Nerve growth factor (NGF) up-regulated steady-state levels of m4 muscarinic acetylcholine receptor (mAChR) mRNA in PC12 cells. Up-regulation of mRNA levels was associated with a corresponding increase in mAChR binding sites. Two other growth factors, basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF), up-regulated m4 mRNA and mAChR binding sites. Treatment of PC12 cells with NGF and bFGF, but not EGF, has previously been demonstrated to result in sustained activation of mitogen-activated protein kinase (MAPK). Analogously, NGF and bFGF, but not EGF, increased the stability of m4 mRNA in PC12 cells. In HER-PC12 cells, a clonal PC12 cell transfectant overexpressing EGF receptors and displaying sustained MAPK activation upon receptor stimulation, EGF treatment stabilized the m4 transcript. A synthetic inhibitor of MAPK kinase, PD98059, inhibited growth factor-induced stabilization of the m4 transcript in both PC12 and HER-PC12 cells. These findings demonstrate that the MAPK pathway is involved in transcript stabilization. Cycloheximide pretreatment abolished the post-transcriptional effect of NGF, indicating that de novo protein synthesis was required for the observed increase in m4 mRNA stability. By contrast, cycloheximide had no discernible post-transcriptional effect if added after NGF treatment, suggesting that an inducible yet stable protein factor was involved in m4 mRNA decay. An unusually well conserved 137 nucleotides of m4 3'-untranslated region has been identified by sequence comparison with other mRNAs that are post-transcriptionally regulated by NGF. In PC12 cells that heterologously overexpress this region, we demonstrate that NGF no longer stabilizes endogenous m4 mRNA. This conserved region probably represents an NGF-responsive element involved in mRNA stability regulation. Finally, transcription of the m4 gene can be induced by all three growth factors but is not dependent on MAPK activity, unlike growth factor-induced m4 mRNA stabilization.

G-protein-coupled receptors (GPCRs)\(^1\) are a major constituent of the signal transduction cascade of cells in the central nervous system. Accordingly, the expression of GPCR genes in neural cells needs to be coordinately regulated in order to integrate the many incoming extracellular cues (for a review, see Ref. 1). Control of GPCR gene expression can potentially be exerted at the level of transcription and post-transcription (i.e., mRNA degradation) (for reviews, see Refs. 2 and 3). The mAChR family, a member of the GPCR gene superfamily, mediates the effects of acetylcholine in the central and peripheral nervous system (4). mAChRs comprise five subtypes (m1–m5), each subtype being derived from a distinct receptor gene and exhibiting disparate patterns and levels of tissue expression (5–8). As such, this receptor family is ideal for studying the regulation of GPCR gene expression (9). Muscarinic agonists, hormones, steroids, and growth factors have been shown to up- or down-regulate steady-state levels of different mAChR subtype mRNAs (9–17). In some instances, the genetic elements controlling mAChR mRNA levels have been identified (18–23). These include transcriptional elements responsible for neural specific expression and cis-acting elements controlling mRNA decay.

In newborn rat brain, nerve growth factor (NGF) has been demonstrated to down-regulate steady-state m1 and m3 mRNA levels while up-regulating m4 mRNA (24). An earlier report revealed that NGF could up-regulate mAChR binding sites in cultured PC12 cells (25). However, the molecular mechanisms responsible for the observed effects of NGF on mAChR gene expression have not been identified. NGF and other peptide growth factors are essential for nerve cell development, differentiation, and survival (26). Growth factor modulation of these cellular processes is accomplished, at least in part, by regulating the expression of specific genes (26). NGF-induced differentiation of adrenal chromaffin-like PC12 cells to a sympathetic neuron-like phenotype has been associated with the differential expression of several hundred mRNA species (27). Several transcripts encoding GPCRs in PC12 cells are regulated by NGF. Examples include the adenosine A2a (27), secretin (27), pituitary adenylate cyclase-activating polypeptide (28), and angiotensin II type 2 receptor mRNAs (29).

