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Structure of the Gene Encoding VGF, a Nervous System-Specific mRNA That Is Rapidly and Selectively Induced by Nerve Growth Factor in PC12 Cells

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Nerve growth factor (NGF) plays a critical role in the development and survival of neurons in the peripheral nervous system. Following treatment with NGF but not epidermal growth factor, rat pheochromocytoma (PC12) cells undergo neural differentiation. We have cloned a nervous system-specific mRNA, NGF33.1, that is rapidly and relatively selectively induced by treatment of PC12 cells with NGF and basic fibroblast growth factor in comparison with epidermal growth factor. Analysis of the nucleic acid and predicted amino acid sequences of the NGF33.1 cDNA clone suggested that this clone corresponded to the NGF-inducible mRNA called VGF (A. Levi, J. D. Eldridge, and B. M. Paterson, Science 229:393–395, 1985; R. Possenti, J. D. Eldridge, B. M. Paterson, A. Grasso, and A. Levi, EMBO J. 8:2217–2223, 1989). We have used the NGF33.1 cDNA clone to isolate and characterize the VGF gene, and in this paper we report the complete sequence of the VGF gene, including 853 bases of 5′ flank. The VGF gene contains two small introns within the 5′ untranslated region of the mRNA, and analysis of the 5′ flank revealed TATAA and CCAAT elements, several GC boxes, and a consensus cyclic AMP response element-binding protein binding site. The VGF promoter contains sequences homologous to other NGF-inducible, neuronal promoters. We further show that VGF mRNA is induced in PC12 cells to a greater extent by depolarization and by phorbol-12-myristate-13-acetate treatment than by 8-bromo-cyclic AMP treatment. By Northern (RNA) and RNase protection analysis, VGF mRNA is detectable in embryonic and postnatal central and peripheral nervous tissues but not in a number of nonneural tissues. In the cascade of events which ultimately leads to the neural differentiation of NGF-treated PC12 cells, the VGF gene encodes the most rapidly and selectively regulated, nervous-system specific mRNA yet identified.

Nerve growth factor (NGF) is critical to the differentiation and survival of sympathetic and sensory neurons in the peripheral nervous system (PNS) (58, 59), and NGF and its receptor have also been identified in discrete regions of the central nervous system (CNS) (1, 16, 96, 102, 105). Investigation of the mechanism of action of NGF has been facilitated through studies of the effects of NGF on the PC12 cell line (39). In the presence of NGF, PC12 cells differentiate from adrenal chromaffin-like cells into sympathetic neuron-like cells. A number of gene products have been identified that are quantitatively induced during the neural differentiation of PC12 cells, including the NGF-inducible large external glycoprotein (66, 85, 86), ornithine decarboxylase (ODC) (33, 65), SCG10 (3, 4), GAP-43 (10, 49), VGF (57), peripherin (55, 56), and the protease transin (63). In addition, several immediate-early genes, some of which encode transcriptional regulatory proteins, are rapidly turned on in PC12 cells in response to NGF or epidermal growth factor (EGF) (21, 22, 25, 35, 52, 69, 70, 98, 108). Since NGF but not EGF triggers the neuronal differentiation of PC12 cells, it has been of great interest to identify genes that are regulated rapidly and selectively by NGF, since these genes may encode proteins that initiate the cascade of biochemical and morphological alterations which result in neural differentiation. To gain further understanding of the mechanisms by which NGF regulates neural differentiation, we have used subtractive screening techniques to isolate cDNA clones which correspond to mRNAs that are regulated in PC12 cells rapidly and to a greater extent by NGF than by EGF.

The nucleotide sequence of one of these cDNA clones, NGF33.1, was found to predict a polypeptide which contained regions of substantial amino acid identity to that of the NGF-inducible clone VGF (80), the nucleotide sequence of which has not been reported. On the basis of detailed sequence analysis of the NGF33.1 clone, we believe that the VGF and NGF33.1 cDNA clones correspond to the same RNA. VGF was originally cloned by Levi et al. on the basis of its induction in PC12 cells by NGF (57); regulation of VGF mRNA levels by other growth factors was not examined. VGF mRNA has been detected in rat brain but not in uterus or liver (101), and the encoded protein has been detected by immunohistochemical techniques in a subset of adult hypothalamic neurons by using antibodies directed against recombinant fusion proteins (101). Of further interest, VGF polypeptide was shown to be stored in secretory vesicles and released from PC12 cells through a regulated secretory pathway (80). We report here the cloning of the rat gene that encodes VGF, including 853 bases of 5′-flanking DNA. We show that VGF mRNA is rapidly induced in PC12 cells to a greater extent by NGF and basic fibroblast growth factor (bFGF) treatment than by EGF treatment and that the mRNA is detectable exclusively in rodent central and peripheral nervous tissues. In the cascade of events which ultimately results in the neural differentiation of PC12 cells in the presence of NGF, the VGF gene encodes the most rapidly and selectively regulated, nervous system-specific mRNA yet identified.

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MATERIALS AND METHODS

cDNA library construction and screening. Total RNA was isolated from PC12 cells treated for 3 h with βNGF (50 ng/ml) and cycloheximide (CHX; 10 μg/ml) (24, 69) as described previously (35, 84), and poly(A)$^+$ RNA was selected by oligo(dT)-cellulose chromatography. First-strand cDNA was synthesized essentially as described previously (40) from 15 μg of poly(A)$^+$ RNA with 70 U of avian myeloblastosis virus reverse transcriptase (Life Sciences) in 40 mM Tris (pH 8.3)–10 mM $MgCl_2$–50 mM KCl–100 μg of oligo(dT)$_{12-18}$ per ml–4 mM sodium pyrophosphate–0.625 mM deoxynucleoside triphosphates at 43°C for 45 min. Second-strand synthesis was carried out in the same tube in 20 mM Tris (pH 7.4)–10 mM NH_4SO_4–5 mM $MgCl_2$–50 μg of bovine serum albumin (BSA) per ml–100 mM KCl–50 μM deoxynucleoside triphosphates–25 U of *Escherichia coli* DNA ligase–5 U of RNase H–115 U of *E. coli* DNA polymerase I. Double-stranded cDNA was blunted with T4 DNA polymerase and Klenow enzyme, ligated to *Eco*RI adaptors (NEN), isolated free of adaptors by Bio-Gel A50 chromatography (Bio-Rad), ligated to *Eco*RI-digested lambda gt10 (Stratagene), and packaged in vitro (Stratagene).

A total of 10^5 PFU of the primary NGF-CHX-treated PC12 cDNA library (2×10^6 PFU) was screened by using a subtractively hybridized probe. ^{32}P-labeled first-strand cDNA was synthesized essentially as described previously (56), using 10 μg of poly(A)$^+$ RNA isolated from PC12 cells treated with βNGF (50 ng/ml) and CHX (10 μg/ml) for 3 h. The RNA template was hydrolyzed with NaOH, and the cDNA was neutralized and coprecipitated in ethanol with 20 μg of poly(A)$^+$ RNA isolated from CHX-treated PC12 cells. The pellet was resuspended in 12 to 15 μl of 0.12 M sodium phosphate (pH 7)–0.82 M NaCl–1 mM EDTA, heat denatured at 85°C for 5 min, and hybridized at 68°C for 18 h (15, 44, 89). The reaction mixture was diluted with 500 μl 0.12 M sodium phosphate–0.1% sodium dodecyl sulfate (SDS) and incubated with 200 mg of hydroxylapatite, preequilibrated in the same buffer, for 1 h at 60°C. Single-stranded cDNA was isolated after centrifugation to remove hydroxylapatite-bound RNA-DNA hybrids, with a recovery of 10 to 20% of the total first-strand synthesis. Replicate nitrocellulose filters were screened with ~10^6 cpm of ^{32}P-labeled subtracted probe per ml and with ~10^7 cpm of ^{32}P-labeled cDNA per ml, synthesized by using poly(A)$^+$ RNA isolated from either CHX- or NGF-CHX-treated PC12 cells, in 5× SSC (1× = 0.15 M NaCl plus 0.015 M sodium citrate)–50% formamide–5× Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA)–100 μg of sonicated salmon sperm DNA per ml–0.1% SDS–20 mM sodium phosphate (pH 7) for 72 h. Filters were washed progressively to a stringency of 0.2× SSC at 65°C. Plaques giving a differential signal were rescreened with ^{32}P-labeled cDNA synthesized from poly(A)$^+$ RNA isolated from NGF-treated, EGF-treated, and untreated PC12 cells and with a battery of ^{32}P-labeled cDNA and oligonucleotide probes complementary to c-*fos*, c-*myc*, c-*fgr*, β-actin, ODC, NGF-IA, and NGF-IB mRNAs. Selected clones were further characterized by Northern (RNA) and Southern analysis.

Northern analysis. Total RNA was prepared from various tissues by using LiCl-guanidinium isothiocyanate (18) and from PC12 cells by detergent lysis and proteinase K treatment (35, 84). RNA was electrophoresed in agarose gels under denaturing conditions as described previously (20) and transferred to nitrocellulose. Probes were labeled by random priming (32), and hybridization and washing were carried out under standard conditions (87). Autoradiograms were optically scanned with a Bio-Rad 620 densitometer, and optical density was determined by using 1-D data analysis software.

