Identification and Characterization of mRNAs Regulated by Nerve Growth Factor in PC12 Cells

DEBRA G. B. LEONARD,¹ EDWARD B. ZIFF,¹ AND LLOYD A. GREENE^{2*}

Department of Biochemistry¹ and Department of Pharmacology,² New York University School of Medicine,
New York, New York 10016

Received 6 April 1987/Accepted 12 June 1987

Differential screening of cDNA libraries was used to detect and prepare probes for mRNAs that are regulated in PC12 rat pheochromocytoma cells by long-term (2-week) treatment with nerve growth factor (NGF). In response to NGF, PC12 cells change from a chromaffin cell-like to a sympathetic-neuron-like phenotype. Thus, one aim of this study was to identify NGF-regulated mRNAs that may be associated with the attainment of neuronal properties. Eight NGF-regulated mRNAs are described. Five of these increase 3- to 10-fold and three decrease 2- to 10-fold after long-term NGF exposure. Each mRNA was characterized with respect to the time course of the NGF response, regulation by agents other than NGF, and rat tissue distribution. Partial sequences of the cDNAs were used to search for homologies to known sequences. Homology analysis revealed that one mRNA (increased 10-fold) encodes the peptide thymosin β_4 and a second mRNA (decreased 2-fold) encodes tyrosine hydroxylase. Another of the increased mRNAs was very abundant in sympathetic ganglia, barely detectable in brain and adrenals, and undetectable in all other tissues surveyed. One of the decreased mRNAs, by contrast, was very abundant in the adrenals and nearly absent in the sympathetic ganglia. With the exception of fibroblast growth factor, which is the only other agent known to mimic the differentiating effects of NGF on PC12 cells, none of the treatments tested (epidermal growth factor, insulin, dibutyryl cyclic AMP, dexamethasone, phorbol ester, and depolarization) reproduced the regulation observed with NGF. These and additional findings suggest that the NGF-regulated mRNAs may play roles in the establishment of the neuronal phenotype and that the probes described here will be useful to study the mechanism of action of NGF and the development and differentiation of neurons.

Nerve growth factor (NGF) plays a major role in the growth, differentiation, and survival of sympathetic and sensory neurons (22, 41). Recent studies have demonstrated the presence of NGF and its receptor in the central nervous system and have shown that certain neurons of the forebrain respond to NGF (35, 51). One in vitro model system used to study the effects and mechanism of action of NGF is the rat PC12 pheochromocytoma cell line (27, 28). In the presence of NGF, PC12 cells differentiate from replicating, chromaffinlike cells to nonreplicating, sympathetic-neuron-like cells. One of the more dramatic aspects of PC12 cell differentiation is the attainment of a neuronal phenotype by the extension of long, branching neurites. Within 1 week of NGF treatment, approximately 90% of the cells have neurites. NGF-treated PC12 cells also develop the ability to generate fast, Na^+ -dependent action potentials (13) and undergo alterations in the levels of a number of specific proteins, many of which change in an RNA synthesis-dependent manner (see, for example, reference 28 for review). Burstein and Greene (6) demonstrated that both RNA synthesis-dependent and -independent events are necessary for neurite outgrowth from PC12 cells in response to NGF. Thus, at least part of the mechanism of action of NGF appears to involve the regulation of the expression of specific genes.

One purpose of the present study has been to detect and characterize mRNAs that are regulated by NGF in PC12 cells. These mRNAs can potentially be used to understand the mechanism of NGF transcriptional control, to identify gene products that are involved in NGF-induced differenti-

ation, and to study the differentiation and development of neurons. An additional goal of this study has been to detect mRNAs which are markers for differentiated chromaffin cells and neurons.

A powerful method for the identification of regulated or differentially expressed mRNAs is by differential screening of cDNA libraries. Developmentally regulated mRNAs have been identified in this manner for a variety of systems including *Dictyostelium discoideum* (58, 68) and *Xenopus laevis* (17). This approach has also been employed to identify mRNAs induced by interferon (21) or by stimulation of growth-arrested fibroblasts with growth factors (8, 43-45). Of particular relevance to the present study are the work of Anderson and Axel (1), who used differential screening to identify two mRNAs which are differentially expressed during normal neural crest cell differentiation and one of which is also regulated by NGF in PC12 cells, and the studies of Levi et al. (40), who have described an mRNA which is induced more than 50-fold in PC12 cells after 5 h of NGF exposure.

In the present study, we describe the construction of cDNA libraries from mRNA from untreated PC12 cells and from PC12 cells treated for 2 weeks with NGF. These libraries were screened with cDNA probes prepared from PC12 cells before and after long-term NGF treatment and from a PC12 cell variant line, F4. This spontaneously arising variant does not respond to NGF and appears to be lacking a number of differentiated properties of PC12 cells. This third probe was included to enhance the probability of identifying clones which are chromaffin cell or neuronal markers. By differential screening we have identified 10

* Corresponding author.

mRNAs of potential interest. Eight of these mRNAs are regulated by NGF: five are increased 3- to 10-fold by NGF, and three are decreased 2- to 10-fold. The characterization of these 10 mRNAs (by the time course and the specificity of regulation and by tissue distribution) indicates that at least several of these mRNAs may play roles in the establishment of a chromaffin or sympathetic-neuron phenotype.

METHODS

Cell culture. PC12 cells were grown as previously described (27) on rat tail collagen-coated dishes in RPMI 1640 medium containing 10% horse serum and 5% fetal bovine serum (KC Biologicals, Lenexa, Kans.). Medium was changed three times per week. When cultures were to be compared after various treatments and times, sister dishes were plated from a single pool of cells. F4 cells are a spontaneously arising mutant line of PC12 cells which no longer bind or respond to NGF and have lost most of the differentiated characteristics of naive as well as NGF-treated PC12 cells. F4 cells were grown as described for PC12 cells except that cells were plated on plastic tissue culture dishes without collagen and required brief treatment with trypsin for removal of the cells from the plate. Mouse 2.5S NGF was extracted from adult male submandibular glands by the method of Mobley et al. (52) and added to NGF-treated cultures to reach a concentration of 50 ng/ml. Human basic fibroblast growth factor (FGF) was purified from term human placentas according to Moscatelli et al. (54) and was generously provided by D. B. Rifkin (Department of Cell Biology, New York University Medical Center). FGF was added at a concentration of 100 ng/ml. Epidermal growth factor was kindly supplied by Fred Maxfield (Department of Pharmacology, New York University Medical Center) and was added to cultures at 5 ng/ml. Insulin and dexamethasone (Sigma) were added at final concentrations of 1 μ g/ml and 10 μ M, respectively. Dibutyryl cyclic AMP (DBcAMP) (Boehringer Mannheim) was used at a final concentration of 1 mM. Phorbol myristate acetate (TPA) was purchased from PL Biochemicals and added to cultures at a 100 nM final concentration. Medium containing elevated K^+ was prepared by adding 1 volume of 160 mM KCl stock solution to 3 volumes of culture medium.

Construction of cDNA libraries. Two cDNA libraries were constructed in the λ gt10 vector (37), as described in greater detail elsewhere (D. G. B. Leonard, Ph.D. thesis, New York University, New York, N.Y., 1987). Cytoplasmic RNA was isolated from untreated or 2-week-NGF-treated PC12 cultures by lysing the cells with 0.5% (vol/vol) Nonidet P-40, centrifuging to remove the nuclei and organelles, and extracting with phenol and chloroform. On average we obtained 260 μ g of cytoplasmic RNA from 10^8 untreated PC12 cells and 1 mg from 10^8 PC12 cells treated for 2 weeks with 50 ng of NGF per ml. Poly(A)⁺ RNA was selected by one cycle of binding to oligo(dT)-cellulose (type 7; P-L Biochemicals) and was approximately 2.5 to 3% of the cytoplasmic RNA loaded onto the column. The first cDNA strand was synthesized with reverse transcriptase (0.5 U/ μ l of reaction volume) using 10 μ g of poly(A)⁺ RNA as a template and in the presence of 100 μ g of actinomycin D (Calbiochem, La Jolla, Calif.) per ml to reduce hairpin formation. The second cDNA strand was synthesized by the procedure of Okayama and Berg (55), using RNase H (P-L Biochemicals), *Escherichia coli* DNA ligase (P-L Biochemicals), and *E. coli* DNA polymerase (New England Biolabs). The double-stranded cDNA was methylated with *Eco*RI

methylase (New England Biolabs) and made blunt-ended by T4 DNA polymerase (Bethesda Research Laboratories) in the presence of 0.1 mM deoxynucleotide triphosphates. *Eco*RI linkers (New England Biolabs) were ligated onto the cDNA, which was then restricted with *Eco*RI (New England Biolabs). The cDNA was separated from the linker fragments on an Aca34 Ultrogel (LKB) column and ligated into the *Eco*RI site of λ gt10. The library was packaged in vitro and amplified in *E. coli* C600 Hfl. The untreated PC12 cell library contained 10^6 independent clones, and the NGF-treated PC12 cell cDNA library contained 4×10^6 clones.

