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Nerve growth factor rapidly regulates VGF gene transcription through cycloheximide sensitive and insensitive pathways

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Nerve growth factor (NGF) initiates and maintains a regulatory cascade, involving gene induction, which results in the neuronal differentiation of PC12 cells. The VGF gene encodes one of the most rapidly induced neuronal mRNAs identified in NGF-treated PC12 cells [Science, 229 (1985) 393–395; Mol. Cell Biol., 11 (1991) 2335–2349]. In this communication we show that NGF-treatment for 60–90 min maximally increases VGF gene transcription by 12- to 14-fold. VGF mRNA half-life was found to substantially decrease in PC12 cells treated with NGF for 9–25 h. Partial inhibition of VGF gene transcription and superinduction of cytoplasmic VGF mRNA levels in the presence of both NGF and cycloheximide suggests that the VGF gene may be regulated through multiple pathways, some of which can be activated in the presence of protein synthesis inhibitors as are the immediate early genes, while others require newly synthesized proteins.

Nerve growth factor; VGF; PC12 cell; Nervous system; Gene expression

1. INTRODUCTION

In the presence of NGF and basic fibroblast growth factor (bFGF) but not epidermal growth factor (EGF), PC12 pheochromocytoma cells differentiate from adrenal chromaffin-like cells into sympathetic neuron-like cells, and consequently these cells have been employed as a model system to investigate the mechanism(s) of action of neurotrophic growth factors [1]. A number of immediate early gene products have been shown to be induced rapidly in PC12 cells in response to either NGF or EGF treatment [2]. In contrast, two PC12 cell mRNAs, VGF [3] and PC3 [4] have been shown to be rapidly and more robustly regulated by NGF in comparison to other growth factors. VGF is expressed in neurons [5] in both peripheral and central nervous systems [3,5] and is released from secretory vesicles [6]. In PC12 cells, VGF mRNA levels increase at least 15- to 30-fold after 3–6 h of NGF treatment and return to basal levels after approximately 48 h of NGF treatment [7,8], while EGF treatment or depolarization result in only 3- to 5-fold inductions of VGF mRNA levels [3,8]. In this report we have examined the mechanisms by which VGF mRNA levels are regulated in PC12 cells.

2. MATERIALS AND METHODS

2.1. *Cell culture*

PC12 cells were grown on collagen-coated dishes as described [1]. Where specified, cultures were treated with 2.5S NGF (B.M), EGF (Collaborative), or recombinant bovine bFGF (B.M) and cells were pretreated for 20 min with either 2.5 μg/ml Act-D (CalBiochem) or 20–100 μg/ml cycloheximide (Sigma) prior to the addition of NGF.

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2.2. *RNA analysis*

PC12 cells were rinsed three times with ice-cold phosphate-buffered saline, lysed in 10 mM Tris (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$ and 0.5% Nonidet P-40, and nuclei and cytosol were fractionated as described [2]. Cytoplasmic RNA [9] and total cellular RNA [10] were isolated, and RNase protection analysis was performed [9] using an antisense VGF exon 3-specific probe [3] to quantify cytoplasmic VGF mRNA levels. Protected RNA fragments were resolved on non-denaturing 5% polyacrylamide gels and after autoradiographic exposure, the bands were excised and quantified by scintillation counting and comparison to standard curves, as previously described [2,9].

2.3. *Run-on transcription analysis*

Nuclei were isolated as described above and labeling of nascent transcripts was carried out in the presence of [^{32}P]UTP by modification of previous protocols [2,9]. Isolated labeled RNA was resuspended in 30 mM Tris (pH 7.4), 0.2% SDS, 5 × Denhardt's solution, 100 μg/ml yeast tRNA and 20 μg/ml proteinase K. ^{32}P-labeled RNA samples were counted and equal numbers of counts of each sample were hybridized to nitrocellulose filters in a final volume of 200 μl of 30 mM Tris (pH 7.4), 0.2% SDS, 0.5 M NaCl, 5 × Denhardt's solution, 100 μg/ml yeast tRNA , 20 μg/ml proteinase K at 65°C for 48–72 h. Nitrocellulose filters bound with either 1 μg of single-stranded antisense VGF-M13mp18 containing ~2.5 kb of VGF coding sequence, 1 μg M13mp18, 5 μg *c-fos*-SP65, or 5 μg β-actin-SP65 were prehybridized for 4 h and hybridized in duplicate. Hybridization efficiency was determined by adding trace amounts of ^{3}H-labeled VGF sense RNA to the hybridization mix [9]. Filters were washed as described [2], the counts per minute (cpm) bound to the M13mp18 filters were subtracted from the specific filters, and the results expressed either as parts-per-million of input radioactivity after correction to 100% binding efficiency, or as fold-induction [9].