RNase protection analysis. PC12 cells were grown on collagen-coated dishes as described previously (39). Untreated PC12 cells and those treated with βNGF (50 ng/ml; R. Stach, University of Michigan), EGF (10 ng/ml; Collaborative), bovine brain bFGF (10 to 100 ng/ml; Boehringer Mannheim), insulin (25 μg/ml; Sigma), 10^{-6} M retinoic acid (Sigma), and acidic fibroblast growth factor (aFGF; 150 ng/ml; Boehringer Mannheim) for the indicated durations were rinsed three times with ice-cold phosphate-buffered saline, and cytoplasmic RNA was isolated as previously described (84) except that the cells were lysed in 10 mM Tris (pH 7.4)–10 mM NaCl–3 mM $MgCl_2$–0.5% (vol/vol) Nonidet P-40 (35). Protection analysis was carried out as previously detailed (84), with the following modifications. Antisense ^{32}P-labeled VGF and cyclophilin RNA probes were hybridized with 5 to 10 μg of total PC12 cell RNA in 80% formamide–40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES)–400 mM NaCl–1 mM EDTA for 18 h at 45°C. Samples were treated for 30 min at 37°C with RNase A (40 μg/ml) and RNase T_1 (2 μg/ml) in 10 mM Tris (pH 7.5)–5 mM EDTA–300 mM NaCl, and the protected RNA fragments were resolved on nondenaturing 5% polyacrylamide gels. After autoradiographic exposure, the bands were excised and quantified by scintillation counting. A standard curve, constructed by hybridizing ^{32}P-labeled antisense VGF RNA with known amounts of sense-strand RNA, was used to convert counts per minute of protected VGF fragment into picograms of VGF mRNA per 10 μg of total RNA.

Southern blot analysis. Genomic DNA was isolated from rat liver and from phage (87). Restriction digests of ~7 μg of genomic DNA and ~5 μg of clone 9-1 DNA were electrophoresed on 0.8% agarose gels and transferred to nitrocellulose (87). The rat liver genomic blot was probed with the entire NGF33.1 cDNA clone, while the blot of the restricted phage 9-1 DNA was sequentially probed with the ~0.6-kb *Eco*RI fragment at the 5′ end of the NGF33.1 cDNA clone and then with the ~2.1-kb *Eco*RI fragment which contains the majority of the coding sequence and includes the 3′ untranslated region of the NGF33.1 cDNA clone. Blots were progressively washed to a stringency of 0.1× SSC–0.1% SDS at 55°C.

Isolation of a VGF genomic clone. Ten genome equivalents (4×10^6 plaques) of an amplified Bonner rat genomic library (partial *Eco*RI digest in Charon 4A) were plated by using the host *E. coli* strain CSH18, and duplicate nitrocellulose filters were screened with the NGF33.1 cDNA clone that was labeled with ^{32}P by random priming (32). Hybridization was carried out in 5× SSC–1× Denhardt's solution–0.1% SDS–100 μg of sonicated salmon sperm DNA per ml–50% formamide for 24 h at 42°C. Filters were progressively washed to a stringency of 0.1× SSC–0.1% SDS at 55°C. Phage were plaque purified by two additional rounds of plating at limited dilution and rescreening.

Sequence analysis of the VGF genomic clone. Fragments of the phage 9-1 insert were subcloned into phage M13mp18 and M13mp19. M13 constructs were transformed into *E. coli* JM109, and single-stranded phage DNA was isolated. Overlapping deletions were created by the method of Dale and Arrow (26). Single-stranded phage DNA was sequenced by the dideoxynucleotide chain termination method (88), using Sequenase and the M13 −40 primer, according to the instructions of the manufacturer (U.S. Biochemicals). In ad-

dition, several primers complementary to the VGF sequence, synthesized on an Applied Biosystems automated DNA synthesizer, were used. A number of regions of the VGF gene were sequenced both by the methods described above and by using an Applied Biosystems model 373A automated sequencer. Nucleotide sequences were analyzed and compared with those in the EMBO, NIH, and PIR data bases, using Intelligenetics IG Suite software.

Primer extension analysis. The transcription start site of the VGF gene was determined by extension of an antisense primer (5′-CCGTTCCGTGGCTGGAGTATGAAAG-3′) complementary to the 5′ end of the NGF33.1 cDNA clone. The primer was labeled with ^{32}P by using polynucleotide kinase and was annealed to poly(A)$^+$ RNA extracted from PC12 cells that had been treated with βNGF (50 ng/ml) for either 3 or 6 h and to yeast tRNA and yeast total RNA. The cDNA was extended using by avian reverse transcriptase, and the products were analyzed on a 6% polyacrylamide-urea gel essentially as described previously (17). Coelectrophoresis of a sequence ladder, obtained by using the same primer and, as a template, single-stranded DNA from an M13 VGF genomic subclone, was used to precisely determine the length of the extended cDNA.

Nucleotide sequence accession numbers. The sequences of the VGF genomic and NGF33.1 cDNA clones have been submitted to GenBank and assigned accession numbers M60522 and M60525, respectively.

RESULTS

A cDNA library of 2×10^6 unamplified recombinants was constructed in lambda gt10 by using mRNA isolated from PC12 cells that had been treated with NGF and CHX to superinduce rapidly regulated mRNAs, as previously described (24, 69). A total of 10^5 clones were screened with subtractively hybridized ^{32}P-labeled cDNA synthesized by using NGF-CHX-treated poly(A)$^+$ RNA as described in Materials and Methods, and 78 independent rapidly regulated clones were identified. These clones were rescreened with the following cDNA and oligonucleotide probes complementary to previously identified rapidly regulated mRNAs: v-*myc*, v-*fos*, c-*fos*, v-*fgr*, NGF-IA/*egr-1*, NGF-IB, β-actin, and ODC (33, 35, 69, 70). Of the initial 78 clones selected, 61 cross-hybridized with NGF-IA. ^{32}P-labeled cDNA, synthesized from poly(A)$^+$ RNA that had been isolated from untreated, NGF-treated, and EGF-treated PC12 cells, was used to rescreen the clones. Two clones, one of which is described in this report, were found on Northern analysis to correspond to mRNAs that are induced to a greater extent by NGF than by EGF. The nucleic acid sequence of the NGF33.1 cDNA clone was determined by single-strand dideoxy sequence analysis (88). The deduced amino acid sequence of NGF33.1 contained regions of identity with that predicted for the NGF-inducible cDNA clone VGF (80). Since the nucleic acid sequence of VGF has not been published, direct comparison with NGF33.1 was not possible, but the predicted amino acid sequence of VGF could be generated almost in its entirety by introducing multiple shifts in the reading frame of the NGF33.1 nucleic acid sequence. On this basis, we concluded that the VGF and NGF33.1 cDNA clones corresponded to the same mRNA, and we have subsequently used the NGF33.1 cDNA clone to further characterize VGF mRNA regulation and to clone the rat VGF gene.

Regulation of VGF mRNA levels in PC12 cells. To ensure that both EGF and NGF treatment of PC12 cells resulted in the rapid stimulation of gene expression, induction of NGF-IA mRNA (69) (also called *egr-1* [95], *zif/268* [23], and *krox 24* [54]), the product of an immediate-early gene that encodes a putative transcriptional factor, was examined. Northern analysis (Fig. 1A and B) showed a rapid, transient induction of NGF-IA mRNA in both NGF-treated PC12 cells as previously described (69, 95) and in EGF-treated PC12 cells. In contrast, the level of expression of VGF, a 2.7-kb mRNA that hybridized to ^{32}P-labeled NGF33.1 cDNA, was found to be much greater in NGF-treated PC12 cells (Fig. 1C) than in those treated with EGF (Fig. 1D). To normalize transfer and hybridization efficiencies, Northern blots were cohybridized with ^{32}P-labeled cyclophilin cDNA (27), which hybridizes to a 1-kb mRNA that is not regulated by NGF treatment (63). On the basis of densitometric scanning of the autoradiograms, after normalization of cyclophilin levels, VGF mRNA levels were maximally induced by approximately 15-fold after 3 to 6 h of NGF treatment, and the mRNA level was found to return to control levels after 48 h of NGF treatment. EGF treatment stimulated VGF mRNA levels by twofold in comparison with untreated cells, with similar kinetics of induction.

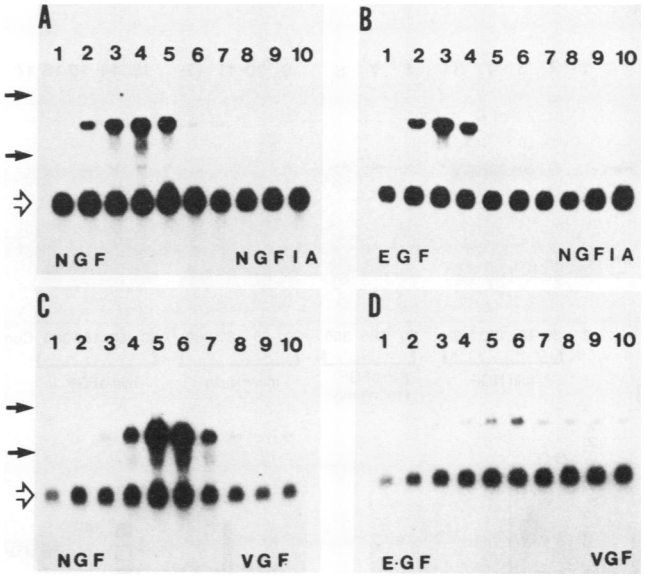


FIG. 1. Northern analysis of total cytoplasmic RNAs isolated from NGF- and EGF-treated PC12 cells. PC12 cells were grown on collagen-coated dishes, and cytoplasmic RNA was isolated as described in Materials and Methods from untreated PC12 cells (lane 1) and those treated with either 50 ng of βNGF per ml (A and C) or 10 ng of EGF per ml (B and D) for 15 min, 30 min, 1 h, 3 h, 6 h, 24 h, 48 h, 4 days, and 7 days (lanes 2 to 10). A 5-μg portion of each sample was electrophoresed on 0.8% agarose gels under denaturing conditions (20), transferred to nitrocellulose, and probed with 10^7 cpm of either ^{32}P-labeled NGF-IA cDNA insert (A and B) or NGF33.1 cDNA insert (C and D) per ml. To control for variation in loading, transfer, and hybridization, blots were cohybridized with ^{32}P-labeled cyclophilin cDNA insert (27) (open arrows). Positions of the 28S and 18S rRNAs are indicated by solid arrows.