Differential screening of the cDNA libraries. Each library was plated at a density of 2×10^3 to 4×10^3 PFU per 15-cm plate in *E. coli* LE392. Three nitrocellulose filter replicas were made from each plate by leaving the filters in contact with the plate until just wet for the first filter, for 2 min for the second filter, and for 4.5 min for the third. The filters were denatured for 2 min in 0.1 M NaOH–1.5 M NaCl, neutralized for 3 min in 0.2 M Tris hydrochloride (pH 7.5)–1 M NaCl–0.3 M sodium citrate–0.26 M KH_2PO_4 –2 mM EDTA, dried at room temperature, and baked at 80°C for 2.5 h. Filters were washed for 1 h at 68°C in 1 M NaCl–50 mM Tris hydrochloride (pH 7.2)–1 mM EDTA–0.1% sodium dodecyl sulfate (SDS), prehybridized for 4 h at 42°C in 10 $\times$ Denhardt solution (1 $\times$ Denhardt solution is 1% Ficoll, 1% polyvinylpyrrolidone, and 1% bovine serum albumin)–5 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.0125 M sodium citrate)–20 mM sodium phosphate (pH 6.5), briefly washed at 42°C in 50% formamide–5 $\times$ SSC–20 mM sodium phosphate (pH 6.5), and hybridized in sealed crystallizing dishes or petri dishes for 30 to 40 h at 42°C in 2.5 ml of 50% formamide–5 $\times$ SSC–20 mM sodium phosphate (pH 6.5)–1 $\times$ Denhardt solution per filter, with 10^7 to 10^8 cpm of 32 P-labeled single-stranded cDNA probe. Each of the three replica filters was hybridized with one of three probes made from either untreated PC12 cells, PC12 cells treated for 2 weeks with NGF, or F4 cells. Probes were synthesized by reverse transcriptase at 2 U/ μ l in the reaction, using 10 μ g of poly(A)⁺ RNA as a template, and in the presence of 100 μ g of actinomycin D per ml to prevent hairpin formation. Labeling was with [32 P]dCTP (800 Ci/mmol; Dupont, New England Nuclear Corp.), at a concentration of approximately 0.1 to 0.2 nM, and unlabeled dATP, dGTP, and dTTP, each at a concentration of 0.5 mM, in a reaction buffer containing 50 mM Tris hydrochloride (pH 8.3) (at 42°C), 50 mM KCl, 8 mM $MgCl_2$, 2 mM dithiothreitol, 30 μ g of oligo(dT) (no. 12116; Collaborative Research) per ml, and 100 μ g of bovine serum albumin (Bethesda Research Laboratories) per ml in a reaction volume of 25 μ l. After synthesis of the cDNA, the RNA was removed by alkaline hydrolysis by the addition of 175 μ l of 0.3 N NaOH–2 mM EDTA and boiling for 1.5 min. The reaction was neutralized by the addition of 25 μ l of 1 M Tris hydrochloride (pH 8) and 25 μ l of 1 N HCl, and the cDNA was ethanol precipitated in the presence of 2 M ammonium acetate. The pellet was suspended in 200 μ l of TE (10 mM Tris hydrochloride, pH 7.4, and 1 mM EDTA) and added to the hybridization solution and filters. After hybridization, the filters were washed in 2 $\times$ SSC–0.1% SDS at room temperature for 1 h, with a change of the wash solution after 30 min, and then washed in 1 $\times$ SSC–0.1% SDS at 68°C for 1 h with one or two changes. Filters were exposed to Kodak XAR-5 film for 2 to 5 days, using an intensifying screen at –70°C. Plaques with different intensities of hybridization signal between the three probes were picked, using the large end of a Pasteur pipette, into 1 ml of SM medium (47). These clones were rescreened and

plaque purified by two subsequent differential screenings as described above, except that 100-mm plates and filters were used and specific plaques were picked with the small end of a Pasteur pipette. The differentially expressed clones described in this paper were obtained by screening approximately 13,000 PFU.

Isolation of cDNA inserts from λ gt10. A viral stock of each differentially expressed clone was made from a plate lysate as described by Maniatis et al. (47). To obtain purified insert DNA, each recombinant virus was grown in liquid culture with infection of a 200-ml culture of *E. coli* LE392 (optical density at 600 nm of 0.25) at a 1:1 ratio. The virus particles were purified by two equilibrium centrifugations in cesium chloride ($\rho = 1.5$). After the final virus band was collected, the CsCl was removed by dialysis against 50 mM Tris hydrochloride (pH 8)–10 mM NaCl–10 mM MgCl₂ at room temperature with stirring. The DNA was released from the viral capsid by digestion with 50 μ g of proteinase K (Boehringer Mannheim) per ml in the presence of 0.5% SDS–20 mM EDTA for 1 h at 65°C. The DNA was sequentially extracted with phenol, phenol-chloroform, and chloroform and ethanol precipitated. Approximately 100 μ g of recombinant viral DNA was restricted with *Eco*RI, ethanol precipitated, and suspended in 0.3 ml of TE. The cDNA insert was separated from the λ gt10 vector by gel electrophoresis on a 5% polyacrylamide gel. The cDNA band was cut out, electroeluted from the gel, and ethanol precipitated. Only one mRNA group of cDNA inserts (clone 15) contained an internal *Eco*RI restriction site and therefore yielded two cDNA inserts (approximately 1,100 and 600 base pairs [bp]), which were both isolated. In general, the larger cDNA fragment was used for Northern blot (RNA blot) analyses although the smaller fragment gave the same Northern blot hybridization pattern for untreated and NGF-treated PC12 cells and F4 cells. The cDNA fragments were suspended in TE and stored at 4°C. For large-scale preparations, the cDNA inserts were cloned into the *Eco*RI site of pBR322 and the recombinant plasmid was grown by standard procedures.

Cross hybridizations. Clones identified by the differential screening were placed into common mRNA groups by a modification of the procedure of Southern (63). Recombinant λ gt10 or pBR322 DNA was restricted with *Eco*RI to release the cDNA insert from the vector. The restricted recombinant viral DNA, the restricted recombinant pBR322 DNA, or purified cDNA inserts for each of the differentially expressed clones were electrophoresed on a 1% agarose gel with 100 ng of an insert per lane. The gel was then soaked in 0.5 M NaOH–1.5 M NaCl for 30 min and neutralized in 0.5 M Tris hydrochloride (pH 7)–3 M NaCl for 90 min. The DNA was transferred to nitrocellulose filters and baked at 80°C for 2.5 h. Filters were prehybridized for 4 to 6 h at 65°C in 6 $\times$ SSC–1 $\times$ Denhardt solution–0.1% SDS–0.2 mg of sonicated salmon sperm DNA per ml. Hybridization was overnight at 65°C in the same buffer as for prehybridization with 10⁶ to 10⁸ cpm of ³²P-nick-translated plasmid or purified insert of one differentially expressed cDNA. Filters were washed for 1 h in 0.5 $\times$ SSC–0.1% SDS at 65°C with one change and exposed to Kodak XAR-5 film with an intensifying screen at –70°C for 10 min to 12 h. The clones described in this paper were the largest cDNA inserts for each mRNA group. This procedure was also used to screen the identified clones with known probes: β actin (human β actin-1; 30), *fos* (*pfos*-1; 12), tyrosine hydroxylase (TH) (*pTH*-1; 38), *Thy*-1 (34), and Anderson and Axel's clone 10 (1).

