters and hybridized with the cDNA clone insert ^{32}P-labeled to a similar specific activity of $\approx 1.5 \times 10^6$ dpm/ng (for a total of 60×10^6 dpm). Arrows indicate the position of the rRNA bands. To control that equal amounts of intact RNA were loaded, filters were separately hybridized to a ^{32}P-labeled mouse H4 histone cDNA fragment (≈ 80–120×10^6 dpm; lower band). The filters were exposed overnight at −70°C to an x-ray film.

Sequence Analysis of PC4 and Isolation of pcD-PC4. The nucleotide sequence of PC4 cDNA was determined. The search for similarities showed that the nucleotide and protein sequences of the clone PC4 were homologous to a putative mouse β-IFN cDNA, a partial sequence originally named pMIF20/11 (12). Because of this finding, a full-length copy of PC4 was cloned. About 100 clones hybridizing to PC4 were found in a cDNA library constructed according to Okayama and Berg (19); the longest clone, pcD-PC4, was 1722 nucleotides in length, plus about 200 nucleotides of the poly(dA) tail, and contained an open reading frame coding for a protein of 449 amino acids (nucleotides 146–1492, Fig. 2 *A* and *B*). The methionine in position 146 is the first residue, since it is preceded by stop codons in all of the frames. pcD-PC4 contains a 5′ untranslated region of 145 nucleotides and a 3′ untranslated region of 230 nucleotides. In the latter region a signal for polyadenylylation was observed at nucleotide 1696, and an (A+T)-rich sequence, considered to be a signal for rapid degradation of the mRNA (24), was observed at nucleotides 1628–1638. This may account for the observed decrease with time in the size of the mRNA hybridizing to PC4 (Fig. 1). A comparison of the protein encoded by the clone pcD-PC4 with the mouse putative β-IFN protein (12) showed 97% identity in 64 overlaps in the COOH-terminal region (residues 386–449, Fig. 3). Interestingly, the same region of the pcD-PC4 protein also showed homology to the rat γ-IFN (ref. 13; Fig. 3), attaining statistical significance with a standard deviation above the scrambled number of +4.5 (ref. 25; R. F. Doolittle, personal communication). This similarity spans the entire γ-IFN sequence, a feature not observed in any other comparison. Some homology of pcD-PC4 to the human β_1-IFN protein was also observed but to a lower degree than with rat γ-IFN (Fig. 3). No evident homology to the mouse β-IFN protein was found (ref. 38; data not shown). The cysteine in position 1 in the rat γ-IFN and position 31 in the human β_1-IFN appears to be conserved