Since treatment of PC12 cells with NGF led to a much greater induction of VGF mRNA levels than did EGF treatment, it was of interest to determine whether VGF mRNA levels were regulated by additional mitogenic or neurotrophic growth factors. Treatment of PC12 cells with both bFGF and aFGF has been shown to result in neural differentiation (81), although the development of a

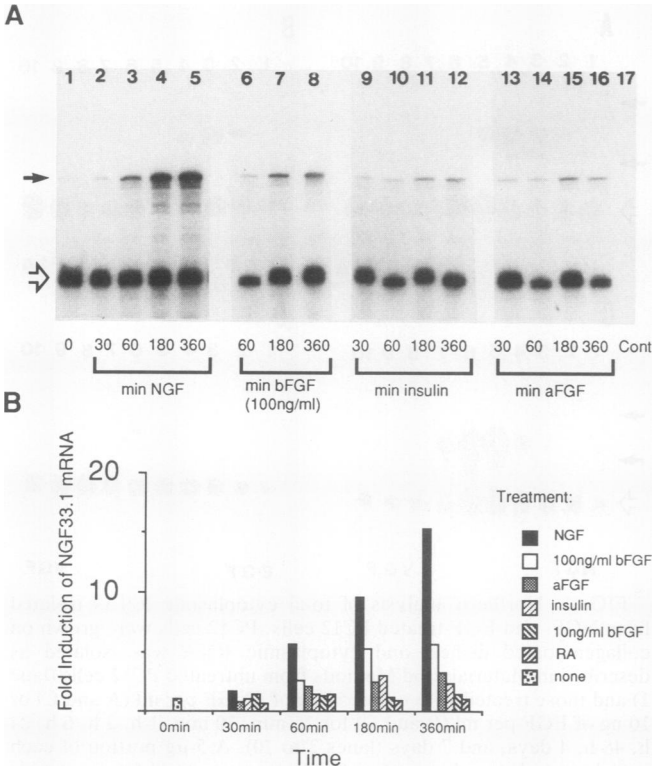


FIG. 2. Effects of treatment with NGF, insulin, aFGF, bFGF, and retinoic acid (RA) on VGF mRNA levels in PC12 cells. PC12 cells were grown on collagen-coated dishes and treated with (per milliliter) 50 ng of βNGF (lanes 2 to 5), 100 ng of bovine brain bFGF (lanes 6 to 8), 25 μg of insulin (lanes 9 to 12), and 150 ng of aFGF (lanes 13 to 17) for the indicated durations. RNase protection analysis was carried out as described in Materials and Methods, using antisense ^{32}P-labeled VGF (NGF33.1) and cyclophilin RNA probes. Protected RNA fragments were resolved on nondenaturing 5% polyacrylamide gels. The solid and open arrows indicate fragments protected by the VGF and cyclophilin antisense RNAs, respectively. After autoradiographic exposure, the bands were excised and quantified by scintillation counting. Assays were run in duplicate, and the results were averaged (duplicates differed by no more than 20% from the average); the results are presented in graphic form in panel B. Fold induction of VGF (NGF33.1) mRNA was calculated as percent control VGF mRNA divided by percent control cyclophilin mRNA.

neuronal phenotype is delayed in comparison with NGF. Furthermore, it has been suggested that bFGF may play a role early in the development of the sympathetic nervous system, prior to the expression of NGF receptors by sympathoadrenal progenitor cells (11). In addition, retinoic acid has been shown to act on the developing CNS in *Xenopus laevis* (29). Therefore, the effects of treatment with these and other growth factors on VGF mRNA levels in PC12 cells were examined by RNase protection analysis. VGF and cyclophilin antisense RNAs were synthesized and hybridized to samples of PC12 cell total RNA, and VGF mRNA levels were quantified as previously described (84). After normalization to cyclophilin RNA levels, VGF mRNA levels were found to increase 13- to 17-fold after 3 to 6 h of NGF treatment and 7-fold after 6 h of bFGF treatment (100 ng/ml) (Fig. 2). Insulin and aFGF were found to increase VGF mRNA by two- to three-fold after 3 to 6 h of treatment, similar in magnitude and kinetics to the effects observed following EGF treatment (Fig. 1D). bFGF at a dose of 10 ng/ml and 10^{-6} M retinoic acid had no effect on VGF mRNA levels (Fig. 2B). Treatment of PC12 cells with either 100 ng of bFGF or 150 ng of aFGF per ml for 2 days resulted in cell flattening and the outgrowth of relatively short neurites (length of 1 to 2 cell diameters) in ~10% of cells and the extension of longer neurites (>2 cell diameters) in 3 to 5% of cells, while PC12 cells treated with 10 ng of bFGF were indistinguishable from untreated cells (86a). In comparison with the preparations of aFGF and bFGF used, treatment of PC12 cells for 2 days with NGF resulted in more extensive cell flattening and process outgrowth (20 to 30% of the cells possessed neurites >2 cell diameters in length), with the time course of process outgrowth and the percentage of neurite-bearing cells similar to results of previous studies (81). In summary, VGF mRNA levels were rapidly induced in PC12 cells by treatment with at least two neurotrophic growth factors, while treatment with several additional growth factors led to markedly less induction.

The mechanism(s) by which VGF mRNA levels are regulated in PC12 cells was investigated by examining the effects of several direct activators of second-messenger pathways, of depolarization, and of washing out and then adding back NGF to long-term NGF-treated PC12 cells. Depolarization stimulates vesicular secretion and increases the expression of select genes, some of which encode secreted proteins (e.g., the pituitary hormones growth hormone [7, 8], prolactin [78], and pro-opiomelanocortin [30, 34]) and some of which are immediate-early genes that encode transcriptional regulatory proteins (9, 37, 76, 82). PC12 cells were treated with several second-messenger activators, including phorbol-12-myristate-13-acetate (PMA) and 8-bromo-cyclic AMP (8-bromo-cAMP), and were depolarized in medium containing 40 mM KCl. Total RNAs were isolated, and Northern analysis carried out as described in Materials and Methods. Blots were cohybridized with ^{32}P-labeled NGF33.1 cDNA and with ^{32}P-labeled cyclophilin cDNA to normalize hybridization and transfer efficiencies. After normalization to cyclophilin mRNA levels, VGF mRNA levels were found to be maximally induced 6.8-fold by PMA after 6 h of treatment, 4.2-fold by 40 mM KCl after 2 to 6 h of treatment, and 1.5-fold by 8-bromo-cAMP after 6 h of treatment (Fig. 3). Overall, treatment with any of these agents alone did not lead to the levels of induction of VGF mRNA that were observed with NGF treatment.

Finally, the effect of washing out and then adding back NGF to long-term NGF-treated PC12 cells was studied by RNase protection analysis. In long-term NGF-treated PC12 cells that had undergone extensive neuronal differentiation, the amount of VGF mRNA was found to have returned to control levels (Fig. 1). Control PC12 cells were treated with NGF for 2 weeks and then either washed and immediately resupplemented with NGF for 4 h (Fig. 4, lane 1) or washed and then deprived of NGF for 4 or 24 h (lanes 2 and 6, respectively), after which the cells were harvested. Comparable VGF mRNA levels were found in all of these controls. Readdition of NGF to deprived cultures was found to result in a five- to eight-fold increase in VGF mRNA levels after 3 to 5 h of NGF treatment (lanes 4, 5, 8, and 9) in comparison with control cultures. The kinetics of induction were similar to those observed after treatment of naive PC12 cells with NGF, but the peak level of VGF mRNA induction observed, both absolute (picograms per 10 μg) and that measured relative to washed, nondeprived controls, was lower than was observed after treatment of naive PC12 cells.

Tissue distribution and developmental expression of VGF

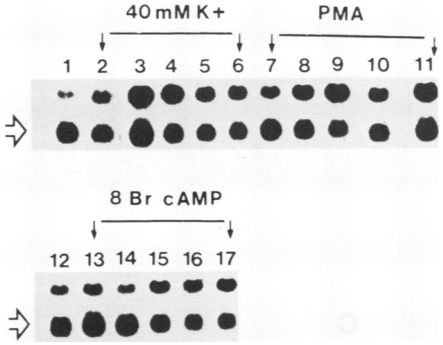


FIG. 3. Effects of depolarization, PMA, and 8-bromo-cAMP on VGF mRNA levels. Samples containing 10 μg of total RNA from untreated PC12 cells (lanes 1 and 12) and those treated for 30 min, 2 h, 6 h, 12 h, and 24 h with 40 mM KCl-supplemented medium (lanes 2 to 6), 10^{-6} M PMA (lanes 7 to 11), and 1 mM 8-bromo-cAMP (lanes 13 to 17) were subjected to Northern analysis as described in Materials and Methods. Nitrocellulose blots were hybridized with 10^7 cpm of ^{32}P-labeled NGF33.1 cDNA insert per ml and with ^{32}P-labeled cyclophilin cDNA to control for variability in loading and transfer (open arrow). Autoradiograms were scanned by optical densitometry, and after normalization to cyclophilin levels, the following relative amounts of VGF mRNA were determined for lanes 1 to 17, respectively, with the level obtained by using untreated PC12 cell total RNA defined as 1: 1, 1, 4.7, 4.2, 3.3, 2.8, 1.7, 3.3, 6.8, 3.2, 1.8, 1, 1, 0.8, 1.5, 1.7, and 1.9.