2.4. *Estimation of VGF mRNA half-life*

Study of mRNA stability was performed essentially as described [11]. PC12 cells (0.5–2 × 10^6 per 35-mm dish) were incubated for 3 h

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in the presence of 50 ng/ml NGF and 1 mCi/ml [^{3}H]uridine. Cultures were rinsed 6 times with serum-free RPMI, supplemented with NGF if the cells were to be chased in the presence of NGF, and then the cultures were incubated with complete medium containing 5 mM uridine and 5 mM cytidine, either in the presence or absence of NGF. Cytoplasmic RNA was prepared, and prehybridization, hybridization, and washing were carried out as described above. Cell growth and/or cell loss was corrected for by determining the DNA concentration per sample [12] as previously described [11] and normalizing all cpm to the unchased sample. DNA values were found to vary from the unchased samples by no more than 10%.

3. RESULTS AND DISCUSSION

3.1. *NGF, bFGF, EGF and depolarization induce VGF gene transcription*

In Fig. 1, panel A, VGF mRNA transcription was measured in untreated PC12 cells, and cells treated with NGF for the indicated times. A maximal 12- to 14-fold increase in VGF transcription was observed after 60–90 min of NGF treatment, in comparison to 3- to 4-fold increases in depolarized PC12 cells or those treated with 10 ng/ml EGF. For comparison, c-*fos* and β-actin transcription rates were found to increase 94- and 10-fold, respectively, after 30 min of NGF treatment (data not shown). We then examined the effect of varying doses of NGF (5 and 50 ng/ml), EGF (2–20 ng/ml) and bFGF (1–100 ng/ml) on VGF transcription rates following treatment for 60 min (Fig. 1, panel B), and on cytoplasmic mRNA levels following treatment for 3 or 6 h (Fig. 1, panel C). 50 ng/ml NGF was found to give 12- to 13-fold stimulation of VGF transcription in comparison to maximal 5- to 6-fold and 6- to 7-fold stimulations in the presence of 5 ng/ml EGF and 100 ng/ml bFGF, respectively. Treatment with the protein synthesis inhibitor CHX (100 μg/ml) for 60 min reduced basal transcription by ~34%, while treatment with CHX + NGF for 60 min reduced the induction in VGF gene transcription to ~5-fold (Fig. 1, panel B) in comparison to c-Fos gene transcription which remained elevated ~100-fold above basal levels (data not shown). The order of efficacy in inducing VGF cytoplasmic mRNA levels, quantified by RNase protection analysis [3] (Figure 1, panel C), and VGF gene transcription rates was found to be NGF > bFGF > EGF.

3.2. *Effect of cycloheximide and actinomycin D treatment on basal and induced levels of VGF mRNA in PC12 cels*

The contribution of newly synthesized proteins to the regulation of VGF mRNA levels was further assessed by RNase protection assay. Cellular protein synthesis was blocked with CHX (20 μg and 100 μg per ml), resulting in decreases of 97% and 99%, respectively, in the incorporation of [^{3}H]leucine into TCA-precipitable counts. Treatment of cultures for 1 h with NGF + 20 μg/ml CHX in comparison to NGF decreased VGF mRNA induction from 6-fold to 4-fold (Fig. 2, panel