Isolation of rat tissue RNA. Adult female rats were sacrificed by rapid CO₂ asphyxiation. The tissues to be studied

were removed from the animal, frozen between blocks of dry ice, and stored at –70°C. Cytoplasmic RNA was isolated by the method of Chirgwin et al. (7). Approximately 0.5 g of each frozen tissue was placed in 7 ml of 4 M guanidine isothiocyanate–14% β -mercaptoethanol–0.1 M Tris hydrochloride (pH 7.5)–5 mM EDTA and disrupted by a Polytron at full speed for 20 to 30 s. The mixture was centrifuged at 5,000 $\times$ g for 10 min. The supernatant was layered onto a 3-ml cushion of CsCl ($\rho = 1.4$) in 0.1 M EDTA–25 mM sodium acetate (pH 5) and centrifuged at 36,000 rpm in an SW41 rotor overnight at room temperature. After centrifugation, the supernatant and part of the cushion were aspirated, the bottom of the tube was cut off, and the remainder of the cushion was drained from the bottom section of the tube. The RNA pellet was suspended in 0.2 ml of 0.3% SDS and ethanol precipitated.

Northern blot analysis. Cytoplasmic RNA was isolated from rat tissues as described above and from culture cells as described by Greenberg and Ziff (25). Cytoplasmic RNA was fractionated on a 1% formaldehyde agarose gel and blotted onto nitrocellulose as described by Greenberg and Ziff (25). From 5 to 10 μ g of cytoplasmic RNA was loaded per lane, with an equivalent amount of RNA per lane on any one blot. Prehybridization and hybridization of the Northern blot were as described by Greenberg and Ziff (25), with 10⁷ to 2 $\times$ 10⁸ cpm of ³²P-labeled cDNA insert. Purified cDNA inserts were ³²P labeled with the large fragment of DNA polymerase I, using random primers as described by Feinberg and Vogelstein (19). Blots were washed at 68°C for successive 30-min periods in 2 $\times$ SSC, 1 $\times$ SSC, 0.5 $\times$ SSC, and, if needed, 0.25 $\times$ SSC, always in the presence of 0.1% SDS. Blots were exposed to XAR-5 film for from 15 min to 1 week with an intensifying screen at –70°C.

Partial sequencing of cDNA clones. Purified cDNA inserts were cloned in both orientations into the *Eco*RI site of the M13mp8 vector (50). Single-stranded DNA templates were prepared as described previously (60), and several hundred nucleotides from each end of the cDNA were determined by the dideoxynucleotide chain termination method (59, 61), using [α -³²P]dATP, and fractionated on TBE buffer gradient polyacrylamide gels (3). The partial sequences were compared with nucleic acid sequences in the Genetic Sequence Data Bank (GenBank) (3a).

RESULTS

Detection of differentially regulated cDNAs. PC12 cell cDNA libraries were made by a modification of the Okayama and Berg method (55), using the λ gt10 vector (37). Two cDNA libraries were constructed, one from untreated PC12 cells and the other from PC12 cells treated for 2 weeks with 50 ng of NGF per ml. Both libraries were used for differential screening so as to enhance the probability of identifying mRNAs which are decreased by NGF treatment (and therefore represented more frequently in the untreated PC12 cell library) as well as mRNAs which are increased in response to NGF (and therefore more abundant in the NGF-treated PC12 cell library). Both of the cDNA libraries are large (at least 10⁶ independent clones), so that most mRNAs are represented. The average insert size is 970 bp, with a range up to 7,000 bp.

The differential screening procedure was designed with an added feature. Not only were NGF-regulated mRNAs to be identified, but also mRNAs which may be specifically associated with the chromaffin or neuronal properties of PC12 cells. Three replica nitrocellulose filters were made from

TABLE 1. Characteristics of the identified clones

Clone	mRNA size (nucleotides)	Long-term NGF response	Level in F4 cells relative to PC12	Sequence homology	Tissue distribution
10a	1,700	3.5 ↑	Lower	Thymosin B ₄	SCG (low in adrenal and brain)
21b	750	10 ↑	Higher		All
63	640	5 ↑	Higher		Lung, placenta, stomach, SCG, heart, kidney
71	820	4.5 ↑	Lower	Intermediate filaments ^a	All
73	1,800	7.5 ↑	Lower		SCG (low in adrenal and brain)
2	940	2.5 ↓	Undetectable		Very low levels in most tissues
10b	1,600 + 5,000	4 ↓ (at 1 week)	Undetectable	TH	Adrenal, E19 brain, placenta
15	1,700	2 ↓	Undetectable		Adrenal, SCG, brain
34b	760		Lower		All
74	1,850 + 2,100		Lower (1,850 mRNA); undetectable (2,100 mRNA)		All, but higher in SCG, brain, muscle, heart

^a Leonard et al., submitted.

each cDNA library plate and were probed with one of three single-stranded, ³²P-labeled cDNA probes. The probes were made from total poly(A)⁺ RNA of untreated PC12 cells, PC12 cells treated for 2 weeks with NGF, and F4 cells. The F4 cell line is a clonally derived, spontaneously arising mutant of PC12 cells which no longer binds or responds to NGF. The cells have lost most of the differentiated chromaffin and sympathetic-neuron characteristics of PC12 cells (48) and have a flattened, fibroblastlike morphology in culture. The hybridization signal to each plaque was then compared for the three probes. Differences in hybridization with the two PC12 cell probes identified mRNAs regulated by NGF, and differences between PC12 cell probes and the F4 cell probe identified potential chromaffin cell- or neuron-specific mRNAs.

A total of 13,000 clones were screened, approximately 10,000 from the untreated PC12 cell library, and 3,000 from the NGF-treated PC12 cell library. Of these clones, 260 (or approximately 2%) had a differential hybridization signal between at least two of the three probes and were picked for rescreening and plaque purification. Only 38 of the original 260 clones continued to show different signal intensities with the three probes after two additional rounds of screening. The cDNA inserts from these clones were then purified and used to probe Northern blots of poly(A)⁺ RNA from untreated and NGF-treated PC12 cells and from F4 cells. A final 23 clones gave a differential hybridization pattern by this analysis. Cross hybridization of these 23 cDNAs by a modified Southern blot analysis has categorized these cDNAs into 10 mRNA groups. Clones 2, 10a, 21b, 34b, 63, 71, and 73 did not cross hybridize, whereas nine cDNAs were independently isolated which cross-hybridized with clone 10b, five for clone 15, and two for clone 74. Within each cross-hybridizing group, the longest cDNA was used for further analyses.

Five mRNAs (recognized by clones 10a, 21b, 63, 71, and 73) were increased 3- to 10-fold in response to NGF. These revealed a variety of expression levels in the F4 cells: clone 10a, 71, and 73 mRNAs were much lower in F4 cells than in PC12 cells, whereas 21b and 63 mRNAs were more abundant in F4 cells. Three mRNAs (recognized by clones 2, 10b, and 15) were decreased 2- to 10-fold by NGF treatment and were undetectable in F4 cells. The levels of two mRNAs (recognized by clones 34b and 74) were not altered by NGF, but were less abundant in F4 cells. The mRNA size(s) corresponding to each clone, as well as some of the characteristics of the clones as discussed below, are summarized in Table 1.

We next characterized the 10 differentially expressed

mRNAs by a variety of methods to establish their relationship to the NGF-induced differentiation of PC12 cells and, more generally, to in vivo neuronal differentiation.

Homologies to known genes. Two approaches were taken to determine whether the regulated mRNAs we detected were homologous to known genes. First, the purified cDNA inserts were cloned into the *Eco*RI site of the M13 mp18 vector in both orientations of the insert. Then, 150 to 300 nucleotides of sequence were determined for each end of the cDNA by the dideoxynucleotide chain termination method (59, 61). These sequences were then compared with the published nucleic acid sequences in the Genetic Sequence Data Bank. By this approach, 9 of the 10 clones showed no significant homologies to known sequences. However, clone 21b was found to be highly homologous to thymosin β₄, a peptide with a wide tissue distribution (23, 69). Clone 21b includes the entire coding region of thymosin β₄, has an additional 21 bp at the 5' end, and extends to nucleotide 419 of the 523-bp published cDNA sequence (23) (Fig. 1A). The 132 nucleotides of clone 21b which encode thymosin β₄ are identical to the published cDNA sequence, except for the substitution of a T for a C at nucleotide 42. This is a silent change of the third nucleotide of the codon for the first serine amino acid of thymosin β₄. By the partial sequence analysis of clone 21b, there is a 5' region which is 99% homologous to the published sequence and a 3' region of 97% homology.

FIG. 1. Comparison of the published sequences of (A) thymosin β₄ (23) and (B) tyrosine hydroxylase (29) cDNAs with clone 21b and 15 cDNAs, respectively. Solid lines indicate noncoding regions. Hatched boxes indicate translated regions of the mRNA. Dotted horizontal lines indicate regions not sequenced.