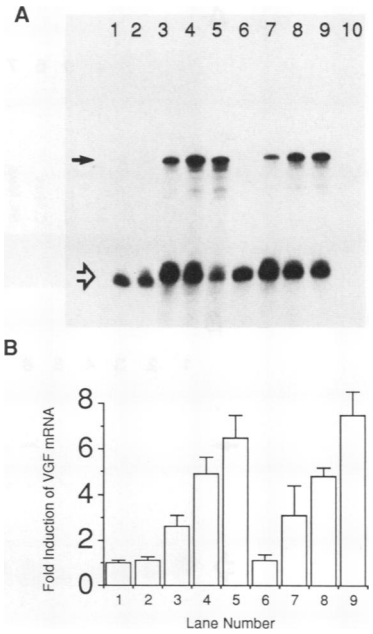


FIG. 4. Effects of deprivation and readdition of NGF to primed PC12 cells. PC12 cells were allowed to differentiate on collagen-coated dishes in the presence of 50 ng of βNGF per ml for 2 weeks. All cultures were rinsed six times with RPMI 1640, supplemented with 10% horse serum and 5% fetal calf serum, to remove NGF (35). NGF was readded in lane 1, and the cells were harvested after 4 h (defined as control for the quantitation in panel B); PC12 cells were cultured in complete medium without NGF for 4 h (lanes 2 to 5) or 24 h (lanes 6 to 9). PC12 cells deprived of NGF for 4 h (lane 2) or 24 h (lane 6) were restimulated with 50 ng of βNGF per ml for 1 h (lanes 3 and 7), 3 h (lanes 4 and 8), and 5 h (lanes 5 and 9). Cytoplasmic RNA was isolated, and RNase protection analysis performed was as described in Materials and Methods, using VGF (solid arrow) and cyclophilin (open arrow) antisense RNAs. Protection analysis was carried out in lane 10 without the addition of cytoplasmic RNA. The assay was performed in triplicate, and the fold induction of VGF, calculated as percent control VGF mRNA divided by percent control cyclophilin mRNA ± SEM, is shown graphically in panel B.

mRNA in the rat. To further understand the role that the VGF gene might play in neuronal differentiation, the distribution of VGF mRNA in the nervous system during development and in a variety of nonneural tissues was examined. RNA was prepared from embryonic and postnatal rat tissues as described in Materials and Methods. Northern analysis was used to detect VGF mRNA in the CNS in adult brain (Fig. 5A, lanes 1 and 14) and embryonic day 18 (e18) brain (lane 10) and in the PNS in the adult superior cervical ganglion (SCG) (lane 11). By Northern analysis (Fig. 5A) and by RNase protection assay (86a), VGF mRNA was not detected in adult liver, spleen, kidney, adrenal gland, lung, cardiac and skeletal muscle, and placenta. The developmental expression of VGF mRNA in the nervous system was then examined in greater detail in the RNase protection assay (Fig. 5B and C). Total RNA was prepared by using guanidinium thiocyanate (18), and VGF mRNA was quantified in samples of brain (e12 to adult), spinal cord (e14, e16, and postnatal day 2 [p2]), SCG (p10), and adrenal gland (p10 and adult). Samples were cohybridized with a cyclophilin antisense RNA to allow comparison of cyclophilin and VGF levels in particular tissues at different developmental ages. Cyclophilin RNA was easily detected in rat brain as early as e12, while VGF mRNA was not detectable in total brain RNA until e18 (12 ± 2 pg standard error of the mean [SEM] VGF mRNA per 10 μg of total RNA) (Fig. 5B). VGF mRNA levels were highest in p10 brain (64 ± 4 pg/10 μg), p10 SCG (~65 pg/10 μg), and p10 cerebellum (55 ± 3 pg/10 μg), with amounts found in the p6 cerebellum, p6 and p10 cerebrum, p10 SCG, p2 spinal cord, and p4 and adult brain that ranged from 34 to 44 pg/10 μg. VGF mRNA was not detectable in adult adrenal gland, with levels in p10 adrenal gland of ~0.3 pg/10 μg of total RNA. These data demonstrate that VGF mRNA is localized in central and peripheral nervous tissues, with peak VGF mRNA levels detected during the first 2 weeks of postnatal development.

Determination of the copy number of the VGF gene and isolation of the VGF genomic clone. The rat NGF33.1 cDNA insert was labeled with ^{32}P and used to probe a Southern blot of rat genomic DNA cut with each of a number of restriction enzymes (Fig. 6A). Detection of a limited number of restriction fragments suggested that the VGF gene was present in a single copy. Two bands were obtained after restriction of genomic DNA with *Eco*RI (arrowheads in Fig. 6A), and three bands were seen after digestion with *Bam*HI, in agreement with the sequence of the NGF33.1 cDNA clone, which contains a single internal *Eco*RI site and two *Bam*HI sites (Fig. 7A). Given that the VGF and NGF33.1 cDNA clones predict amino acid sequences which contain several extended regions of identity (see Fig. 9), the Southern analysis is further evidence that the two cDNAs correspond to the same mRNA transcribed from a single gene.

A rat Bonner genomic library was screened with the NGF33.1 cDNA clone, and a single positive clone, 9-1, was obtained on rescreening. Restriction analysis of this clone revealed an insert of ~12.5 kb in length containing two internal *Eco*RI sites. A Southern blot of phage 9-1 DNA was hybridized with the two *Eco*RI restriction fragments of the NGF33.1 cDNA clone (Fig. 7A), labeled with ^{32}P by using random primers (the 5′ fragment, bases 1 to 434, corre-

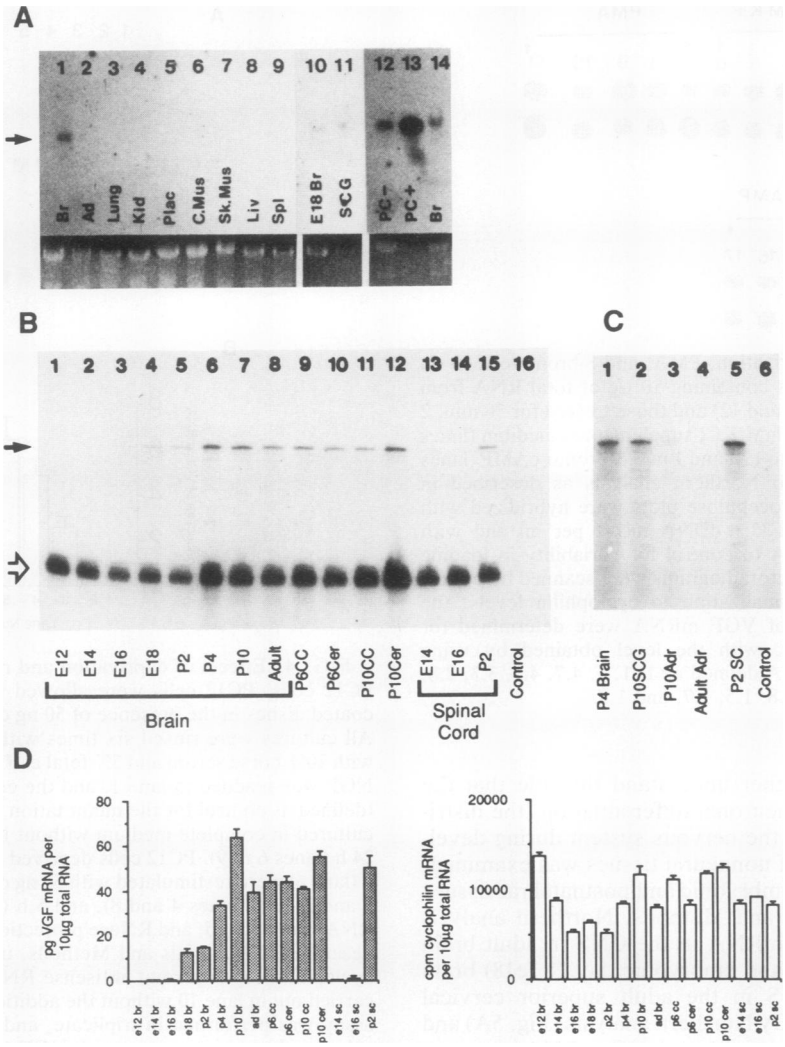


FIG. 5. Tissue distribution of VGF mRNA in the developing and adult rat. (A) Northern analysis carried out by using 10 μg of total RNA (exceptions noted below) isolated as described in Materials and Methods. The following samples were electrophoresed, transferred to nitrocellulose, and probed with ^{32}P-labeled NGF33.1 cDNA in lanes 1 to 14, respectively: adult brain, adrenal gland, lung, kidney, placenta, cardiac muscle, skeletal muscle, liver, spleen, e18 brain, 5 μg of adult SCG, 3-h CHX-treated PC12 cell RNA, 2 μg of 3 h NGF-CHX-treated PC12 cell RNA, and adult brain. To control for variation in loading, the corresponding ethidium bromide-stained 18S and 28S ribosomal subunits in lanes 1 to 14 are shown below the autoradiogram. The distribution of VGF mRNA in the nervous system during development was studied by using RNase protection analysis. Ten micrograms of total RNA from brain, cerebral cortex (CC), cerebellum (Cer), spinal cord (SC), and adrenal gland (Adr), 5 μg of total RNA from SCG (C) and 0 μg for the controls (B, lane 16; C, lane 6) were hybridized with both VGF and cyclophilin antisense RNA probes (B) or with VGF RNA probe alone (C). Assays were performed in triplicate, and mean VGF mRNA levels were quantified as picograms of VGF mRNA per 10 μg of total RNA ± SEM. The values obtained, based on analysis of the samples in panel B, are shown graphically in panel D for lanes 1 to 15, respectively: 0, 0.3 ± 0.3, 0.7 ± 0.3, 12 ± 2, 15 ± 0.3, 34 ± 2, 64 ± 4, 39 ± 5, 44 ± 4, 44 ± 3, 41 ± 1, 55 ± 3.0, 0.3 ± 0.3, 1 ± 1, and 50 ± 5.