In the present report, the regulation of m4 gene expression in cultured PC12 cells by NGF and two other growth factors, basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF), is described. Both post-transcriptional and transcriptional events are implicated as mechanisms regulating growth factor-induced changes in m4 mRNA levels.
Mechanisms Regulating m4 mAChR Gene Expression

EXPERIMENTAL PROCEDURES

Materials—Rat PC12 pheochromocytoma cells were obtained from American Type Culture Collection (Rockville, MD). HER-PC12 cells were a generous gift of Dr. Philip Cohen at the University of Dundee. Plasmid Rm4(cRMR1)p11 (6) was a generous gift of Dr. T. I. Bonner at the National Institutes of Health. The mammalian expression vector pcDNA3 was purchased from Invitrogen Corp. (San Diego, CA). [3H]Quinuclidinyl benzilate ([3H]QNB) (44.9 Ci/mmol) and GeneScreen Hybridization Transfer Membranes were from NEN Life Science Products. RNase A and T1 nuclelease, protein K, actinomycin D, and G-50 Sephadex columns were from Boehringer Mannheim. PD98059 was purchased from New England Biolabs. Cycloheximide was from Calbiochem. T7 and SP6 RNA polymerase, mouse 2.5 S NGF, human recombinant bFGF, and human recombinant EGF were from Promega (Madison, WI). Restriction endonucleases, G-418 (Geneticin), RPMI 1640 medium, fetal bovine serum, and heat-inactivated horse serum were from Life Technologies, Inc.

Northern Blot Analysis—PC12 cells were maintained in culture as described previously (27). In cases where NGF, bFGF, EGF or other treatments were included, the medium was changed every second day, and fresh growth factor was added. At the indicated times, PC12 cells were washed three times with phosphate-buffered saline (PBS) (pH 7.4), and total cellular RNA was isolated by a single-step guanidinium-isoamyl alcohol procedure (27). In cases where NGF, bFGF, EGF or other treatments were included, the medium was changed every second day, and fresh growth factor was added. At the indicated times, PC12 cells were washed three times with phosphate-buffered saline (PBS) (pH 7.4), and total cellular RNA was isolated by a single-step guanidinium-isoamyl alcohol procedure (27).

Construction of Riboprobe Templates—The construction of plasmid 118GADPHspSP73 for generating an antisense riboprobe of the GADPH mRNA has previously been described (18). The antisense riboprobe for the m4 mRNA was constructed as follows. PCR primers, 5'-GAATTCGGCCGAAGTGCATAGAGC-3' and 3'-CTCGAGATCTGTCT-9 and 5'-UTR (nt 1741–2040) from Rm4(cRMR1)p11. The resulting PCR product was agarose gel-purified and then incubated with actinomycin D (5 μg/ml) to arrest transcription as described (9, 18). At the indicated times, total RNA was isolated from cultured cells, and an RNase protection assay was performed as described by Lee et al. (18). Briefly, EcoRI-linearized 286Rm4spSP73 and StyI-linearized 118GADPHspSP73 were incubated separately with T7 RNA polymerase and [32P]CTP (50 μCi) at 37 °C for 60 min to generate 32P-labeled antisense riboprobes. The resulting riboprobes were purified from unincorporated nucleotides on a G-50 Sephadex column. 32P-labeled m4 and GADPH riboprobes (5× 10^6 cpm each) were incubated simultaneously with total RNA (50 μg) in 30 μl of hybridization buffer for 18 h. At 50 °C (18). Upon completion of hybridization, 350 μl of ribonuclease digestion buffer (10 μg Tris-HCl (pH 7.5), 300 mM NaCl, and 5 mM EDTA) containing RNase A (40 μg/ml) and T1 (2 μg/ml) was added to each assay tube, followed by incubation at 30 °C for 60 min. RNase digestion was stopped by the addition of 10 μl of 20% SDS and 5 μl of proteinase K (10 mg/ml), and RNase-resistant RNA was ethanol-precipitated and loaded on a 5% acrylamide-7 M urea gel. RNase-resistant m4 hybrid levels were normalized to GADPH hybrid levels.

Nuclear Run-on Transcription Assay—PC12 cells were treated with NGF, bFGF, EGF, or saline vehicle at the indicated times. Following three washes with phosphate-buffered saline, nuclei were isolated as described by Greenberg and Ziff (31). The isolated nuclei (∼ 5× 10^6) were resuspended in 200 μl of glycogen storage buffer (50 mM Tris-HCl (pH 8.3), 40% glycerol, 5 mM MgCl2, and 0.1 mM EDTA) and stored at −70 °C prior to use. Nuclei (200 μl) were combined with 100 μCi of [3H]UTP and 200 μl of 2× reaction buffer mix (10 mM Tris-HCl (pH 8); 5 mM MgCl2; 0.3 mM KCl; 25 mM dithiothreitol; and 5 mM ATP, CTP, and GTP) for a final volume of 410 μl and incubated for 30 min at 30 °C. Following incubation of nuclear run-on reaction, the reaction mix was incubated with RNase-free DNase for 5 min at 30 °C, followed by the addition of 200 μl of SDS/Tris buffer (5% SDS, 0.5 μg Tris-HCl (pH 7.4), 0.1% EDTA) and 10 μl of proteinase K for 30 min at 42 °C. The radiolabeled RNA was isolated by the single-step guanidinium-thiocyanate/phenol-chloroform extraction procedure (30) and ethanol-precipitated. The pellet was dissolved in 1 ml of hybridization buffer and immobilized onto a GeneScreen hybridization membrane. Membranes were washed as described by Greenberg and Ziff (31). All wash buffers contained RNase A and T1. Upon completion of the final wash, filters were treated with RNase for an additional 30 min at 37 °C. Blots were analyzed by scanning on a Molecular Dynamics PhosphorImager.