FIG. 2. Time course of mRNA regulation by NGF. Northern blot analyses of F4 and PC12 cell cytoplasmic RNA (5 μ g per lane) probed with 32 P-labeled cDNA inserts. Sister cultures of PC12 cells were treated with NGF for the indicated lengths of time and then used for preparation of cytoplasmic RNA for Northern blot analysis. Autoradiographic exposure time was 6 h for all blots except 10a (19 h) and 10b (2.5 h). The line at the top left of each blot indicates the origin, and the two arrows indicate the positions of the 28S (top) and 18S (bottom) rRNA bands.

Since the published sequence was from a rat spleen-derived cDNA and clone 21b was derived from a rat cell line, this degree of homology confirms the identification of clone 21b as encoding thymosin β_4 . Of note is the fact that clone 21b mRNA (encoding thymosin β_4) is increased approximately 10-fold in PC12 cells in response to NGF.

The second approach for identifying homologies was to test nucleic acid probes of known genes for hybridization with the regulated cDNAs. Although our study was not exhaustive, we screened the following: β actin (30), which is rapidly and transiently transcriptionally activated by NGF in PC12 cells (24); tyrosine hydroxylase (TH; 38), whose activity is decreased by NGF in PC12 cells (28); *v-fos* (12), whose cellular homolog is rapidly and transiently up-regulated by NGF in PC12 cells (11, 24); Thy-1 (34), the protein (57) and mRNA (14; S. A. Drexler and L. A. Greene, Soc. Neurosci. Abstr., vol. 12, part 1, 1986, p. 215) levels of which are increased by NGF in PC12 cells; and Anderson and Axel's clone 10 (1), which was demonstrated to be increased by NGF in PC12 cells. One of our clones (clone 40, not otherwise described in this paper) cross hybridized with the actin probe. Clone 40 mRNA was decreased twofold by 2

weeks of NGF treatment and was more abundant (two- to threefold) in F4 cells. The TH probe cross hybridized with clone 15 cDNA (Fig. 1B). The 5' and 3' ends and the regions 5' and 3' to the internal *Eco*RI site of clone 15 cDNA were sequenced. Comparison of these results with the published sequence for rat TH mRNA (29) revealed that the insert in clone 15 begins at the G of the AUG start codon and is complete through the poly(A) tail (Fig. 1B).

v-fos and Thy-1 probes did not cross hybridize with any of the 10 cDNA clones. The Anderson and Axel clone 10 (1) hybridized very weakly with clones 15 (TH) and 10b; however, clones 15 and 10b did not cross hybridize even weakly with each other, and both mRNAs were decreased approximately twofold by 4 days of NGF treatment, whereas the mRNA recognized by Anderson and Axel's clone 10 was increased after 4 days of NGF treatment. In addition, the Anderson and Axel clone 10 is not related to TH (clone 15) by sequence analysis (D. Anderson, personal communication). Therefore we conclude that there is no identity between any of our cDNAs and the Anderson and Axel clone 10.

Time course analysis of the regulation by NGF. The clones described here were originally identified by the changes in the relative abundances of the corresponding mRNAs after 2 weeks of NGF treatment. To further investigate the time course of these changes in detail, we performed Northern blot analyses of total cytoplasmic RNA from PC12 cells exposed to NGF for various times up to 2 weeks. Most of the identified cDNAs hybridized to a single mRNA species (Fig. 2). However, clones 10b and 74 consistently recognized more than one mRNA species. The multiple upper mRNA bands of clone 10b always hybridized less than the lower, smaller mRNA, but followed the same regulation patterns observed for the smaller band. While the two mRNAs recognized by clone 74 in PC12 cells were generally of equal intensity, in F4 cells only the smaller mRNA was observed and at lower relative abundance than in PC12 cells. In addition, clone 15 occasionally hybridized to a larger mRNA (approximately 4,200 nucleotides) that produced a band much fainter than the 1,800-nucleotide TH mRNA. This larger mRNA changed in parallel with the 1,800-nucleotide TH mRNA in PC12 cells and was not detected in any of the tissues in which TH mRNA was detected (see below). For these three clones, which hybridize to more than one mRNA species, the smallest band was always used for the analysis of the regulation of mRNA levels.

The Northern blots for one experiment are shown in Fig. 2, and the mRNA levels as determined by quantitative densitometric scanning of the autoradiographs for two independent experiments are shown in Fig. 3. Whereas clone 63 and 71 mRNAs responded with increased levels at as early as 4 h of NGF treatment, the other three induced mRNAs (clones 10a, 21b, and 73) had a more delayed response to NGF at 12 h to two days of treatment, which corresponds to the beginning of neurite extension from PC12 cells (27). Clone 63 mRNA reached maximal levels somewhat earlier than the others, and clone 71 mRNA levels increased gradually over the 2 weeks. The maximal decrease for clone 2 mRNA occurred between 4 and 12 h of NGF treatment. Clone 10b and 15 (TH) mRNAs each showed somewhat more complex time courses of regulation (Fig. 2 and 3). Both mRNAs reached a maximal level (twofold over controls) at 4 h and then decreased to a level below that in untreated PC12 cells. PC12 cells continued to have lowered levels of the clone 15 mRNA (TH mRNA) for the remainder of the 2 weeks, while in contrast, clone 10b mRNA showed a mini-

FIG. 3. Time course of mRNA regulation by NGF. Quantitative densitometric scans of the autoradiographic films of two independent time-course experiments (one of which is shown in Fig. 2), plotted in arbitrary units of mRNA level versus time of NGF exposure. The line represents the average of the two data points at each time point. The identification number for each clone is noted in the upper left-hand corner of each graph. All points were arbitrarily scaled so that the 0 time point value equals 1 (clones 10a, 21b, 63, 71, 73, 34b, and 74) or 5 (clones 2, 10b, and 15).

num at 1 week of NGF exposure and then increased back to a level just below that in untreated PC12 cells by 2 weeks. The two clones detected by their lower abundance in F4 cells (34b and 74) showed no reproducible change in response to NGF when untreated and 2-week-NGF-treated PC12 cells were compared. However, both mRNAs had a small but reproducible minimum between 12 h and 1 day of treatment (Fig. 2 and 3).

Regulation by other factors. PC12 cells respond to agents other than NGF in a variety of ways. FGF was initially reported to induce transient process outgrowth and ornithine decarboxylase activity in PC12 cells (64, 65) and has more recently been shown to reproduce the entire spectrum of changes induced by NGF, including the production of stable neurite outgrowth (R. E. Rydel and L. A. Greene, J., in press). PC12 cells have surface receptors for epidermal growth factor and respond by an increase in ornithine decarboxylase activity (36), rapid cell surface ruffling (9), and changes in protein phosphorylation (31), but not by

induction of neurite outgrowth. PC12 cells also respond to DBcAMP by exhibiting short, spiky processes and several alterations in protein synthesis, but do not undergo neurite outgrowth or a variety of other biochemical responses that are triggered by NGF (see reference 28 for review). Dexamethasone also elicits responses from PC12 cells, including a severalfold increase in TH activity (56, 62) and mRNA levels (2, 42). In addition, Greenberg et al. (24, 26) have observed that the proto-oncogene *c-fos* is transcriptionally regulated in PC12 cells by a number of factors in addition to NGF (i.e., epidermal growth factor, FGF, insulin, increased extracellular K^+ , DBcAMP, the phorbol ester TPA, and nicotine). Therefore, we asked whether the changes in mRNA level observed for our clones were specific to NGF and therefore possibly related to neuronal differentiation, or could occur in response to other factors or growth conditions that do not cause neuronal differentiation. Sister PC12 cultures were treated for 1 week with one of a variety of factors and growth conditions (listed in Fig. 4).

FIG. 4. Regulation of PC12 cell mRNAs by NGF and other agents. Sister cultures of PC12 cells were treated for 1 week with the agents described below. Also included is RNA from PC12 cells grown in suspension culture (SUSP.) with or without NGF. Cytoplasmic RNA was isolated and used for Northern blotting. Blots were probed with ^{32}P -labeled cDNA inserts. Autoradiographic exposure time was 2.5 h for all blots except clones 10b, 15, and 34b (45 min) and 10a (21 h). The line at the top left of each blot indicates the origin, and the two arrows indicate the positions of the 28S (top) and 18S (bottom) rRNA bands. Additions to lanes: NGF, 50 ng/ml; DEX, 10 μM dexamethasone; FGF, 100 ng/ml; EGF, 5 ng of epidermal growth factor per ml; Insulin, 1 μg /ml; DBcAMP, 1 mM; TPA, 100 nM.