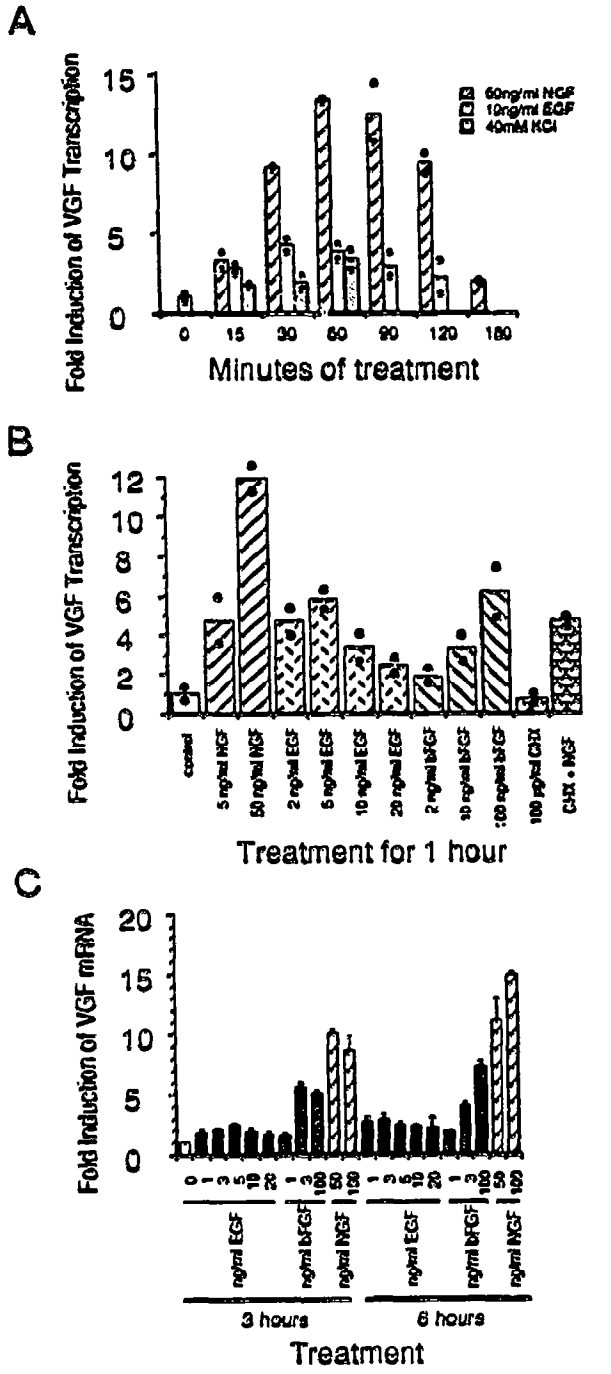

Fig. 1. Run-on analysis of VGF gene transcription. In panel A, VGF transcription, measured in untreated PC12 cells and those incubated with 50 ng/ml NGF, 10 ng/ml EGF and 40 mM KCl, has been expressed as fold-induction with basal transcription defined as 1. Rates of VGF gene transcription ranged from 80–100 ppm in untreated PC12 cells and from 840–1300 ppm in PC12 cells treated for 60–90 min with NGF. Each bar is the average of duplicate filters, represented by the open circles. In panel B, the effect of NGF, EGF, bFGF and CHX on VGF gene transcription in PC12 cells treated for 1 h was measured. Values of VGF gene transcription in control PC12 cells ranged from 78–128 ppm compared to 1098–1211 ppm in PC12 cells treated with NGF for 60 min. VGF cytoplasmic mRNA levels were determined by RNase protection analysis (panel C). Assays were run in triplicate, and the results expressed as fold-induction of VGF mRNA with control defined as 1. The bars represent the mean ± S.E.M.