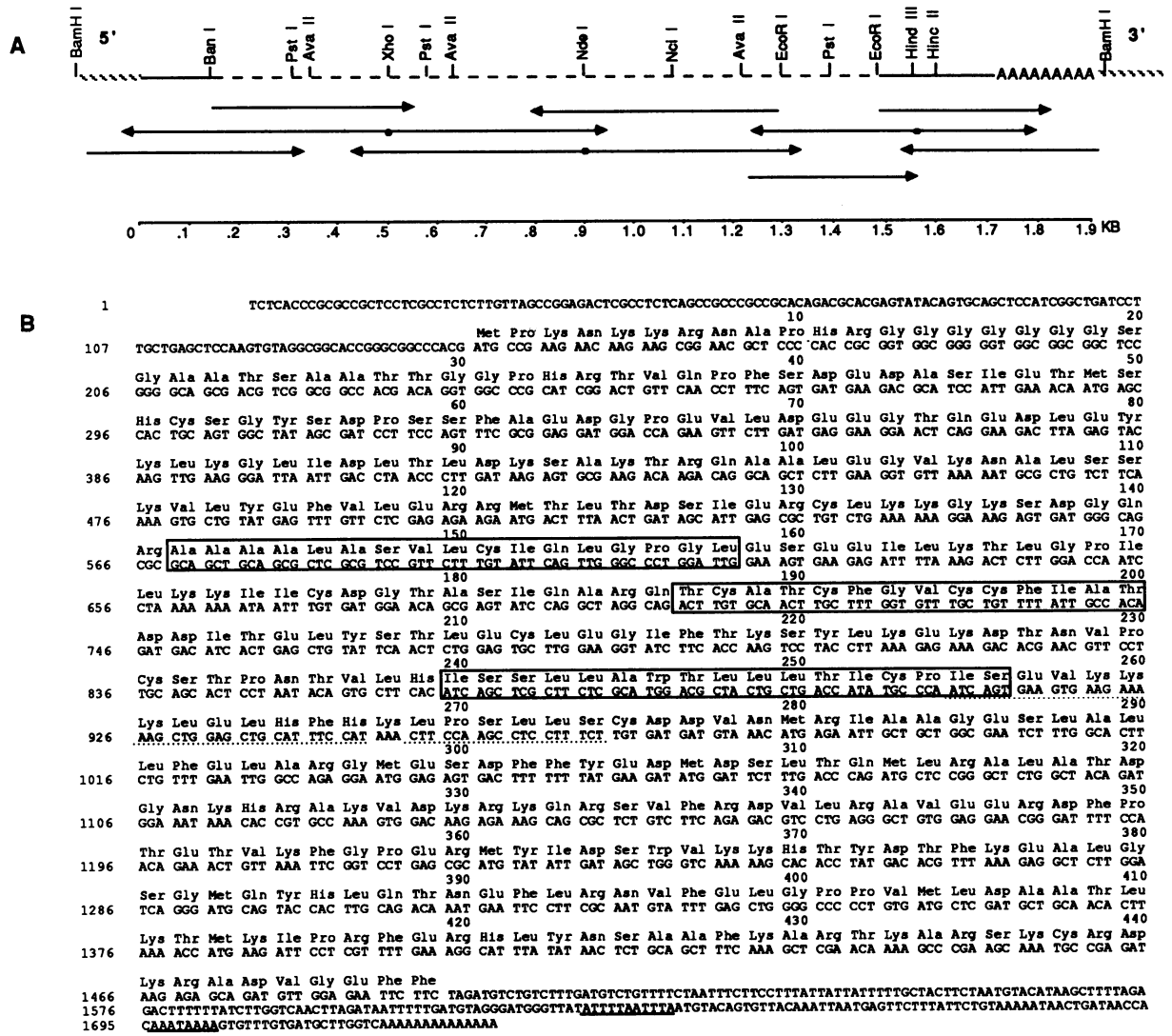

FIG. 2. Restriction map and sequencing strategy (*A*) and nucleotide and predicted amino acid sequence (*B*) of pcD-PC4 cDNA. (*A*) Continuous line, untranslated regions; dotted lines, translated region; thick hatched lines, plasmid pcD; arrows, DNA fragments sequenced. (*B*) Nucleotide sequence of pcD-PC4 is presented in the 5′ to 3′ orientation. The translational open reading frame is shown above the sequence. Adenosine residues are found at the 3′ end (about 200). Numbers above the translated sequence indicate amino acid residues. Nucleotide numbers are on the left of the sequence. The poly(A) addition signal and the (A+T)-rich sequence are underlined. Dots indicate the region of homology to the rat γ-IFN signal peptide sequence. The major hydrophobic regions are boxed.

in PC4 (residue 276). The hydropathic profile of pcD-PC4 obtained by the Kyte and Doolittle algorithm (26) shows three extended regions of hydrophobicity, between residues 142–158, 187–200, and 240–256; the latter region ends where the homology with the rat γ-IFN's signal peptide begins (Fig. 3; ref. 13). The NH_2-terminal region (first 140 residues) is essentially hydrophilic. An interesting feature of this portion of the molecule is the presence of an oligoglycine stretch (residues 13–19), encoded by the *pen* repeat, which consists of clusters of GGN triplets (27). This repeat is preceded by a sequence of basic and hydrophilic residues and is followed further downstream by an acidic region (residues 41–80, 15% aspartic acid and 20% glutamic acid). No active enzyme or potential N-glycosylation sites were identified along the sequence of pcD-PC4.

Expression of pcD-PC4 in COS-1 Cells. The plasmid pcD-PC4 was used to transfect COS-1 cells, a simian virus 40-transformed monkey kidney cell line (28). Northern blot analysis (Fig. 4) of RNA from transiently transfected (48 hr) cells showed high levels of PC4 mRNA, about 2 kb in size. This size is consistent with a transcript beginning few nucleotides before the initiation of the 5′ noncoding region of PC4—at the 16 S intron splicing site of the pcD plasmid