sponds to the 5′ untranslated sequence and the portion of the mRNA that encodes the amino terminus of the VGF polypeptide, while the 3′ fragment, bases 429 to 2595, corresponds to the remainder of the coding sequence and the 3′ untranslated sequence). Clone 9-1 was restricted with *Eco*RI, and three fragments derived from the inserted genomic DNA, with molecular sizes of approximately 9, 2.7, and 0.8 kb, were obtained (arrowheads in Fig. 6B and C). Southern blot analysis revealed that the 3′ NGF33.1 cDNA probe hybridized predominantly to the 2.7-kb *Eco*RI fragment and less strongly to the ~9-kb *Eco*RI fragment (Fig. 6B). The blot was stripped and reprobed, and the 5′ NGF33.1 cDNA probe was found to hybridize primarily to the larger ~9-kb fragment (Fig. 6C). Inability to completely strip the previously bound 3′ probe and the lower specific activity of the 5′ probe than of the 3′ probe are most likely responsible for the visualization of the 2.7-kb *Eco*RI fragment seen in Fig. 6C. Southern blots (Fig. 6B and C) demonstrate that the phage 9-1 insert contains the two *Eco*RI fragments of the VGF gene that were detected by genomic Southern analysis and that the ~9-kb fragment contains the 5′-flanking sequence while the 2.7-kb fragment contains the majority of the coding sequence (Fig. 7B).

Sequence analysis of the gene encoding VGF. Restriction fragments of the VGF genomic clone were subcloned into M13mp18 and M13mp19 vectors, and nested deletions were

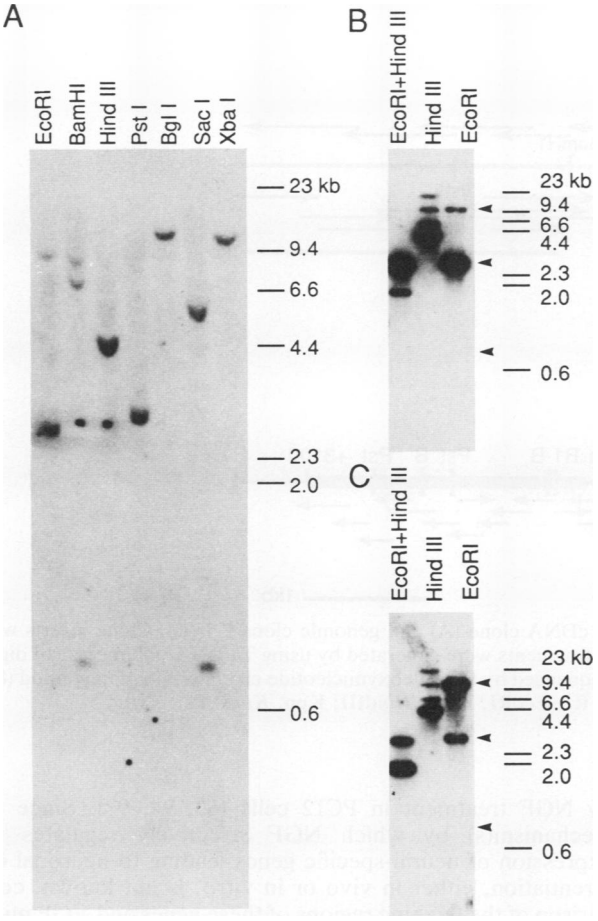


FIG. 6. Southern analysis of genomic and phage 9-1 DNA. (A) Rat liver DNA (7 μg), cut with seven different restriction endonucleases, was electrophoresed on a 0.8% agarose gel, transferred to nitrocellulose, hybridized with ^{32}P-labeled NGF33.1 cDNA, and autoradiographed. The arrowheads indicate the ~9- and ~2.7-kb *Eco*RI fragments that hybridized. Positions of the molecular size markers are shown to the right. (B and C). Phage 9-1 DNA (5 μg) was restricted as shown, and the blot was hybridized with the ^{32}P-labeled 3′ *Eco*RI fragment of the NGF33.1 cDNA clone which contains the majority of the VGF coding sequence and includes the 3′ untranslated region (B). After exposure to film, the blot was stripped and rehybridized with the ^{32}P-labeled 5′ *Eco*RI fragment of the NGF33.1 cDNA clone (C). Arrowheads indicate locations of the ~9-, ~2.7-, and ~0.8-kb *Eco*RI fragments of the phage 9-1 insert; positions of molecular size markers are shown on the right.

constructed by using T4 DNA polymerase as described by Dale and Arrow (26). Single-stranded M13 DNA, isolated after transformation of the host *E. coli* JM109, was used as a template for sequencing by the dideoxynucleotide chain termination method (88). Partial restriction maps of the NGF33.1 cDNA and VGF genomic clones and the sequencing strategies are shown in Fig. 7. The sequence of the VGF genomic clone contains 853 bases of the 5′ flank, the entire coding sequence, and 55 bases of the 3′ flank.

Determination of the mRNA cap site. The precise position of the transcription start site, 78 bases upstream to the 5′ end of the NGF33.1 cDNA clone, was determined by primer extension and coelectrophoresis of the reverse transcript with a sequencing ladder obtained by using the identical primer and a single-stranded M13 VGF genomic subclone as the template (Fig. 8). Although RNA polymerase II prefers to initiate at purines (5), initiation at a pyrimidine was suggested on the basis of primer extension studies. However, purine residues do flank the assigned start site at +2, +3, and −3 (Fig. 8). A TATAAA box is positioned at −24, 20 to 30 bases upstream of the putative mRNA cap site, indicating that there are no intron sequences 5′ to the NGF33.1 cDNA sequence and supporting the contention that this is the true start site of transcription.

Structure of the VGF gene. The nucleotide sequence of the VGF gene and the amino acid sequence deduced from NGF33.1 cDNA and VGF genomic clones are presented in Fig. 9. The dark bars underline those portions of the amino acid sequence predicted from the NGF33.1 cDNA and VGF genomic clones which are shared with the amino acid sequence predicted by the VGF cDNA clone (80, 101). The VGF genomic sequence and the NGF33.1 cDNA sequence are predicted to encode a 617-amino-acid polypeptide with a molecular mass of 68,000 Da; the most common consensus ribosome binding site was not found, but the A at position +4 in relation to the ATG start codon (nucleotide 241) and the G at position −3 (nucleotide 235) are not unfavorable to translation (51). A VGF polypeptide of 92,000 Da was previously identified by immunoprecipitation and in vitro translation of hybrid-selected RNA (57, 80); discrepancy of the size predicted by the VGF cDNA clone (76,175 Da) with the observed migration of VGF polypeptide on SDS-polyacrylamide gels was thought to be due to a high relative number of proline residues (57), a characteristic shared by the deduced amino acid sequences of the NGF33.1 cDNA and VGF genomic clones. A region of weak amino acid homology between amino acids 360 and 450 of the predicted VGF (NGF33.1) sequence and a number of cytoskeletal proteins, including the neurofilament L and M proteins (NF-L and NF-M), troponin T, tropomyosin, and microtubule-associated protein 2, was noted (Fig. 10) (IFIND algorithm [Intelligenetics]; score range = 10 to 13, standard deviation = 5.94 to 9.02). Hydrophobicity analysis using the Kyte-Doolittle algorithm (53) demonstrates a hydrophobic N-terminal domain, a possible signal peptide that might direct internalization of the polypeptide into the lumen of the endoplasmic reticulum (Fig. 10). Sequence analysis of the VGF genomic clone and comparison with the NGF33.1 cDNA clone demonstrated the presence of two relatively short introns, 261 bp (intron A) and 124 bp (intron B) in length. The two introns interrupt the VGF gene in a region that encodes the 5′ untranslated sequence of the mRNA. This pattern of introns and exons is distinct from that found for the neurofilament genes, NF-L and NF-M, the encoded proteins of which share weak homology with the amino acid sequence predicted by the NGF33.1 cDNA and VGF genomic clones (Fig. 10). A consensus AATAAA polyadenylation signal (nucleotide 3005), 12 nucleotides 5′ to the start of the poly(A) tail found in the corresponding NGF33.1 cDNA clone, and the putative destabilization sequence ATTTA (nucleotide 2871) were found. The AUUUA sequence is found near the 3′ end of several rapidly turned over mRNAs, including those encoded by the immediate-early genes c-*fos*, c-*jun*, and NGF-IA, and has been shown to increase the rate of mRNA degradation (13, 90).