Cytoplasmic RNA was then isolated from the cells and analyzed by Northern blotting. The autoradiographs of the Northern blots from one experiment are shown in Fig. 4, and the mRNA levels as determined by quantitative densitometric scanning of the autoradiographs from two independent experiments are shown in Fig. 5.

Certain general points are apparent from this study. First, FGF was the only factor tested which could alter the abundance of all these mRNAs to the same or greater extent than NGF (Fig. 4 and 5). This is consistent with the work of Rydel and Greene (in press) showing that acidic and basic FGF can mimic all the NGF-induced biochemical responses tested in PC12 cells. Second, the effect of 10 μM dexamethasone on these mRNAs was opposite to the effects of NGF and FGF for all the NGF-regulated clones except 2 and

10b (compare lanes 1, 2, and 4 in each panel of Fig. 4 and 5). This is of interest, since it has been suggested that NGF and glucocorticoids may have opposing roles in the differentiation decision of neural crest cells toward the neuronal or chromaffin phenotypes (4, 16, 39). Cultures were also treated with NGF and dexamethasone simultaneously, since glucocorticoids have also been proposed to antagonize the actions of NGF in some systems (15, 16, 66). In the PC12 system, however, corticosteroids do not block NGF-induced neurite outgrowth (27). For most of the mRNAs, the combination of NGF and dexamethasone yielded an intermediate level of regulation, indicating a reciprocal antagonism. For clone 2 mRNA, which is decreased by either agent alone, the combination may have an even greater inhibitory effect.

To test whether neurite outgrowth is required for the NGF-induced mRNA changes, PC12 cells were grown in suspension culture with NGF. Under this condition, the cells do not morphologically differentiate in the presence of NGF; however, they do undergo certain transcriptional-dependent events necessary for neurite outgrowth (an effect called priming; 6). We found that the same NGF-dependent regulation occurred in suspension culture (last two lanes of each panel in Fig. 4 and 5) as on a collagen substrate, and we therefore conclude that regulation of these identified mRNAs is not dependent upon neurite outgrowth.

DBcAMP caused a twofold increase of clone 15 (TH), 10a, and 73 mRNAs, as well as a threefold decrease of clone 2 mRNA. TPA decreased the levels of clone 15 (TH) and clone 2 mRNAs. Finally, small changes were induced by other factors which were not reproducible.

Rat tissue distribution. As a first step toward relating the PC12 cell-derived probes to *in vivo* differentiation, and as an additional step in their characterization, we have asked where these mRNAs are expressed in rats. Northern blots of total cytoplasmic RNA from PC12 cells and various rat tissues were probed with ^{32}P -labeled cDNA inserts (Fig. 6). The mRNAs recognized by clones 21b (thymosin β_4) and 15 (TH) had expected distribution patterns. Thymosin β_4 protein has been reported to be present in all tissues and cell lines tested (32; 33; 70). The present study demonstrated the presence of thymosin β_4 mRNA (clone 21b) in all tissues of the rat which were examined (Fig. 6), as well as in PC12 and F4 cells. TH mRNA (clone 15), which encodes the rate-limiting enzyme of the catecholamine biosynthetic pathway, was detected in the adrenal gland, the superior cervical ganglion (SCG), and the brain (seen with autoradiographic exposures longer than shown in Fig. 6), as expected, but not in other tissues. The low level of expression of TH in whole brain is presumably due to the dilution of a strong signal from a small subpopulation of catecholaminergic neurons by the rest of the mostly noncatecholaminergic brain cells.

Several of the mRNAs recognized by our other probes exhibited novel tissue distributions. Clone 10a and 73 mRNAs, which were both increased by NGF and undetectable in F4 cells, were present at very high levels in the SCG, barely detectable in brain and adrenal gland, and not detectable in all other tissues examined. The mRNAs that hybridized with the NGF-inducible clones 63 and 71 also yielded strong signals in the SCG and were either undetectable or observable only after long autoradiographic exposures in the adrenal gland. However, these mRNAs were present in a variety of other tissues as well. The mRNA recognized by clone 71 was present in all other tissues examined, including the brain, where there was a stronger hybridization signal at embryonic day 19 (E19) than in the adult. Clone 63 gave a positive signal in placenta, kidney, lung, stomach, and heart,

FIG. 5. Effects of various agents on mRNA levels. The quantitative densitometric scans of the autoradiographic films of two independent experiments as described in the legend of Fig. 4 are plotted in arbitrary units of mRNA level for each condition tested. The bars represent the average of the two data points. The identification number for each clone is noted in the upper left-hand corner of each graph. The additions are as indicated in the legend of Fig. 4. All points were arbitrarily scaled so that the "untreated" value (first column of each panel) equals 1.

but not in brain or in liver, spleen, thymus, and skeletal muscle.

Of the mRNAs that decreased in response to NGF treatment, that which hybridized to clone 10b was more abundant in the adrenal gland than in the SCG. This mRNA was also present in E19 brain and placenta but, interestingly, was only just detectable in adult brain and undetectable in F4 cells and all other tissues examined. Another down-regulated mRNA, recognized by clone 2, was detectable in a variety of tissues, but at levels much lower than in PC12 cells.

Finally, clone 74, which was not substantially regulated by NGF, hybridized with an 1,850-nucleotide mRNA which was most abundant in the SCG, brain, skeletal muscle, and heart and was found at considerably lower levels in all other tissues assayed. In addition to the 1,850-nucleotide mRNA, clone 74 also hybridized equally well with a 2,100-nucleotide

message in PC12 cells. This species, however, was not detected in rat tissues or in F4 cells.

DISCUSSION

Strengths and limitations of the differential screening approach. The aim of the present study has been to define a set of cDNA probes that can be used to study the mechanism by which NGF regulates mRNA levels, to identify proteins that may play roles in NGF-promoted cellular differentiation, and to identify mRNAs and proteins which may serve as cell-specific markers. PC12 cell cDNA libraries were differentially screened to detect mRNAs with different abundances in untreated PC12 cells, long-term-NGF-treated PC12 cells, and F4 cells. We have identified cDNAs for a number of NGF-regulated mRNAs, including five previously uncharacterized messages, and for two previously identified

FIG. 6. Rat tissue distribution of mRNA expression. Northern blot analyses of rat tissue cytoplasmic RNA (7 μ g per lane) probed with 32 P-labeled cDNA inserts. Autoradiographic exposure time was 1.5 days for all the blots. With longer exposure times, clone 73 mRNA was also detectable in the adrenal gland and the adult brain. The line at the top left of each blot indicates the origin, and the two arrows indicate the position of the 28S (top) and 18S (bottom) rRNA bands.

mRNAs (thymosin β_4 and TH), one of which was not known to be NGF regulated (thymosin β_4). By further sequence analysis, clone 73 mRNA was found to encode a protein that had significant homology to, but was not identical with, all known intermediate filament proteins (D. G. B. Leonard, J. D. Gorham, P. Cole, L. A. Greene, and E. B. Ziff, manuscript submitted).

Several features of this differential screening method preclude the identification of certain NGF-regulated mRNAs. First, we compared untreated and long-term-NGF-treated PC12 cells to favor the detection of mRNAs that are specifically related to neuronal differentiation. Therefore, we did not identify mRNAs that undergo transient regulation by NGF, such as the messages encoding *c-fos*, *c-myc*, actin, or ornithine decarboxylase (25). Thy-1 protein (57) and its mRNA (14; Drexler and Greene, Soc. Neurosci Abstr., vol. 12, part 1, 1986, p. 215), which were increased in PC12 cells within several days of NGF treatment and then returned to lower levels by 2 weeks of NGF exposure, were also not detected. Second, because only a limited number of clones were screened (in this case approximately 13,000), it is likely

that other NGF-regulated mRNAs have escaped our detection. For instance, as based on a comparison of mRNA sizes and the extent of NGF regulation, we did not detect the NGF-regulated message recognized by Anderson and Axel's clone 10 (1) or the highly induced clone VGF8a mRNA (40). Third, differential screening is unlikely to detect very low-abundance mRNAs. In a comparable type of study on platelet-derived growth factor-induced mRNAs in fibroblasts, Cochran et al. (8) estimated that at least 500 mRNA copies per cell were required for detection by their protocol. Indeed, our Northern blot experiments indicate that the mRNAs in the present study are all relatively abundant.