RESULTS

NGF Regulates Steady-state Levels of m4 mRNA and mAChR Density—The effects of chronic NGF treatment (50 ng/ml) on mAChR binding sites and m4 mRNA expression in PC12 cells were investigated. Total cellular mAChR binding sites were assessed by receptor binding assays in whole cells. Scatchard analysis of [3H]QNB binding to PC12 cells under basal conditions indicated that mAChR density was 40 ± 3 fmol/mg of total cellular protein with a Kd value of 0.11 ± 0.04 nM (n = 2). Since the affinity of [3H]QNB did not change significantly with 12-day NGF treatment (Kd = 0.10 ± 0.02 nM; n = 3), subsequent time course experiments on mAChR density were measured using a saturating radioligand concentration of 2 nM. Incubation of PC12 cells with 50 ng/ml NGF induced a time-dependent up-regulation of mAChR density that was evident by day 3 and maximal (3-fold increase) by days 12–15 (Fig. 1). The observed changes in whole cell mAChR binding sites following NGF treatment were absent in saline-treated PC12 cells maintained in culture for up to 15 days (data not shown). These findings agree with a previous study demonstrating that increases in mAChR binding sites in plasma membrane homogenates of PC12 cells depended on NGF and not the duration of culture (29). Quantification of mAChR mRNA levels by Northern blot analysis demonstrated that the up-regulation of mAChR binding sites was accompanied by an induction of steady-state mRNA levels (Fig. 1). The time courses for induction of mRNA and protein levels were nearly identical. NGF-induced up-regulation of m4 mRNA levels was significant (p < 0.05) by day 3 (1.8-fold increase) and maximal by days 12–15 (3–4-fold). GADPH mRNA levels were unaffected by NGF treatment (Fig. 1). As seen for mAChR density, steady state m4...
mRNA levels were not affected in cells treated with saline for 0–15 days (data not shown).

**NGF Increases m4 mRNA Stability**—Changes in steady-state mRNA levels may be attributable to alterations in the degradation rate of a transcript and/or rate of gene transcription (3). Hence, the relative contribution of a post-transcriptional mechanism in the modulation of m4 mRNA levels by NGF was addressed. For mRNA decay experiments, PC12 cells pretreated with NGF (50 ng/ml) or saline were incubated with actinomycin D (5 μg/ml) to arrest transcription. The decay of m4 mRNA was followed by RNase protection assay. The RNase-resistant hybrids are shown in Fig. 2A. In control PC12 cells treated with saline (15 days), m4 mRNA displayed a basal half-life of 1.4 h (Fig. 2B; Table I). This half-life value was not significantly different from those obtained in control cells treated with saline for 3 and 12 days (data not shown). Therefore, the basal half-life of m4 mRNA was not influenced by the length of time cells were maintained in culture. In contrast, the basal half-life of the m4 mRNA significantly increased (p < 0.05) by ~4-fold to 5.6, 5.3, and 4.6 h following 3, 9, and 15 days of NGF treatment, respectively (Fig. 2B; Table I).

**bFGF and EGF Regulate m4 mRNA and mAChR Binding Sites**—The effects of two other growth factors on m4 gene expression were examined for comparison with NGF-induced changes. Both bFGF (10 ng/ml) and EGF (50 ng/ml) increased [3H]QNB binding in intact PC12 cells with maximal increases occurring by day 12 and 9, respectively (Figs. 3 and 4). Maximal up-regulation of mAChR density was 4.5- and 2-fold with bFGF and EGF treatment, respectively. While induction of steady-state m4 mRNA levels coincided with the up-regulation of mAChR density following NGF treatment (Fig. 1), induction of m4 mRNA levels preceded receptor up-regulation when cells were treated with either bFGF or EGF (Figs. 3 and 4). For instance, mRNA levels were maximally increased 4- and 3-fold by day 3 of bFGF and EGF treatment, respectively (Figs. 3 and 4). However, both growth factors had only marginal effects, if any, on mAChR density during this same time period. The temporal regulation of steady-state m4 mRNA levels appeared to be biphasic when cells were treated with either bFGF or EGF; nevertheless, mRNA levels remained significantly elevated for up to 15 days (p < 0.05).