Structure of the VGF promoter. VGF mRNA levels are rapidly regulated by NGF and PMA treatment and by depolarization (Fig. 1 to 3). Since the observed induction of VGF mRNA may be at least in part due to an effect on gene transcription, and the mRNA is expressed exclusively in neural tissues, it was of interest to sequence the 5′ flank and

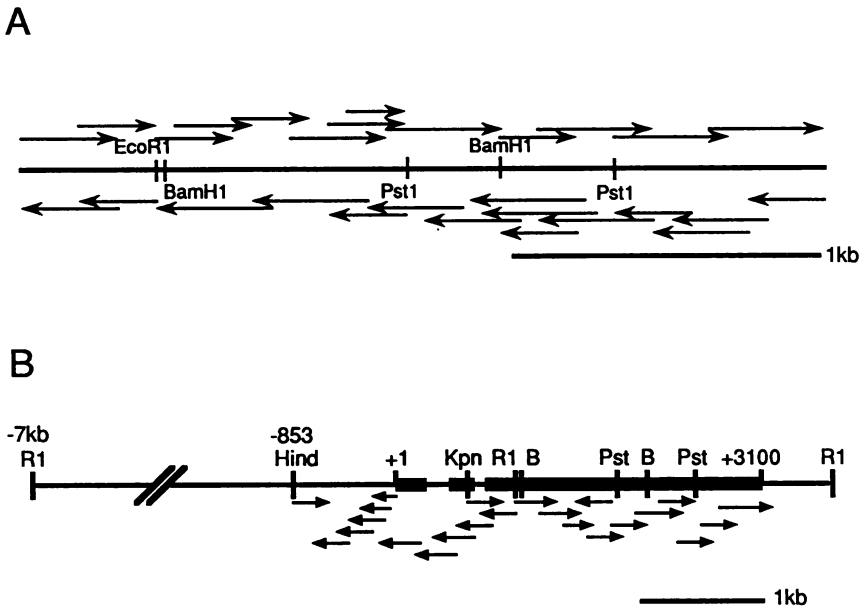


FIG. 7. Partial restriction maps and sequencing strategies of NGF33.1 cDNA clone (A) and genomic clone 9-1 (B). Clone inserts were restricted and subcloned into M13mp18 and M13mp19. Sequential deletion fragments were generated by using T4 DNA polymerase to digest linearized single-stranded M13 subclones (26). Single-stranded DNA was sequenced by the dideoxynucleotide chain termination method (88). In panel B, exons are shown as dark boxes. Restriction sites: B, *Bam*HI; R1, *Eco*RI; Hind, *Hin*dIII; Kpn, *Kpn*I; Pst, *Pst*I.

promoter region of the VGF gene as the initial step in understanding its regulation. The sequence of the 5′ flank was examined both for the presence of consensus binding elements of a number of known eukaryotic transcription factors (48, 106) and for homologies to the promoter regions of other neuronal, NGF-inducible genes. Consensus TATAAA and CCAAT elements were identified at −24 and −133, respectively. It is also of interest that in the VGF promoter, the CCAAT motif is found in the context of an element with dyad symmetry (−138 TCCAATCATTGGA −126). In addition, a consensus binding site for the cAMP response element-binding protein (CREB) was found at position −71 (73, 74). Further analysis of the VGF promoter revealed four GC box motifs (−62, −99, −253, and −605) and two elements (−252 and −39) which closely resemble the AP-2 binding site consensus sequences (47, 72). A consensus serum response element (SRE), a motif with dyad symmetry that is required for the induction of c-*fos* transcription by serum and growth factor treatment (36, 99, 100), was not found in the VGF 5′ flank, but a sequence with substantial dyad symmetry that resembles an SRE was identified at −511.

Although the precise cellular distribution of VGF mRNA within the nervous system has not yet been determined, our studies have shown that VGF mRNA is localized within the brain, spinal cord, and SCG, and previous studies have demonstrated VGF mRNA in brain and VGF polypeptide in hypothalamic neurons (101). As an initial step in examining whether nucleotide sequences within the 5′ VGF flank might either specify the cellular distribution of the VGF mRNA or control the induction of VGF mRNA by NGF, the sequence of the VGF promoter was compared with the flanking sequences of a number of neural genes, including those encoding NF-L and -M (60, 61), neuron-specific enolase (83), peripherin (97), and SCG10 (77). The transcription of a subset of these genes has further been shown to be regulated by NGF treatment in PC12 cells (62, 94, 97). Since the mechanism(s) by which NGF selectively regulates the expression of neural-specific genes leading to neuronal differentiation, either in vivo or in vitro, is not known, comparison of the flanking regions of these genes and VGF might reveal common, functionally important sequences. These shared sequence elements could hypothetically be recognized by an NGF-dependent transcription factor(s) leading to the selective activation of genes that are involved in neuronal differentiation or are required to maintain neuronal function. Regions of sequence homology were detected between VGF and NF-M, in a region which is in itself homologous to NF-L (60), and between VGF and peripherin (Fig. 11). Interestingly, the two regions of homology between the VGF and peripherin promoters are located comparable distances from their respective transcription start sites. It is not known at present whether these shared sequences are functionally involved in the regulation of VGF mRNA levels by NGF or in determining the neural expression of VGF mRNA.

DISCUSSION

This report establishes that the VGF gene can be distinguished from the class of rapidly inducible immediate-early genes both on the basis of its rapid but selective regulation by the neurotrophic growth factors NGF and bFGF and by its expression exclusively in tissues of the CNS and PNS. The kinetics of induction of VGF mRNA, with peak levels in PC12 cells observed after 3 to 6 h of NGF treatment, are similar to those reported for ODC mRNA (33, 65). However, EGF treatment of PC12 cells has been shown to increase ODC gene transcription (35) and enzyme activity (41), although ODC mRNA levels were found to be induced quantitatively to a greater extent by NGF treatment than by EGF treatment (65). In any case, the induction of ODC by NGF

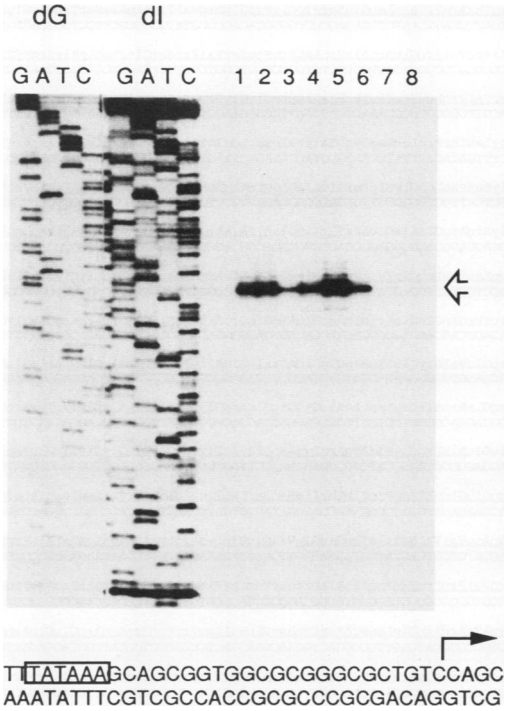


FIG. 8. Primer extension mapping of the 5′ end of VGF mRNA. A ^{32}P-labeled oligonucleotide, complementary to the 25 nucleotides at the 5′ end of the NGF33.1 cDNA clone, was annealed to poly(A)$^+$ RNA isolated from PC12 cells treated with NGF for 3 h (lanes 4 to 6) or 6 h (lanes 1 to 3) and with yeast tRNA (lane 7) and yeast total RNA (lane 8). The amounts of oligonucleotide and RNA, respectively, in lanes 1 to 3 and 4 to 6 were varied as follows: lanes 1 and 4, 1 ng and 2 μg; lanes 2 and 5, 3 ng and 2 μg; lanes 3 and 6, 5 ng and 1 μg; lanes 7 and 8, 3 ng and 50 μg. Sequence ladders, generated by using the same oligonucleotide primer, single-stranded DNA from a genomic VGF M13 subclone, and labeling mixes that contained either dGTP or dITP, were coelectrophoresed with the primer extension products (indicated by the open arrow) to precisely determine the site of transcription initiation. The position of the TATAAA box in relation to the transcription start site is shown.

treatment has been disassociated from the actions of NGF that result in the neuronal differentiation of PC12 cells (38), and the distribution of ODC mRNA is not limited to nervous tissues.

There is substantial evidence that growth factor treatment causes the rapid induction of several immediate-early genes which encode proteins that either regulate transcription, such as c-*fos* (25, 35, 52) and c-*jun* (108), or are homologous to the Zn finger-containing transcription factors (23, 54, 70, 95) and steroid hormone receptors (71), all of which bind to DNA. Since induction of the immediate-early genes is not specific to neurotrophic growth factors, their precise function in the cascade of events leading to neuronal differentiation remains unclear. The mechanism by which VGF gene expression is regulated selectively by NGF is likely to be distinct from that of genes that are regulated by both EGF and NGF, such as the immediate-early genes. It is possible, however, that these recognized and putative transcription factors are involved in the induction of VGF, much as c-*fos* and c-*jun* appear to participate in the regulation of proenkephalin gene expression (93).

Immunohistochemical analysis using antibodies generated to a VGF–β-galactosidase fusion protein has localized VGF polypeptide in a subpopulation of neurons in the adult hypothalamus (101). In addition to establishing that VGF mRNA is detectable in nervous tissues but not in a variety of nonneural tissues, the data presented here demonstrate that VGF mRNA is expressed widely, throughout the developing and adult CNS and PNS. Further experimentation is needed to investigate whether VGF polypeptide levels parallel VGF mRNA levels in neurons of the developing and adult cerebellum, cerebrum, spinal cord, and PNS. It is of interest that VGF mRNA levels were highest in p10 total brain and cerebellum, with slightly lower levels found in p6 cerebellum, p6 and p10 cerebrum, p10 SCG, p2 spinal cord, and p4 and adult brain. During the first 3 weeks of postnatal cerebellar development in the rodent, Purkinje cell differentiation and dendritic outgrowth, extensive granule cell migration, and synaptogenesis occur (2, 42, 43, 68). In the cerebellum in particular, as well as in the nervous system in general, VGF mRNA levels peak during a critical period of morphological and functional development.