Inclusion of the F4 cell probe proved to be an important factor for the detection of NGF-regulated clones. In the original screening, clones 2, 10a, and 15 were detected as clones which were not regulated by NGF, but were lower or undetectable in F4 cells. Only after the Northern blot analyses did the NGF regulation of these three clones become apparent. Therefore, the addition of a third probe from a less differentiated cell type was useful for the identification of regulated mRNAs by differential screening.

Properties of the identified mRNAs. Since each of the mRNAs detected in the present study showed a somewhat different pattern of characteristics, it is useful to consider each individually.

(i) **Thymosin β_4 .** The mRNA encoding thymosin β_4 was found to be induced approximately 10-fold in response to treatment with NGF or FGF, but not in response to a number of other treatments. Thymosin β_4 was originally identified in extracts of calf thymus and was reported (46) to induce terminal deoxynucleotidyltransferase, an early marker of thymocyte maturation. This led to the proposal that thymosin β_4 is a secreted thymic hormone (46). However, thymosin β_4 mRNA does not encode a signal peptide (23, 69). Thymosin β_4 protein (32, 33, 70) and mRNA (Fig. 6) have a ubiquitous cell and tissue distribution. Thymosin β_4 mRNA is induced by alpha interferon in a human neuroblastoma cell line (21) and a lymphoblastoid cell line (49). Wodnar-Filipowicz et al. (69) have hypothesized that thymosin β_4 may be a component of the cytoskeleton. While such a role for thymosin β_4 is intriguing given the extensive morphological changes involved in PC12 neuronal differentiation and the induction of this mRNA by NGF, the precise physiologic role of thymosin β_4 in NGF-responsive as well as in other cell types remains to be determined.

(ii) **TH.** We have isolated a near-full-length cDNA clone of TH mRNA which encodes this rate-limiting enzyme in catecholamine biosynthesis. Two effects of NGF on TH mRNA levels were observed here. There was first a transient, twofold induction of TH mRNA at 4 h of NGF treatment, followed by a long-term twofold decrease from the control levels of TH mRNA. The latter observation correlates well with previous reports that long-term exposure of PC12 cells to NGF brings about a threefold decrease of the specific activity of TH (18, 27). Thus here, as in several other cases (5, 42), there appears to be a good correlation between changes in TH mRNA and TH activity or TH protein levels.

Several agents in addition to NGF have been found to regulate TH activity or mRNA in PC12 cell cultures. Such regulation is of potential importance given the rate-limiting role of TH in catecholamine biosynthesis. Previous studies have reported that dexamethasone induces TH activity (56; 62) and mRNA levels (2; 42) by severalfold. We have also observed an induction of TH mRNA levels by dexamethasone. Lewis et al. (42) reported TH mRNA level increases

in a PC12 cell subclone within 4 to 24 h of exposure to DBcAMP; in the present study, the relative abundance of TH mRNA doubled by 1 week of treatment with this analog. Finally, of note is the intriguing observation that TH mRNA was observed here to decrease by severalfold after a week of exposure to the tumor promoter TPA.

(iii) Clones 10a and 73. Among the clones described in this study, clones 10a and 73 appear to recognize mRNA(s) with the most neuronally restricted tissue distributions. Both are very abundant in the SCG, with low levels in the adrenal gland and the brain, and are undetectable in other tissues. These thus represent one of the types of mRNAs sought in this study. We hypothesize that clones 10a and 73 correspond to the 3' and 5' ends, respectively, of the same mRNA. The two mRNAs are approximately the same size, have a similar time course of induction by NGF, show the same pattern of responses to agents other than NGF, and exhibit the same tissue distribution. These two cDNAs may not cross hybridize because clone 10a cDNA is very small and includes the 3' poly(A) tail, while clone 73 cDNA does not encode a poly(A) tail. Therefore, these two cDNA clones could arise from the same mRNA but not overlap in sequence. This hypothesis will only be verified when full-length cDNAs of these clones are identified. A more detailed characterization of 73 cDNA and its corresponding mRNA and encoded protein will be presented elsewhere (Leonard et al., submitted) and reveals that the encoded protein appears to be a novel intermediate filament protein and that the mRNA is distributed only within a restricted subset of central and peripheral nervous system neurons.

(iv) Clones 63 and 71. Both clones 63 and 71 detect mRNAs that are induced approximately fivefold in response to long-term NGF treatment. Clone 71 mRNA was detectable in all rat tissues surveyed, while the mRNA detected by clone 63 showed a more restricted tissue distribution. Clone 63 mRNA showed an induction by NGF within 4 h of treatment and peaked by 1 day, whereas clone 71 mRNA increased more gradually over the 2-week course of NGF treatment. These results provide further evidence that NGF induces a complex temporal progression of mRNA regulation events, rather than a single, coordinated pattern of mRNA synthesis. Some mRNAs are maximally induced by NGF within 1 h, such as *c-fos* and actin (24), whereas others require several hours, such as ornithine decarboxylase (20, 24) and TH (Fig. 3). More greatly delayed mRNA level changes are observed at 12 h to 1 day (e.g., clone 63; Fig. 3) or after even longer times of NGF treatment, such as occurs for Thy-1 (14; Drexler and Greene, Soc. Neurosci. Abstr. vol. 12, part 1, 1986, p. 215) and clones 10a, 71, and 73 (Fig. 3).

(v) Clones 2 and 10b. Clone 2 mRNA was maximally decreased after only 12 h of NGF treatment and was the only mRNA with only low-level expression in all the tissues screened. The very low relative abundance of clone 2 mRNA in F4 cells and all the tissues screened raises the possibility that this mRNA is related to the neoplastic behavior of PC12 cells. As was the case for TH mRNA, the mRNAs detected by clone 10b transiently increased and then decreased after a day of NGF treatment. The observation that this NGF-decreased mRNA was in much higher abundance in the adrenals and in PC12 cells than in the SCG suggests that it could encode a protein that may be a marker for the developmental divergence of chromaffin cells and sympathetic neurons. The relatively higher level of this mRNA in E19 brain and placenta as compared to adult brain and other tissues except the adrenals is intriguing, but presently difficult to interpret. Clearly it will be of interest to obtain further

sequence and distribution information for these messages as well as for the proteins they encode.

Regulation by factors other than NGF. To examine the relationship of the mRNA regulation observed with NGF to neuronal differentiation, we also tested a number of other agents to which PC12 cells respond. Of the agents which are not able to promote PC12 cell neuronal differentiation, none reproduced the same pattern of mRNA regulation as NGF. For instance, while DBcAMP decreased clone 2 mRNA to a similar degree as did NGF, it had no effect on clones 10b, 21b, 63, and 71 and produced an effect opposite to that of NGF for clone 15 (TH) mRNA. TPA, an activator of protein kinase C and a potent tumor promoter, reproduced the effect of NGF on clone 15 (TH) mRNA and had almost the same effect as NGF on clone 2 mRNA, but had no significant effect on any of the mRNAs that were increased by NGF. These two sets of observations are of particular interest in light of the hypothesis that protein kinases A and C may play roles in the mechanism of NGF-induced differentiation (see, for example, reference 10).

The one agent tested that caused the same pattern of mRNA regulation as NGF was FGF. This is also the only other factor that has been shown to promote PC12 cell neuronal differentiation (64, 65; Rydel and Greene, in press). This observation, along with the inability of nondifferentiating factors to regulate the same mRNAs, supports the possibility that the mRNA regulations we have identified may be correlated with neuronal differentiation. These specific changes contrast with the finding that the rapid, transient regulation by NGF and FGF of *c-fos*, *c-myc*, actin, and ornithine decarboxylase genes is mimicked by a variety of nondifferentiating factors (24).

In addition to promoting neurite outgrowth, FGF induces the same spectrum of biochemical responses in PC12 cells as NGF does (Rydel and Greene, in press). Our findings extend this similarity of NGF and FGF to the mRNA level (see also Drexler and Greene, Soc. Neurosci. Abstr., vol. 12, part 1, 1986, p. 215). FGF also enhances the extent of neurite outgrowth and cell survival in cultures of at least several types of central nervous system neurons (53, 67) and thus could play a neurotrophic role in the nervous system. The mRNA probes we have identified may be useful probes for the study of the role of FGF in neuronal differentiation and its mechanism of action.