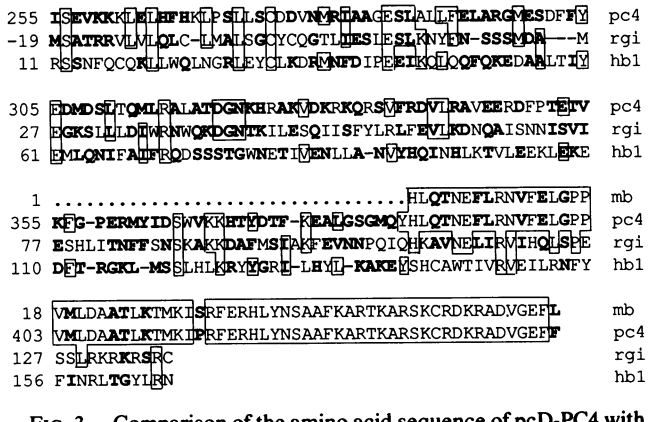

FIG. 3. Comparison of the amino acid sequence of pcD-PC4 with the IFN proteins. The computer program FASTP was used to generate binary (pairwise) alignments with gaps of pcD-PC4 with IFN's sequences. The region of homology of pcD-PC4 (residues 255–449, pc4) to the rat γ-IFN (rgi), to the human β_1-IFN (hb1), and to the partial sequence of the mouse β-IFN (mb) is indicated. Open boxes, identical amino acids; bold characters, conservatively substituted amino acids.

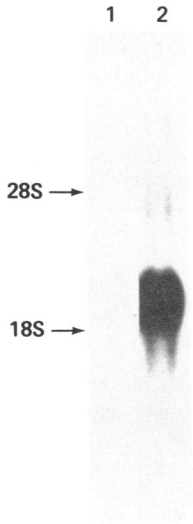

FIG. 4. Expression of PC4 in transiently transfected COS-1 cells. RNA blots of 8 μg of total RNA per lane isolated from the monkey kidney cell line COS-1 were hybridized to ^{32}P-labeled PC4 cDNA. Lane 1, control cells; lane 2, transfected cells. The time of exposure was 5 hr.

(19)—and terminating at the cDNA 3′ terminus. No expression was observed in control cells (transfected with pcD without insert). The antiviral assay of the culture medium and cellular extracts did not show significant activity (the standard rat IFN used for reference—a mixture of the α and β forms—gave 280 units/ml).

Tissue Expression of PC4. To define the specificity of PC4, it was hybridized to total RNA from the brain and several other nonneural tissues of the rat (Fig. 5). PC4 RNA appeared in tissues such as placenta and amnion, which are actively proliferating and secreting various growth factors (29). It was also expressed in skeletal muscle, heart, and spleen. The level of the PC4 transcript in the spleen was lower compared to that observed in the other organs. Little or no expression was observed in liver, kidney, and adult brain.

The expression of PC4 in embryonic and postnatal brain was also examined. As shown in Fig. 6, PC4 mRNA expression began in the neural tube at embryonic day 12.5 (E12.5), was maximal at E13.5, but rapidly decreased within the next 2 days and was very low at later stages.

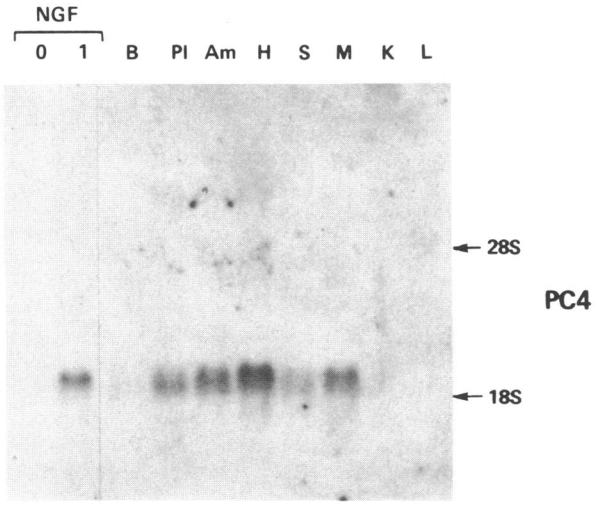

FIG. 5. Expression of PC4 in adult rat tissues. Eight micrograms of total RNA per lane, obtained from PC12 cells, control or treated 1 hr with NGF (100 ng/ml), and from adult rat brain (B), placenta (Pl), amnion (Am), heart (H), spleen (S), skeletal muscle (M), kidney (K), and liver (L), was hybridized with the ^{32}P-labeled cDNA. Amnion and placenta were from 14-day pregnant animals. The lanes 0 hr NGF and 1 hr NGF were exposed for about 1/15th of the time of the other lanes.