**bFGF but Not EGF Stabilizes m4 mRNA**—In a manner analogous to NGF, bFGF (10 ng/ml) was able to stabilize m4 mRNA in PC12 cells (Fig. 5; Table I). The temporal regulation of m4 mRNA stability by bFGF was biphasic in nature, which probably accounts for the biphasic regulation of steady-state m4 mRNA levels by the same growth factor (Figs. 3 and 5; Table I). The stability of the m4 mRNA increased from a basal half-life of 1.4 h to 4.9 and 5.2 h following 3 and 9 days of bFGF treatment, respectively. By day 12 of bFGF treatment, the stability of m4 mRNA (t½ = 1.9) had reverted back to its basal state (Fig. 5; Table I). In contrast to NGF and bFGF, EGF (50 ng/ml) had no effect on the half-life of the m4 mRNA (Fig. 5; Table I). The half-life values were 1.9, 1.9, and 1.0 h following 3, 9, and 12 days of EGF treatment, respectively.

**MAPK Activity Stabilizes m4 mRNA—HER-PC12 cells are a clonal line derived from PC12 cells transfected to overexpress the EGF receptor (33). Unlike in native PC12 cells, stimulation of EGF receptors in HER-PC12 cells leads to sustained activation of the Ras/Raf/MEK/MAPK pathway and neuronal differentiation (33). Hence, the effect of EGF in HER-PC12 cells was investigated to establish whether prolonged activation of MAPK was required for stabilization of m4 mRNA. In cultures of HER-PC12 cells treated with saline, the basal half-life of the m4 mRNA was 1.2 h (Fig. 6A; Table I). Exposure of these cells to EGF (50 ng/ml) for 3 days led to a stabilization of the transcript with a corresponding half-life of 4.5 h (Fig. 6A; Table I). The half-life of the m4 transcript remained elevated for up to 12 days of EGF treatment (data not shown). These findings are in contrast to those obtained in PC12 cells, where EGF did not stabilize the m4 mRNA (Fig. 5; Table I).

Inhibition of MEK by the synthetic compound PD98059 blocks NGF-induced MAPK activity and MAPK-dependent neurite formation in native PC12 cells (34). Since overexpression of the EGF receptor in HER-PC12 cells leads to sustained activation of MAPK (33), the effects of PD98059 on EGF-induced neurite formation and m4 mRNA stabilization were assessed. PD98059 inhibited EGF-mediated neurite formation in a dose-dependent manner. Whereas cells treated with EGF alone (50 ng/ml for 3 days) had extended long neurites (>200
mRNA was 1.4 h in cells treated with 50 ng/ml NGF (50 ng/ml). Cells were washed three times with phosphate-buffered saline and incubated with 5 μg/ml actinomycin D to arrest transcription. At the indicated times, cells were harvested, and total RNA was isolated. The decay of m4 mRNA was determined by an RNase protection assay as described under “Experimental Procedures.”

Data shown are the mean of 2–4 independent experiments. S.E. values were <5% of the means.

**Table I**

### Half-lives of the m4 mRNA

| Concentrations | Mean ± S.E. (n) | p value |
|----------------|-----------------|---------|
| PC12 Saline, 15 days | 1.4 ± 0.2 (4) | <0.05<sub>a</sub>, <0.05<sub>b</sub>, <0.05<sub>c</sub>, <0.05<sub>d</sub> |
| NGF, 3, 9, or 15 days | 5.6 ± 0.3 (2), 5.3 ± 0.2 (3), 4.6 ± 0.1 (4) | <0.05<sub>a</sub>, <0.05<sub>b</sub>, <0.05<sub>c</sub>, <0.05<sub>d</sub> |
| bFGF, 3, 9, or 15 days | 4.9 ± 0.5 (3), 5.2 ± 0.2 (3), 1.9 ± 0.3 (3) | <0.05<sub>a</sub>, <0.05<sub>b</sub>, <0.05<sub>c</sub>, <0.05<sub>d</sub> |
| EGF, 3, 9, or 15 days | 1.9 ± 0.2 (3), 1.9 ± 0.4 (3), 1.0 ± 0.1 (3) | NS<sub>a</sub>, NS<sub>b</sub>, NS<sub>c</sub> |
| Cycloheximide, 3 days | 1.3 ± 0.1 (3) | NS<sub>a</sub> |
| Cycloheximide, 3 h pretreatment + EGF, 3 days | 1.2 ± 0.3 (3) | NS<sub>a</sub> |
| Cycloheximide, 12 h | 1.5 ± 0.3 (2) | NS<sub>a</sub> |
| NGF, 3-day pretreatment + cycloheximide, 12 h | 4.2 ± 0.2 (3) | <0.05<sub>a</sub> |
| HER-PC12 Saline, 3 days | 1.2 ± 0.4 (3) | NS<sub>a</sub> |
| EGF, 3 days | 4.5 ± 0.1 (3) | <0.05<sub>a</sub> |
| PD98059, 3 days | 1.4 ± 0.2 (3) | NS<sub>a</sub> |
| PD98059, 0.5-h pretreatment + EGF, 3 days | 1.3 ± 0.4 (3) | NS<sub>a</sub> |
| PC12utr1 Saline, 15 days | 1.0 ± 0.4 (3) | NS<sub>a</sub> |
| EGF, 3 days | 4.8 ± 0.5 (3) | <0.05<sub>a</sub> |
| PD98059, 3 days | 1.4 ± 0.2 (3) | NS<sub>a</sub> |
| PD98059, 0.5-h pretreatment + EGF, 3 days | 1.3 ± 0.4 (3) | NS<sub>a</sub> |
| PC12utr10 Saline, 15 days | 4.6 ± 0.4 (3) | <0.05<sub>a</sub> |
| EGF, 3 days | 4.3 ± 0.1 (3) | NS<sub>a</sub> |