Another factor with effects on many of the mRNAs detected by this study is dexamethasone. NGF and glucocorticoids have been suggested to play opposing roles in the decision of neural crest-derived cells to become either chromaffin cells or sympathetic neurons (4, 39). In culture, precursor or chromaffin cells may be induced by NGF to acquire neuronal characteristics, whereas glucocorticoids enhance their chromaffin cell properties (15, 16). In this light, it is of interest that NGF and dexamethasone had opposite effects on six of the eight NGF-regulated mRNAs studied here. We have also observed a reciprocal antagonism with the combination of NGF and dexamethasone. Thus, these cDNA probes could be used to investigate the mechanisms of mRNA regulation by NGF and glucocorticoids, the interactions of these two agents, and their roles in the environmental signaling of neural crest cell differentiation.

In summary, this study is the initial phase of investigations to understand the mechanism of action of NGF in gene regulation and neuronal differentiation, as well as to identify neuron-specific markers. The cDNA probes described here have already raised a number of interesting issues and promise to be of continued usefulness in future studies.

ACKNOWLEDGMENTS

This study was supported in part by grants to L.A.G. from the Public Health Service (NS 16036) and the March-of-Dimes Birth Defects Foundation and to E.B.Z. from the Dysautonomia Foundation, the American Parkinson Disease Foundation, and the Mental Health Clinical Research Center (grant MH 35976). D.G.B.L. was supported by Public Health Service Training Grant 5 T32 GM 07308 from the National Institute of General Medical Sciences.

We thank Adriana Rukenstein for her skillful aid with cell culture, Michael Greenberg for his advice in many phases of this work, and Patrick Cole for technical assistance with DNA sequencing. We also thank Russell Rydel for sharing with us his findings on FGF prior to their publication.

LITERATURE CITED

- Anderson, D. J., and R. Axel. 1985. Molecular probes for the development and plasticity of neural crest derivatives. *Cell* 42:649-662.
- Baetge, E. E., B. B. Kaplan, D. J. Reis, and T. H. Joh. 1981. Translation of tyrosine hydroxylase from poly(A)-mRNA in pheochromocytoma cells is enhanced by dexamethasone. *Proc. Natl. Acad. Sci. USA* 78:1269-1273.
- Biggin, M. D., T. J. Gibson, and G. F. Hong. 1983. Buffer gradient gels and ³⁵S label as an aid to rapid DNA sequence determination. *Proc. Natl. Acad. Sci. USA* 80:3963-3965.
- Bilofsky, H. S., C. Burks, J. W. Fickett, W. B. Good, F. I. Lewitter, W. P. Windome, C. D. Swindell, and C.-S. Tung. 1986. The GenBank genetic sequence database. *Nucleic Acids Res.* 14:1-4.
- Bjerre, B., and A. Bjorklund. 1973. The production of catecholamine-containing cells *in vitro* by young chick embryos: effects of "nerve growth factor" (NGF) and its antiserum. *Neurobiology* 3:140-161.
- Black, I. B., D. M. Chikaraishi, and E. J. Lewis. 1985. Trans-synaptic increase in RNA coding for tyrosine hydroxylase in a rat sympathetic ganglion. *Brain Res.* 339:151-153.
- Burstein, D. E., and L. A. Greene. 1978. Evidence for RNA synthesis-dependent and -independent pathways in stimulation of neurite outgrowth by nerve growth factor. *Proc. Natl. Acad. Sci. USA* 75:6059-6063.
- Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18:5294-5299.
- Cochran, B. H., A. C. Reffel, and C. D. Stiles. 1983. Molecular cloning of gene sequences regulated by platelet-derived growth factor. *Cell* 33:939-947.
- Connolly, J. L., S. A. Green, and L. A. Greene. 1984. Comparison of rapid changes in surface morphology and coated pit formation of PC12 cells in response to nerve growth factor, epidermal growth factor, and dibutyl cyclic AMP. *J. Cell Biol.* 98:457-465.
- Cremins, J., J. A. Wagner, and S. Halegoua. 1986. Nerve growth factor action is mediated by cyclic AMP- and Ca²⁺/phospholipid-dependent protein kinases. *J. Cell Biol.* 103:887-893.
- Curran, T., and J. I. Morgan. 1985. Superinduction of *c-fos* by nerve growth factor in the presence of peripherally active benzodiazepines. *Science* 229:1265-1268.
- Curran, T., G. Peters, C. Van Beveren, N. M. Teich, and I. M. Verma. 1982. FBJ murine osteosarcoma virus: identification and molecular cloning of biologically active proviral DNA. *J. Virol.* 44:674-682.
- Dichter, M. A., A. S. Tischler, and L. A. Greene. 1977. Nerve growth factor-induced change in electrical excitability and acetylcholine sensitivity of a rat pheochromocytoma cell line. *Nature (London)* 268:501-504.
- Dickson, G., H. Prentice, J.-P. Julien, G. Ferrari, A. Leon, and F. S. Walsh. 1986. Nerve growth factor activates thy-1 and neurofilament gene transcription in rat PC12 cells. *EMBO J.* 5:3449-3453.
- Doupe, A. J., S. C. Landis, and P. H. Patterson. 1985. Environmental influences in the development of neural crest derivatives: glucocorticoids, growth factors and chromaffin cell plasticity. *J. Neurosci.* 5:2119-2142.
- Doupe, A. J., P. H. Patterson, and S. C. Landis. 1985. Small intensely fluorescent (SIF) cells in culture: role of glucocorticoids and growth factors in their development and phenotypic interconversions with other neural crest derivatives. *J. Neurosci.* 5:2143-2160.
- Dworkin, M. B., and I. B. Dawid. 1980. Use of a cloned library for the study of abundant poly(A)⁺ RNA during *Xenopus laevis* development. *Dev. Biol.* 76:449-464.
- Edgar, D. H., and H. Thoenen. 1978. Selective enzyme induction in a nerve growth factor responsive pheochromocytoma cell line (PC12). *Brain Res.* 154:186-190.
- Feinberg, A. P., and B. Vogelstein. 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity: addendum. *Anal. Biochem.* 137:266-267.
- Feinstein, S. C., S. L. Dana, L. McConlogue, E. M. Shooter, and P. Coffino. 1985. Nerve growth factor rapidly induces ornithine decarboxylase mRNA in PC12 rat pheochromocytoma cells. *Proc. Natl. Acad. Sci. USA* 82:5761-5765.
- Friedman, R. L., S. P. Manly, M. McMahon, I. M. Kerr, and G. R. Stark. 1984. Transcriptional and posttranscriptional regulation of interferon-induced gene expression in human cells. *Cell* 38:745-755.
- Goedert, M., U. Otten, S. P. Hunt, A. Bond, D. Chapman, M. Schlumpf, and W. Lichtensteiger. 1984. Biochemical and anatomical effects of antibodies against nerve growth factor on developing rat sensory ganglia. *Proc. Natl. Acad. Sci. USA* 81:1580-1584.
- Goodall, G. J., M. Richardson, Y. Furuichi, A. Wodnar-Filipowicz, and B. L. Horecker. 1985. Sequence of a cloned 523-bp cDNA for thymosin β_4 . *Arch. Biochem. Biophys.* 236:445-447.
- Greenberg, M. E., L. A. Greene, and E. B. Ziff. 1985. Nerve growth factor and epidermal growth factor induce rapid transient changes in proto-oncogene transcription in PC12 cells. *J. Biol. Chem.* 260:14101-14110.
- Greenberg, M. E., and E. B. Ziff. 1984. Stimulation of 3T3 cells induces transcription of the *c-fos* proto-oncogene. *Nature (London)* 311:433-438.
- Greenberg, M. E., E. B. Ziff, and L. A. Greene. 1986. Stimulation of neuronal acetylcholine receptors induces rapid gene transcription. *Science* 234:80-83.
- Greene, L. A., and A. S. Tischler. 1976. 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.
- Greene, L. A., and A. S. Tischler. 1982. PC12 pheochromocytoma cultures in neurobiological research. *Adv. Cell. Neurobiol.* 3:373-414.
- Grima, B., A. Lamouroux, F. Blanot, N. F. Biguet, and J. Mallet. 1985. Complete coding sequence of rat tyrosine hydroxylase mRNA. *Proc. Natl. Acad. Sci. USA* 82:617-621.
- Gunning, P., P. Ponte, H. Okayama, J. Engel, H. Blau, and L. Kedes. 1983. Isolation and characterization of full-length cDNA clones for human α -, β -, and γ -actin mRNAs: skeletal but not cytoplasmic actins have an amino-terminal cysteine that is subsequently removed. *Mol. Cell. Biol.* 3:787-795.
- Halegoua, S., and J. Patrick. 1980. Nerve growth factor mediates phosphorylation of specific proteins. *Cell* 22:571-581.
- Hannappel, E., and W. Leibold. 1985. Biosynthesis rates and content of thymosin β_4 in cell lines. *Arch. Biochem. Biophys.* 240:236-241.
- Hannappel, E., G.-J. Xu, J. Morgan, J. Hempstead, and B. L. Horecker. 1982. Thymosin β_4 : a ubiquitous peptide in rat and mouse tissues. *Proc. Natl. Acad. Sci. USA* 79:2172-2175.
- Hedrick, S. M., D. I. Cohen, E. A. Nielsen, and M. M. Davis. 1984. Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. *Nature (London)* 308:149-153.
- Honegger, P., and D. Lenoir. 1983. Nerve growth factor (NGF) stimulation of cholinergic telencephalic neurons in aggregating cell cultures. *Dev. Brain Res.* 3:229-238.
- Huff, K. R., and G. Guroff. 1979. Nerve growth factor-induced reduction in epidermal growth factor responsiveness and epidermal growth factor receptors in PC12 cells: an aspect of cell