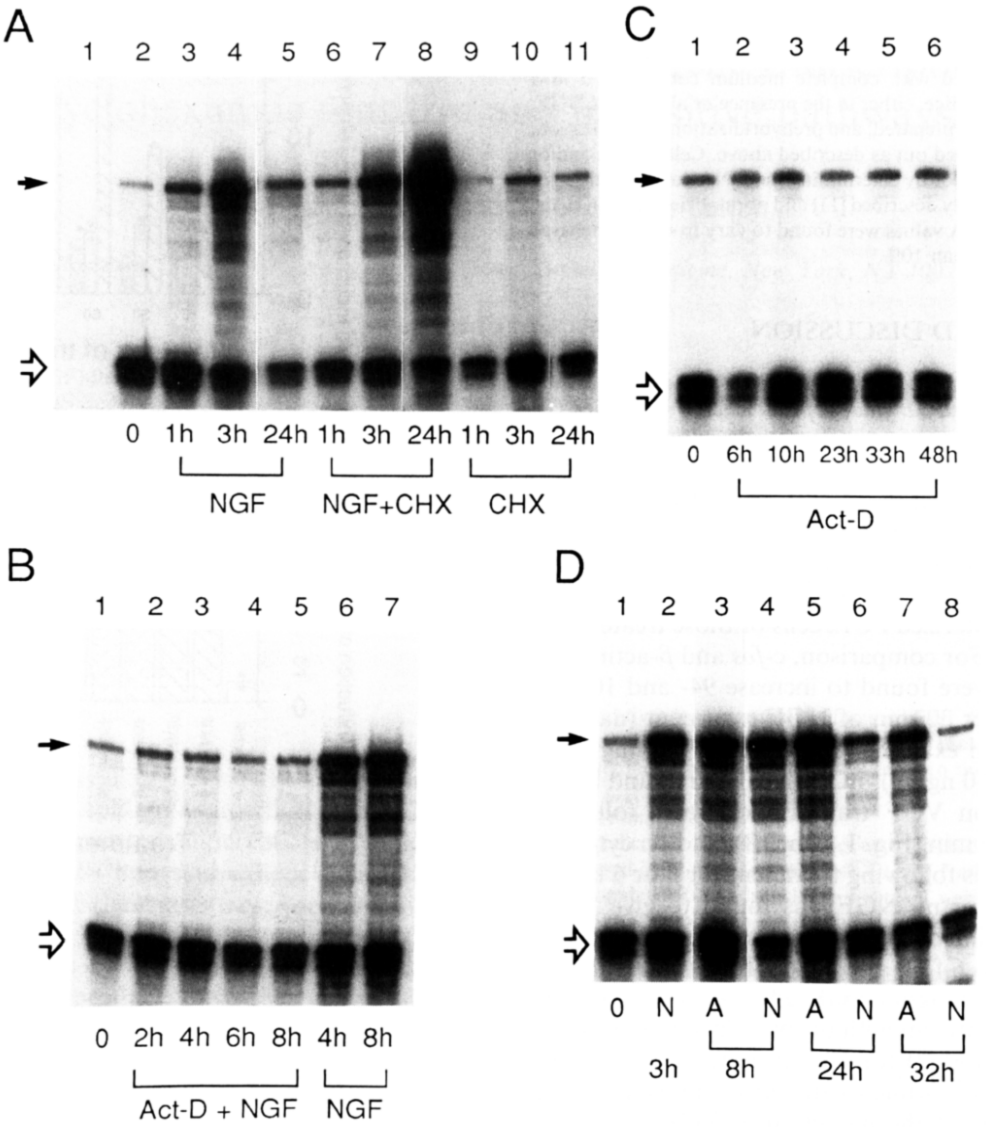

Fig. 2. Effects of cycloheximide and actinomycin-D treatment on VGF mRNA levels. RNase protection assays in panels A–D were carried out in triplicate on 10 μg samples of total RNA using VGF and cyclophilin antisense probes, and the protected bands, indicated by the solid (VGF) and open (cyclophilin) arrows, were resolved on non-denaturing polyacrylamide gels, visualized by autoradiography, and quantified by scintillation counting. In panel A, cytoplasmic RNA was isolated from untreated PC12 cells (lane 2) and those treated with NGF (lanes 3–5), NGF + 20 μg/ml cycloheximide (CHX) (lanes 6–8), or 20 μg/ml CHX (lanes 9–11) for 1, 3 and 24 h. As a control, no RNA was added to the hybridization analyzed in lane 1. In panel B, cytoplasmic RNA was isolated from untreated PC12 cells (lane 1), PC12 cells treated with NGF for 1 h (lane 2) or 3 h (lane 3), and from PC12 cells pre-treated for 20 min with 2.5 μg/ml actino D prior to the addition of NGF for 1 h (lane 4) or 3 h (lane 5). In panel C, cytoplasmic RNA was isolated from untreated PC12 cells (lane 1), and those treated with 2.5 μg/ml Act-D for 6–48 h (lanes 2–6). In panel D, total RNA was isolated from untreated PC12 cells (lane 1), and those treated with NGF for 3, 8, 24 and 32 h (lanes 2, 4, 6 and 8, respectively, labeled 'N'). In lanes 3, 5 and 7, PC12 cells were treated with NGF for 3 h, and then 2.5 μg/ml actino D was added and the cultures harvested 8, 24 and 32 h (lanes 3, 5 and 7, respectively, labeled 'A') after the initiation of treatment with NGF.