* Relative to PC12 (saline, 15 days).
* Relative to PC12 (cycloheximide, 12 h).
* Relative to HER-PC12 (saline, 3 days).
* Relative to PC12utr1 (saline, 15 days).
* Relative to PC12utr10 (saline, 15 days).

PD98059 had similar effects on mRNA stability in native PC12 cells. Pretreatment of cells with 50 μM PD98059 for 30 min abolished stabilization of m4 mRNA following 50 ng/ml NGF treatment for 3 days (data not shown). Stabilization of the m4 mRNA Is Dependent on de Novo Synthesis of a Stable Protein Factor—Cycloheximide at concentrations of 0.5–10 μg/ml has previously been demonstrated to effectively inhibit protein synthesis in PC12 cells by >95% within 8–12 h (35, 36). The effect of NGF (50 ng/ml, 3 days) on m4 mRNA stability in PC12 cells pretreated with cycloheximide (1 μg/ml cycloheximide, 4 h) was assessed (Fig. 6C; Table I). Cycloheximide treatment alone (3 days) resulted in a half-life measurement of 1.3 h for the m4 mRNA. This value did not change following the administration of NGF for 3 days in the cycloheximide-pretreated PC12 cells (t₁/₂ = 1.2 h). Both cycloheximide-derived half-life values were not significantly differ-
ent ($p > 0.05$) from the value obtained in saline-treated cells ($t_{1/2} = 1.4$ h; see Fig. 2B; Table I).

Next, the effect of cycloheximide on mRNA stability in NGF-pretreated PC12 cells was assessed. The measured half-life of the m4 mRNA was 1.5 h in cells treated for 12 h with 1 $\mu$g/ml cycloheximide alone (Table I). In parallel experiments, cultures were pretreated with NGF for 3 days followed by cycloheximide treatment for 12 h. Under these conditions, the m4 mRNA half-life was 4.2 h (Fig. 6C; Table I). A similar half-life value was observed when 3-day NGF-pretreated cells were incubated with cycloheximide for up to 3 days (data not shown). These data demonstrate that inhibition of protein synthesis before but not after NGF treatment impairs NGF-induced stabilization of m4 mRNA.

Analysis of the m4 3' UTR—A short 137-nt segment of the m4 3' UTR (nt 1826–1962) exhibits unusually high sequence identity (52%) with a 151-nt segment of the GAP-43 3' UTR (Fig. 8). Such high sequence conservation may reflect a common functional role between these two 3' UTRs in regulating mRNA stability (37). Several studies have provided evidence that a region of the GAP-43 3' UTR (which encompasses the conserved 151-nt segment) is a cis-acting element, and this element is bound by a 65-kDa factor (38–40). To ascertain whether the 137-nt segment of the m4 mRNA (nt 1826–1962) acts as a cis-acting element, a 300-nt fragment of the m4 3' UTR (nt 1741–2040) was subcloned into a mammalian expression vector pcDNA. The resulting construct, m4utr-pcDNA3, was stably transfected into PC12 cells. The expression level of the 300-nt m4 3' UTR relative to endogenous m4 mRNA in transfected cells was determined by RNase protection with a $^{32}$P-labeled
Cultured cells were treated with 50 ng/ml EGF or saline for 3 days. When total RNA from untransfected PC12 cells was subjected to RNase protection, a single RNase-resistant hybrid of 300 nt was visible (data not shown). When total RNA from clonal cell lines transfected with m4utr-pcDNA3 was subjected to RNase protection, two RNase-resistant hybrids of 300 and 377 nt were visible (data not shown). The 300-nt hybrid corresponds to the endogenous m4 mRNA, while the 377-nt hybrid represents the heterologously expressed 300-nt m4 3'UTR (and 77 nt of pcDNA3 polylinker sequence). Two clonal lines were selected for study, PC12utr1 (molar ratio 1:1 of 300-nt m4 3'UTR to endogenous m4 mRNA) and PC12utr10 (molar ratio 10:1 of 300-nt m4 3'UTR to endogenous m4 mRNA).