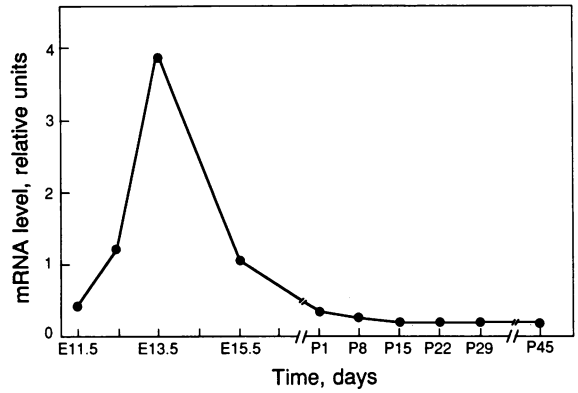

FIG. 6. Developmental expression of PC4 in the rat brain. Northern blot analysis of 8 μg of total RNA per lane isolated from the brain of embryos or of postnatal animals was performed. Represented are the values of the densitometry scanning of each autoradiographic band, normalized by using β-actin as reference probe. P, postnatal day.

DISCUSSION

The homology of pcD-PC4 with the partial sequence of a virus-inducible mouse gene (pMIF20/11; ref. 12), at the protein and nucleotide level, suggests that PC4 is the analogous form of this gene in the rat. Skup *et al.* (12) showed that the protein obtained by hybrid-translation from pMIF20/11 had antiviral activity and some degree of homology to the human β_1-IFN and thus defined it as a new mouse β-IFN. However, expression of the full-length pcD-PC4 cDNA in the monkey cell line COS-1 did not provide evidence for the expected antiviral activity. To clarify this point it will be necessary to express the full-length cDNA clone of pMIF20/11, which is not yet available. The same region of the deduced protein sequence of pcD-PC4 cDNA that is homologous to pMIF20/11 is also genuinely related to the rat γ-IFN protein more than to a β-IFN, either mouse or human (the sequence of the rat β-IFN is unfortunately not yet available). One of the three extended hydrophobic regions of the protein sequence (residues 240–256) immediately precedes the signal peptide of the rat γ-IFN. Whether this is an internal signal peptide remains to be seen. Moreover, the conservation of a cysteine residue in the same NH_2-terminal region of the rat γ-IFN and human β_1-IFN, in position 276 of PC4, suggests common functional domains. The unusual glycine stretch at the NH_2-terminal of PC4 could also have functional significance, although not related to the IFN homology, possibly as a region of flexibility positioned between two domains of the molecule with different charge.

The relation of PC4 to IFNs and lymphokines is intriguing, especially because of the known actions of these molecules on cell differentiation and proliferation (30, 31). Indeed, PC4 is expressed in proliferating tissues, such as amnion and placenta, and in the embryonic brain (neural tube) at E13.5, in a period mainly related to neuroblast division, which, in the rat, continues until the second week of embryonic life (32). On the other hand, between E11 and E13–E14, while neuroblast proliferation is still active, the postmitotic neurons begin to differentiate (33). Furthermore, cells positive to a neuronal marker (neurofilament protein) have been detected in rat brain as early as E12 (34). Thus it is possible that PC4 plays a role in the proliferative or/and in the differentiative processes in the embryonic brain. The expression of PC4 mRNA in skeletal muscle could be due to the satellite cells

(myoblasts), which express the NGF receptor (35). However this does not explain the expression of PC4 in the heart, where no satellite cells are present. PC4 appears moreover to be regulated by NGF through pathways that are cAMP-independent.

It is of considerable interest that rat γ-IFN, to which PC4 is related, has been recently shown to facilitate the NGF-induced neuronal differentiation in PC12 cells, possibly via a more rapid entry into a G_0 state (36). Indeed, γ-IFN is a lymphokine that inhibits cell proliferation to a greater extent, relative to its antiviral activity, than the other types of IFN (37). Our data are compatible with the idea that PC4 could also be a member of the lymphokine family but one that is devoid of antiviral activity. Since γ-IFN is not regulated by NGF in PC12 cells (unpublished results), this raises the question as to whether PC4 is its physiological counterpart controlling the proliferative and/or differentiative pathways induced by NGF in PC12 cells. If such an action occurs, then, by analogy to the effects of the lymphokines and IFNs (30, 31), it could be by regulating the expression of other genes, possibly after autocrine secretion and binding to receptors specific for PC4 on PC12 cells membrane. It remains to be determined whether PC4 expression is under the control of NGF *in vivo*.

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