Only a fraction of the hypothalamic neurons that express VGF polypeptide were found to also express detectable levels of NGF receptor (101). As a consequence of the data presented here, one explanation for this observation is that VGF gene expression is being regulated in these hypothalamic neurons by neurotrophic growth factors other than NGF, such as bFGF or possibly by the NGF-like growth factors brain-derived neurotrophic factor (6) and neurotrophin-3 (45, 64), although regulation of VGF mRNA levels by the last two has not yet been experimentally tested. Alternatively, there is evidence suggesting that transient expression of NGF receptors occurs early in the developing cerebellum and olfactory bulb (16). In addition, sympathoadrenal progenitor cells of the developing PNS express bFGF receptors and respond to bFGF prior to the expression of NGF receptors (11). Should NGF not be required for continued expression of VGF in the adult hypothalamus, then transient expression of NGF and NGF receptor or of bFGF and bFGF receptor early in development could result in VGF mRNA induction and polypeptide synthesis. The VGF gene could potentially be regulated in the adult by neurotrophic growth factors other than those initially involved in its induction early in development. The failure of treatment with the neurotrophic growth factor aFGF to induce VGF mRNA to levels similar to those observed following NGF or bFGF treatment of PC12 cells is somewhat puzzling in light of previous findings that long-term treatment with aFGF causes neuronal differentiation (81). However, the response of PC12 cells to long-term treatment with aFGF, as measured by the outgrowth of neurites and the induction of acetylcholinesterase activity, was much more dependent on the presence of heparin (50 μg/ml) than was the response of PC12 cells to either NGF or bFGF treatment. Since the effect of aFGF treatment on VGF mRNA levels was measured in the absence of heparin, it is possible that induction of VGF mRNA to comparatively greater levels might occur in the presence of heparin.

Initial analysis suggests that VGF gene expression is regulated in part by protein kinase C and phosphoinositol hydrolysis rather than through a protein kinase A pathway, since PMA was a more potent inducer of VGF mRNA levels than was 8-bromo-cAMP. Down-regulation of the protein kinase C pathway by prolonged stimulation of PC12 cells with PMA (24 h) decreased but did not eliminate the induction of VGF mRNA levels that was seen in control cultures after treatment with NGF (86a). Further experimentation is necessary to clarify the role of Ca^{2+} in the induction VGF

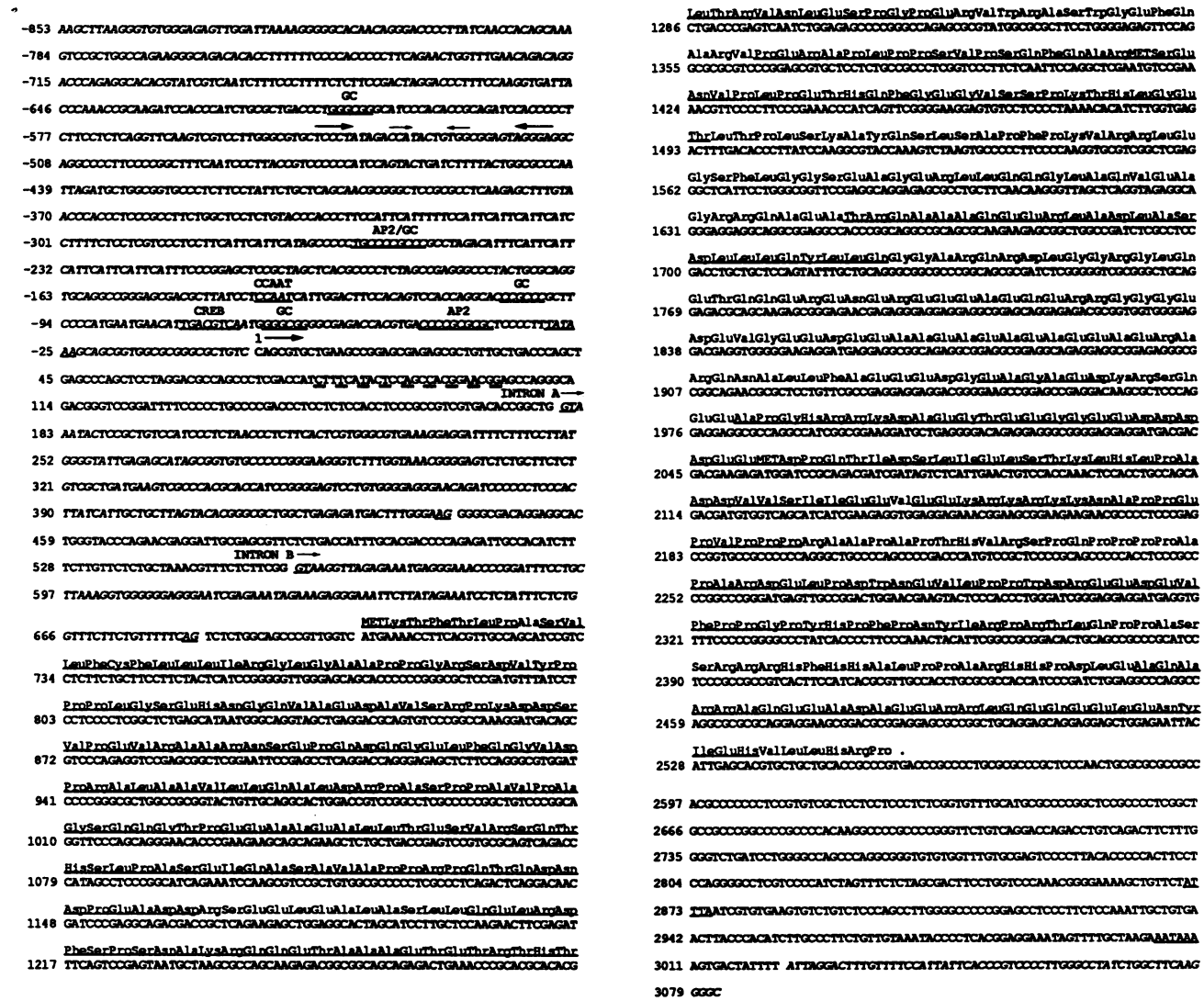
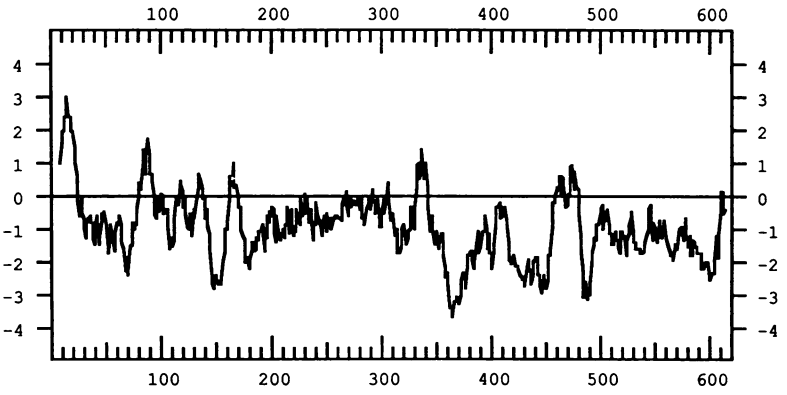


FIG. 9. Genomic sequence of VGF. The sequence is numbered relative to the presumptive transcription start site at position 1. The TATAAA, GC, CCAAT, AP-2, and CREB elements are underlined. A sequence with substantial dyad symmetry that resembles an SRE is identified at −511 by the arrows. Consensus splice site dinucleotides are underlined. The sequence at the 5′ end of the NGF33.1 cDNA clone, complementary to the oligonucleotide used for primer extension studies, is indicated by the dashed underline. The predicted amino acid sequence of VGF (NGF33.1) is shown; underlined amino acids are identical to the deduced amino acid sequence of the VGF cDNA clone (80). Consensus 3′ polyadenylation (AATAAA) and mRNA destabilization (ATTTA) sequences are underlined.

mRNA levels. Depolarization, which leads to a rapid four-fold increase in VGF mRNA levels, triggers calcium influx through voltage-gated calcium channels. Genes regulated by depolarization include the immediate-early genes (9, 37, 75, 76, 82, 107) and those encoding neuropeptide and neurotransmitter biosynthetic enzymes (12, 31, 50, 103). Since VGF polypeptide is secreted through a regulated pathway in PC12 cells and its release is stimulated by depolarization (80), the elevation in VGF mRNA levels that was observed after depolarization may be a response to depletion of cellular VGF stores, much as the pituitary genes that encode growth hormone, pro-opiomelanocortin, and prolactin respond to stimulated secretion of the respective peptide hormones by increasing the rate of gene transcription (7, 8, 30, 34, 78). In addition to being induced by depolarization in vitro, a number of immediate-early genes have been found to be regulated in the CNS in vivo by seizures (75, 82, 107), kindling (28, 104), and sensory stimulation (46), although a similar induction in VGF mRNA levels in the hippocampus following seizure activity, as measured by RNase protection analysis, has not been observed (86b), further suggesting that the predominant mechanisms underlying VGF and immediate-early gene regulation by depolarization may differ.