In PC12utr10 cells, the m4 mRNA was found to decay with a basal half-life of 4.6 h, which is significantly longer than the basal half-life of 1.4 h measured in wild-type PC12 cells (Table I). When the PC12utr10 cell line was treated with NGF for 15 days (50 ng/ml), the decay of the m4 mRNA was unaffected, with a measured half-life of 4.3 h (Table I). By contrast, the half-life of the m4 mRNA in PC12utr1 cells under basal conditions was 1.0 h and was significantly increased to 4.8 h in the presence of NGF for 15 days (Table I).

**Regulation of m4 Gene Transcription by Growth Factors—** Having identified a post-transcriptional mechanism for NGF-induced regulation of m4 gene expression in PC12 cells, we examined the effect of NGF on gene transcription. To measure new m4 mRNA synthesis, a nuclear run-on transcription assay was performed on cell nuclei isolated from NGF (50 ng/ml)- and saline (basal)-treated cells (Fig. 9A). At all time points investigated, there was no significant change in the rate of GAPDH gene transcription following NGF treatment ($p > 0.05$). Therefore, GAPDH transcriptional rates served as an internal control for normalizing the rate of m4 gene transcription (Fig. 9B).

Treatment of PC12 cells with NGF for 3 and 6 days did not affect m4 gene transcription ($p > 0.05$). In contrast, the rate of new m4 mRNA synthesis was significantly increased about 2.5-fold over basal levels following 12 days of NGF treatment ($p < 0.05$). Pretreatment with 50 μM PD98059 did not inhibit induction of m4 gene transcription following 12 days of NGF treatment (2.3 ± 0.3-fold over basal level (cells treated with 50 μM PD98059 only); $n = 3$).

The increase in m4 gene transcription mediated by NGF was also apparent with either bFGF (10 ng/ml) or EGF treatment (50 ng/ml), although the degree and time course of induction were notably different among these three growth factors (Fig. 9B). Transcription of the m4 gene increased 2.2-fold over basal level at day 3 of bFGF treatment and remained in this elevated state for up to 12 days. The induction of m4 gene transcription by EGF was analogous to the effects of bFGF at the early time points, where the rate of transcription increased approximately 2-fold in PC12 cells treated with EGF for 3 and 6 days (Fig. 9B). However, the rate of transcription declined slightly to 1.6-fold over basal level following 12 days of EGF treatment, whereas this rate remained elevated at day 12 of bFGF treatment. As demonstrated for NGF induction of m4 gene transcription, 50 μM PD98059 pretreatment did not inhibit induction following 3-day treatment of either bFGF (2.2 ± 0.1-fold over cells treated with 50 μM PD98059 only; $n = 3$) or EGF (2.4 ± 0.4-fold; $n = 3$).

**DISCUSSION**

PC12 cells express functional tyrosine kinase receptors for NGF, FGF, and EGF (26). In general, stimulation of these receptors leads to tyrosine phosphorylation and activation of the signaling proteins Src-homologous collagen protein, phospholipase C-γ1, and phosphatidylinositol-3 kinase (41). Src-
homologous collagen protein recruits ancillary proteins to stimulate Ras, resulting in sequential activation of protein kinases Raf, MEK, and MAPK. The present data demonstrate that steady-state m4 mRNA levels in PC12 cells are subject to up-regulation by growth factors. NGF- and FGF-dependent up-regulation is characterized by an increase in both mRNA stability and gene transcription, while EGF treatment results only in an induction of the latter. It is also evident from this study that regulation of m4 mRNA stability and gene transcription involves different signaling pathways.