- differentiation. *Biochem. Biophys. Res. Commun.* **89**:175–180.
37. Huynh, T. H., R. A. Young, and R. W. Davis. 1985. Construction and screening cDNA libraries in λ gt10 and λ gt11, p. 49–78. In D. Glover (ed.), *DNA cloning techniques*, vol. 1: A practical approach. IRL Press, Oxford, U.K.
38. Lamouroux, A., N. Faucon Biguet, D. Samolyk, A. Privat, J. C. Salomon, J. F. Pujol, and J. Mallet. 1982. Identification of cDNA clones coding for rat tyrosine hydroxylase antigen. *Proc. Natl. Acad. Sci. USA* **79**:3881–3885.
39. Landis, S. C., and P. H. Patterson. 1981. Neural crest cell lineages. *Trends Neurosci.* **4**:172–175.
40. Levi, A., J. D. Eldridge, and B. M. Paterson. 1985. Molecular cloning of a gene sequence regulated by nerve growth factor. *Science* **229**:393–395.
41. Levi-Montalcini, R., and P. U. Angeletti. 1968. Nerve growth factor. *Physiol. Rev.* **48**:534–569.
42. Lewis, E. J., A. W. Tank, N. Weiner, and D. M. Chikaraishi. 1983. Regulation of tyrosine hydroxylase mRNA by glucocorticoid and cyclic AMP in a rat pheochromocytoma cell line. *J. Biol. Chem.* **258**:14632–14637.
43. Linzer, D. I. H., S.-J. Lee, L. Ogren, F. Talamantes, and D. Nathans. 1985. Identification of proliferin mRNA and protein in mouse placenta. *Proc. Natl. Acad. Sci. USA* **82**:4356–4359.
44. Linzer, D. I. H., and D. Nathans. 1983. Growth-related changes in specific mRNAs of cultured mouse cells. *Proc. Natl. Acad. Sci. USA* **80**:4271–4275.
45. Linzer, D. I. H., and D. Nathans. 1984. Nucleotide sequence of a growth-related mRNA encoding a member of the prolactin-growth hormone family. *Proc. Natl. Acad. Sci. USA* **81**:4255–4259.
46. Low, T. L. K., S.-K. Hu, and A. L. Goldstein. 1981. Complete amino acid sequence of bovine thymosin β_4 : a thymic hormone that induces terminal deoxyribonucleotidyl transferase activity in thymocyte populations. *Proc. Natl. Acad. Sci. USA* **78**:1162–1166.
47. Maniatis, T. F., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
48. McGuire, J. C., and L. A. Greene. 1979. Rapid stimulation by nerve growth factor of amino acid uptake by clonal PC12 pheochromocytoma cells. *J. Biol. Chem.* **254**:3362–3367.
49. McMahon, M., G. R. Stark, and I. M. Kerr. 1986. Interferon-induced gene expression in wild-type and interferon-resistant human lymphoblastoid (Daudi) cells. *J. Virol.* **57**:362–366.
50. Messing, J., and J. Vieira. 1982. A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments. *Gene* **19**:269–276.
51. Mobley, W. C., J. L. Rutkowski, G. I. Tennekoon, K. Buchanan, and M. V. Johnston. 1985. Choline acetyltransferase activity in striatum of neonatal rats increased by nerve growth factor. *Science* **229**:284–287.
52. Mobley, W. C., A. Schenker, and E. M. Shooter. 1976. Characterization and isolation of proteolytically modified nerve growth factor. *Biochemistry* **15**:5543–5551.
53. Morrison, R. S., A. Sharma, J. De Vellis, and R. A. Bradshaw. 1986. Basic fibroblast growth factor supports the survival of cerebral and cortical neurons in primary culture. *Proc. Natl. Acad. Sci. USA* **83**:7537–7541.
54. Moscatelli, D., M. Presta, and D. B. Rifkin. 1986. Purification of a factor from human placenta that stimulates capillary endothelial cell protease production, DNA synthesis, and migration. *Proc. Natl. Acad. Sci. USA* **83**:2091–2095.
55. Okayama, H., and P. Berg. 1982. High-efficiency cloning of full-length cDNA. *Mol. Cell. Biol.* **2**:161–170.
56. Otten, U., and M. Towbin. 1980. Permissive action of glucocorticoids in induction of tyrosine hydroxylase by nerve growth factor in a pheochromocytoma cell line. *Brain Res.* **193**:304–308.
57. Richter-Landsberg, C., L. A. Greene, and M. L. Shelanski. 1985. Cell surface thy-1-cross-reactive glycoprotein in cultured PC12 cells: modulation by nerve growth factor and association with the cytoskeleton. *J. Neurosci.* **5**:468–476.
58. Rowekamp, W., and R. A. Firtel. 1980. Isolation of developmentally regulated genes from Dictyostelium. *Dev. Biol.* **79**:409–418.
59. Sanger, F., and A. R. Coulson. 1978. The use of thin acrylamide gels for DNA sequencing. *FEBS Lett.* **87**:107–110.
60. Sanger, F., A. R. Coulson, B. G. Barrell, A. J. H. Smith, and B. Roe. 1980. Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. *J. Mol. Biol.* **143**:161–178.
61. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5468.
62. Schubert D., M. LaCorbiere, F. G. Klier, and J. H. Steinbach. 1980. The modulation of neurotransmitter synthesis by steroid hormones and insulin. *Brain Res.* **190**:67–79.
63. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503–517.
64. Togari, A., D. Baker, G. Dickens, and G. Guroff. 1983. The neurite-promoting effect of fibroblast growth factor on PC12 cells. *Biochem. Biophys. Res. Commun.* **114**:1189–1193.
65. Togari, A., G. Dickens, H. Kuzuya, and G. Guroff. 1985. The effect of fibroblast growth factor on PC12 cells. *J. Neurosci.* **5**:307–316.
66. Unsicker, K., B. Kirsch, U. Otten, and H. Thoenen. 1978. Nerve growth factor-induced fiber outgrowth from isolated rat adrenal chromaffin cells: impairment by glucocorticoids. *Proc. Natl. Acad. Sci. USA* **75**:3498–3502.
67. Walicke, P., W. M. Cowan, N. Ueno, A. Baird, and R. Guillemine. 1986. Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension. *Proc. Natl. Acad. Sci. USA* **83**:3012–3016.
68. Williams, J. G., and M. M. Lloyd. 1979. Changes in the abundance of polyadenylated RNA during slime mould development measured using cloned molecular hybridization probes. *J. Mol. Biol.* **129**:19–35.
69. Wodnar-Filipowicz, A., U. Gubler, Y. Furuichi, M. Richardson, E. F. Nowoswiat, M. S. Poonian, and B. L. Horecker. 1984. Cloning and sequence analysis of cDNA for rat spleen thymosin β_4 . *Proc. Natl. Acad. Sci. USA* **81**:2295–2297.
70. Xu, G.-J., E. Hannappel, J. Morgan, J. Hempstead, and B. L. Horecker. 1982. Synthesis of thymosin β_4 by peritoneal macrophages and adherent spleen cells. *Proc. Natl. Acad. Sci. USA* **79**:4006–4009.