Since (i) the distribution of VGF mRNA is restricted to tissues of the CNS and PNS, (ii) the expression of VGF mRNA appears to be developmentally regulated within the nervous system, and (iii) VGF mRNA is relatively selectively inducible by NGF and bFGF, we decided to clone and characterize the VGF gene and to examine the structure of the promoter. We found that the VGF promoter has a canonical TATAA box and a CCAAT element. Four GC box sequences and a consensus CREB site (5′-TGACGTCA-3′) were identified. The CREB site and one of the SP1 elements

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Neurofilament triplet M protein - Chicken (fragment)
  380       390       400       410       420       430       440       450       460
GGEDEVGEEDEEAAEAEAEAEEAERARQNALLFAEEEDGEAGAEDKRSQEEAPGHRRKDAEGTEEGG---EEDDDDEEMDPQTIDSLI
          || || ||  |||           |||  |  ||     |||      ||   ||||   ||    ||
AAKAQEEEQEEEKAEEEAVEEEAVSEKAAEQAAEEEEKEEEEAEE----EEAAKS---DAA--EEGGSKKEEIEEKEEREEAEEEEAE
        140       150       160       170              180           200

Neurofilament triplet L protein - Mouse
 360       370       380             390       400       410       420       430       440       450
TQQERENEREEEAEQERRGGGEDEVG----EEDEEAAEAEAEAEEAERARQNALLFAEEEDGEAGAEDKRSQEEAPGHRRKDAEGTEEGGEEDDDDEEMDPQ
         |||  |    || |      || ||   ||   ||           ||| ||   ||    ||             ||  ||
VEETIEATKAEEAKDEPPSEGEAEEEEKEKEEGEEEEGAE---EEEAAKDESEDTKEEEEGGEGEEEDTKESEE-----------EEKKEESAGEEQVAKK
  460       470       480       490             500       510       520                 530       540

Troponin T, cardiac muscle, major isoform - Rabbit
 360       370       380             390       400       410       420       430
ETQQERENEREEEAEQERRGGGEDEVGEEDEEA---AEAEAEAEEAERARQNALLFAEEEDGEAGAEDKRSQEEAPGHR
         ||  |||         ||| |||   |||||| ||             ||||    |||
DLEEVVEEYEEEQEEQEA--------GEE-EEAGGGAEAEAETEETQA----------EEDGQEE-EDKEDEDGPVEES
        10                20        30        40                50        60
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FIG. 10. Analysis of hydropathicity and regions of amino acid sequence homology detected by data base search. Kyte-Doolittle analysis (53) was carried out on the amino acid sequence predicted by using the longest open reading frame of the NGF33.1 cDNA and VGF genomic clones. A hydrophobic N-terminal region, consistent with a signal sequence, was detected. Comparison of the amino acid sequence predicted by the NGF33.1 cDNA and VGF genomic sequences to the NBRF-PIR data base revealed a region that was weakly homologous to the NF-L and -M proteins, troponin T, tropomyosin, and microtubule-associated protein 2 (IFIND algorithm [Intelligenetics]; score range = 10 to 13, standard deviation = 5.94 to 9.02).

are found between −62 and −78. In eukaryotic promoters, functional GC box sequences, which interact with SP1 to enhance transcription, are usually found 40 to 70 nucleotides upstream of the RNA initiation site (14), while the CCAAT element is generally located 60 to 80 nucleotides upstream of the transcription start site (67). In general, the CREB site has been found to function in the regulation of gene expression through interaction with either protein kinase A or calcium-calmodulin-dependent protein kinase pathways (73, 74, 92). The c-*fos* gene in particular has been shown to be activated by depolarization and calcium agonists, through a pathway that phosphorylates CREB (92), which in turn interacts with an element in the c-*fos* promoter (5′-TGACGTTT-3′) that is functionally indistinguishable from a CREB site (91). The c-*fos* Ca^{2+} response element at −60 and the VGF CREB element at −71 are positioned similarly with respect to the transcription start site. The CREB element may therefore be important in the regulation of the VGF gene by depolarization, and functional studies are in progress to test this hypothesis. Although somewhat puzzling in light of the identification of a potential CREB binding site in the VGF 5′ flank, experimental evidence does not strongly implicate the cAMP-dependent protein kinase A pathway in regulation of the VGF gene, since treatment of PC12 cells with either 8-bromo-cAMP, forskolin, or 3-isobutyl-1-methylxanthine resulted in maximal ~2-fold increases in VGF mRNA levels after 3 to 6 h (Fig. 4; 86a). Further studies are necessary to define the contributions of these sequence elements to the regulation of VGF mRNA levels.

Two AP-2-like sites were identified which closely resemble the published consensus sequences 5′-(T/C)(C/G)C/G)CC(A/C)N(G/C)(G/C)(G/C)-3′ (47) and 5′-CC(C/G)C(A/G)GGC-3′ (72). The site between −261 and −252, 5′-TGCCCCGCCC-3′, overlaps one of the GC boxes, and the other site, 5′-CCCCGCGC-3′, is found between −48 and −39, in close proximity to the TATAA box, CREB, and SP1

```
HOMOLOGY 1:  RAT PERIPHERIN  75%

rat VGF          -314  T C A T T C A T T C A T C C T T T T C T -295
                       |   | | |     |   |   | | | | | | | | |
rat peripherin   -337  T A A T T T T T A C C T C C T T T T C T -318

HOMOLOGY 2:  RAT PERIPHERIN  75%

rat VGF          -326  T C A T T T T T C C A T  -315
                       |   | | | | | | |   |   |
rat peripherin   -337  T A A T T T T T A C C T  -326

HOMOLOGY 3:  MOUSE NF-M  74%

rat VGF          -754  T T T T C C C C A C C C C C T T C A G  -736
                       | |     | | | | | | | | | | |     |   |
mouse NF-M       -120  T T C C C C C C A C C C C C G G C C G  -102
```

FIG. 11. Regions of homology between the VGF promoter and the promoters of other NGF-inducible genes. Nucleotide numbers indicate the positions of the homologies relative to the respective promoters. Genomic sequences for peripherin and mouse NF-M were obtained from references 97 and 60, respectively.

elements. AP-2 appears to mediate transcriptional activation in response to both the phorbol ester-diacylglycerol-activated protein kinase C and the cAMP-dependent protein kinase A pathways (47, 72). Should PMA treatment lead to an induction in VGF mRNA levels through an increase in VGF gene transcription, then this response might be mediated through interactions with sequence elements within the VGF 5′ flank, in particular the AP-2 sites or even possibly the CREB binding site, since a consensus AP-1 binding site was not found. In vitro-translated Jun proteins have been shown to bind to the CRE sequence -TGACGTCA-, but with much lower affinity than to the similar AP-1 binding site -TGAGTCA- (79). Functional studies, however, suggest that AP-1 (Fos/Jun) is not involved in mediating depolarization-induction of c-*fos* through the calcium response element, nor was this element able to confer 12-*O*-tetradecanoyl phorbol-13-acetate inducibility (92). Thus, an understanding of the mechanism(s) by which phorbol esters regulate VGF mRNA levels is likely to be dependent on functional analysis of the VGF promoter.

Regulation of the c-*fos* gene by NGF and serum stimulation is dependent on the presence of the SRE (99). Similar SRE-like elements have been identified in the 5′ flank of the NGF-IA gene, another immediate-early gene that is rapidly induced by growth factor treatment (19). VGF mRNA levels do not appear to be regulated by serum stimulation (86a), nor was an SRE found in the 832 bp of sequenced 5′ flank. To try to identify additional elements in the VGF gene which might be involved in the regulation of VGF mRNA by NGF and bFGF and in the selective expression of the VGF gene in the nervous system, the VGF promoter was compared with the promoters of those NGF-induced genes whose genomic sequences are available. Two regions of ~75% homology between the VGF and peripherin promoters were found to be similarly positioned, between −295 and −340, with respect to the transcription start site. We did not detect substantial homology between the VGF and peripherin promoters in the regions of homology previously detected between the peripherin promoter and the promoters of the NGF-inducible genes encoding calcyclin, NF-L, and NF-M (97). Studies to assess the functional importance of these homologous elements in the induction of VGF mRNA by NGF and in the expression of VGF mRNA in neural tissues are in progress.

We have determined the complete coding sequence of the VGF gene. Comparison of the NGF33.1 cDNA and VGF genomic sequences and of the predicted polypeptide sequences with sequences in the NIH, EMBO, and PIR data bases revealed no strong homology to any known gene. One region of the VGF (NGF33.1) protein sequence (amino acids 370 to 450) does show weak homology to a number of structural proteins, including the neurofilaments, which may be primarily due to the relatively high glutamic acid content of the region. In light of the localization of VGF predominantly within the secretory vesicles of PC12 cells (80) rather than the cytoplasm, it is unlikely that VGF is functionally or structurally related to the neurofilament proteins. In addition, the intron-exon pattern of the VGF gene, composed of two relatively small introns within the 5′ untranslated sequence of the gene, is clearly distinct from the intron-exon structure of the intermediate filament genes. The positioning of introns A and B in the 5′ untranslated region (i.e., the absence of introns within the translated sequence) and the sequence identity shared between the N-terminal polypeptide sequences predicted by the VGF and NGF33.1 cDNA clones suggest that the two cDNAs correspond to the same mRNA, not to alternatively spliced forms of the same primary transcript.

Although the function and mechanism(s) of regulation of the VGF gene are unknown, the demonstration that VGF mRNA is expressed exclusively in the nervous system, with peak mRNA levels measured during a crucial phase of neuronal development, suggests that VGF may be involved in the regulation of cell-cell interactions or in synaptogenesis during the maturation of the nervous system. The observation that VGF mRNA levels are reinduced following NGF deprivation and readdition also suggests that VGF polypeptide may be functionally involved in the early events that precede neurite outgrowth and regeneration. As noted previously (80) and shown above, the amino acid sequences deduced from the VGF cDNA clone and from the NGF33.1 cDNA and VGF genomic clones predict polypeptides that are rich in dibasic amino acids. If these potential sites for proteolytic cleavage are recognized, the possibility exists that VGF is the precursor of one or more biologically active peptides. Cloning of the VGF gene will enable the distribution of VGF mRNA within the nervous system to be fully characterized, the regulatory elements responsible for the neural expression of this gene to be dissected, and experiments to assess the function of the encoded polypeptide to be attempted. Studies of the regulation of VGF gene expression that are in progress may ultimately clarify the potential role of VGF in neuronal differentiation and the mechanism(s) by which NGF selectively and rapidly induces VGF mRNA levels in PC12 cells.

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