In native PC12 cells, the ability of NGF and FGF, but not EGF, to stabilize m4 mRNA (present study) parallels the ability of these growth factors to induce neurite extension (26). A striking difference between the effects of NGF and EGF is the observation that activation of MAPK is persistent with NGF stimulation but transient with EGF (33, 41). It has been postulated that growth factor-mediated differentiation of PC12 cells requires prolonged activation of the Ras/Raf/MEK/MAPK signaling cascade (33, 41). Accordingly, EGF treatment of HER-PC12 cells (PC12 cells transfected to overexpress EGF receptors 50-fold) leads to prolonged activation of MAPK and neurite extension (33). Similarly, stimulation of overexpressed insulin receptors or heterologously expressed platelet-derived growth factor receptors in PC12 cell transfectants leads to differentiation that is associated with sustained activation of MAPK (42, 43). To determine if m4 mRNA stabilization is dependent on the duration of MAPK activation, the effects of EGF were studied in HER-PC12 cells. In contrast to the effects of EGF in native PC12 cells, EGF triggered stabilization of m4 mRNA in HER-PC12 cells, implying that prolonged MAPK activity is critical for the observed post-transcriptional event. Consistent with this premise, we demonstrate that the MEK inhibitor PD98059 is capable of inhibiting m4 mRNA stabilization in both native PC12 cells treated with NGF and HER-PC12 cells treated with EGF. PD98059 has previously been shown to block MAPK phosphorylation and activation and inhibit PC12 cell differentiation by inhibiting MEK activity (34).

In HER-PC12 cells, PD98059 at concentrations that inhibited m4 mRNA stabilization inhibited EGF-induced neurite extension. Taken together, these data suggest that MAPK activity promotes stabilization of m4 mRNA. In separate studies, we have analyzed other components of the Ras/Raf/MEK/MAPK signaling pathway in the regulation of m4 mRNA. Analysis of a PC12 subline, 17.26 (44), stably overexpressing a dominant inhibitory form of p21^{ras}, reveals that NGF-mediated regulation of m4 mRNA levels is Ras-dependent.

Sustained activation of MAPK in PC12 cells is associated...

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2 R. L. Malek and N. H. Lee, unpublished observations.
with the translocation of MAPK to the nucleus (33, 43). Translocated MAPK phosphorylates transcription factors leading to the expression of late response genes for PC12 cell differentiation (45). The MAPK-dependent nature of NGF-induced m4 mRNA stabilization would be consistent with de novo synthesis of a regulatory protein factor involved in mRNA stability. Protein factor interactions with cis-acting elements of mRNA molecules are thought to underlie the regulation of mRNA stability (46). Among the most intensely studied interactions are those involving the adenine/uridine-rich binding factors and the instability-conferring adenine/uridine-rich element found on the 3'-UTR of cytokine and early response mRNAs (46). Inhibition of protein synthesis with cycloheximide has profound effects on regulated mRNA turnover. For example, induction of AU-B expression, an adenine/uridine-rich binding factor implicated in regulating cytokine mRNA degradation, is inhibited by cycloheximide in anti-CD3-stimulated T cells (47). Conversely, it is possible that inhibiting protein synthesis will not affect regulated mRNA turnover. This would imply that phosphorylation of a constitutively expressed factor provides the link between receptor activation and mRNA turnover (48). To ascertain if m4 mRNA stabilization requires de novo protein synthesis, PC12 cells were treated with cycloheximide before and after NGF treatment. When PC12 cells were pretreated with cycloheximide, NGF was unable to stabilize m4 mRNA. In contrast, cycloheximide did not affect stabilization of m4 mRNA when added after NGF treatment. These findings suggest that NGF induces de novo synthesis of a stable protein factor(s) necessary for m4 mRNA stabilization, but further experiments are necessary to confirm and extend these observations. At present, it is unclear whether NGF-induced stabilization of GAP-43 and NF-L mRNAs in PC12 cells requires induction of protein factors (35, 49).

Cis-acting elements controlling mRNA stability can be found anywhere along the length of an mRNA molecule (46). To date, the regulatory cis-acting elements of GPCR mRNAs have been localized to the 3'-UTR (3, 18, 50, 51). In a recent study, a 261-nt segment of 3'-UTR, designated the muscarinic agonist response element, has been shown to facilitate carbachol-mediated destabilization of the m1 mRNA via a protein kinase C-dependent mechanism (3, 18). The muscarinic agonist response element exhibits modest structural and sequence homology with the thyrotropin-releasing hormone response element found in the thyrotropin-releasing hormone receptor (TRH-R) 3'-UTR (3). This conservation is particularly noteworthy, since the thyrotropin-releasing hormone response element functions in a manner analogous to the muscarinic agonist response element by facilitating TRH-mediated destabilization of the TRH mRNA via protein kinase C (50). By searching for homologous sequences in noncoding regions, it may be possible to identify families of cis-acting elements involved in post-transcriptional regulation (37).

To identify candidate element(s) mediating the post-transcriptional effects of NGF, the m4 3'-UTR was compared with other NGF-stabilized mRNAs, such as the GAP-43 and NF-M (35, 49). Included in the sequence comparisons were mRNAs recently implicated as targets for NGF regulation (27). The resulting analysis has identified a conserved 137-nt segment of the m4 3'-UTR (nt 1826–1962) that exhibits 52% sequence identity with a 151-nt segment of the GAP-43 3'-UTR (nt 735–885) (Fig. 8). Such an unusually high sequence match is unexpected for two nonhomologous genes unless these 3'-UTR segments function as regulatory elements (37). This possibility is further supported by the observation that the sequence identity between the m4 and GAP-43 3'-UTRs prominently decreases to about 30% after masking the conserved regions. By comparison, the estimated sequence identity between the 3'-UTRs of homologous genes from mammals and birds in the absence of selective pressure is 30%, or approximately the same percentage of identity between unrelated sequences (37).

In PC12utr10 cells (10:1 molar ratio of 300-nt m4 3'-UTR to m4 mRNA), the presence of a heterologously transcribed 300-nt fragment of the m4 3'-UTR (nt 1741–2040), encompassing conserved nt 1826–1962, stabilizes endogenous m4 mRNA in a NGF-independent manner. Furthermore, the transcribed fragment effectively abrogates the ability of NGF to stabilize the m4 mRNA. Our findings are analogous to recent observations that deletion of the corresponding homologous region in the GAP-43 3'-UTR results in a mutant GAP-43 mRNA (missing nt 734–909) that is more stable than the wild-type transcript (40). In another study, deletion of nt 619–910 renders the mutant GAP-43 mRNA resistant to stabilization by NGF (38, 52). In both examples, the deleted nucleotides encompass a region of GAP-43 3'-UTR (nt 735–885) that has a counterpart in the m4 3'-UTR (nt 1826–1962) (Fig. 8). Interestingly, GAP-43 3'-UTR mutants missing this homologous region (nt 734–909) are no longer capable of binding to a 65-kDa factor (40). Nucleotides 734–909 represent one of at least two or three distinct cis-acting elements in the GAP-43 3'-UTR controlling GAP-43 mRNA stability (38, 40, 52). Each element is bound by a different trans-acting factor (39, 40, 53).

Based on the above findings, it seems plausible that the transcribed 300-nt fragment in PC12utr10 cells affects endogenous m4 mRNA stability by either competing with the endog-
enous mRNA for a stability-modifying trans-acting factor or disrupting mRNA secondary structure and subsequent mRNA-protein interactions. The inability of the transcribed 300-nt 3'–UTR in PC12utr1 cells (1:1 molar ratio of 300-nt m4 3'–UTR to m4 mRNA) to modify basal m4 mRNA stability or abrogate NGF-induced stabilization of the m4 mRNA provides further support for this premise. In addition, our experiments with cycloheximide provide indirect evidence of a protein factor that modifies m4 mRNA stability. We are tentatively designating conserved nt 1826–1962 of the m4 3'–UTR a putative NGF-responsive element. It should be noted that our experimental paradigm does not distinguish between competition for trans-acting factors versus disruption of secondary structure. Notwithstanding, our experimental approach has the advantage of studying m4 mRNA stability in a native PC12 cell environment. By contrast, the popular deletion mutagenesis approach would involve studying mutant m4 mRNAs heterologously expressed in non-PC12 cell lines to avoid interference with the endogenous mRNA (3). With the identification of a strong NGF-responsive element candidate (nt 1826–1962) in the m4 3'–UTR, the subject of future studies will be to identify a core sequence controlling m4 mRNA stability.

Finally, we have demonstrated a MAPK-independent facet of trophic factor regulation of m4 mRNA levels. All three trophic factors, NGF, bFGF, and EGF induced a delayed response activation of m4 gene transcription that occurred in the presence of the MEK inhibitor PD98059. These findings complement the recent characterization of the m4 mACHr promoter, which is devoid of AP-1 consensus elements, cAMP-responsive elements, and serum-responsive elements (20, 21). These three enhancer elements are found in the promoter region of many genes induced by NGF-signaling where induction is dependent on the Ras/Raf/MEK/MAPK pathway (45). The m4 mACHr promoter does contain five Sp1 binding sites (20, 21), and NGF induction of the p21 and cyclin D1 genes in PC12 cells involves the Sp1 transcription factor (54). The signaling pathways by which NGF activates Sp1 sites are not yet known. If induction of the m4 gene involves at least in part Sp1 enhancer sites, our data suggest that activation of the Sp1 transcription factor can occur independently of MAPK activity.

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