A). VGF mRNA levels in cells treated with either NGF or NGF + CHX for 3 h were very similar, but after 6 h (not shown), treatment with NGF + CHX resulted in a 30-fold induction in VGF mRNA levels in comparison to 15-fold for NGF alone. Levels of VGF mRNA in PC12 cells treated for 24 h with CHX + NGF were induced 50- to 70-fold above control levels, compared to a 6-fold induction in PC12 cells treated with NGF alone. A similar apparent increase in mRNA stability in the presence of CHX has been seen for the mRNA products of a number of immediate early genes [13]. Identical results were obtained using cultures treated with NGF + 100 μg/ml CHX (not shown). During the preparation of this communication, Possenti et al. [14] reported that NGF-treatment for 2 h resulted in an 11- to 12-fold increase in VGF gene transcription, and that treatment with CHX + NGF for 2 h substantially decreased the induction of VGF mRNA levels measured

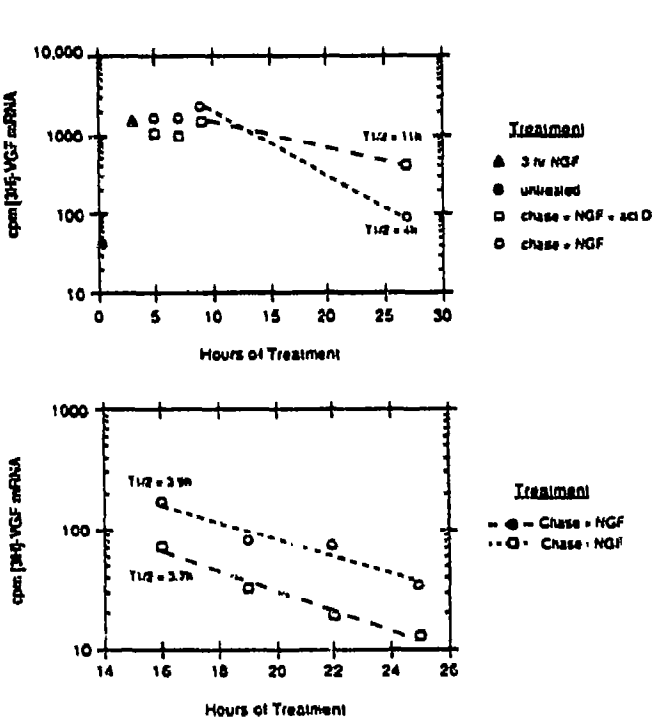

Fig. 3. Estimation of VGF mRNA half-life by [^{3}H]uridine pulse/chase analysis-PC12 cells were labeled for 3 h in complete media supplemented with 1 mCi/ml [^{3}H]uridine. In panel A, total cytoplasmic [^{3}H]RNA was isolated from naive PC12 cells (10^6 cells per dish) (solid circle), PC12 cells treated with NGF for 3 h (solid triangle), and PC12 cells treated with NGF for 3 h and chased with 5 mM uridine and cytidine for an additional 2, 4, 6 and 24 h in either the presence (open squares) or absence (open circles) of 2.5 μg/ml Act-D. PC12 cell [^{3}H]RNA was hybridized to both VGF-M13 and M13 DNAs immobilized on filters, and the results are presented as cpm [^{3}H]VGF mRNA, determined as the difference in [^{3}H]RNA bound to VGF and control M13 filters. Decay curves were approximated by assuming that VGF mRNA levels decreased logarithmically between 9 and 27 h after the initiation of labeling. VGF mRNA half-life ($T_{1/2}$) was calculated to be 4 h in the presence of NGF and 11 h in the presence of NGF + Act-D. In panel B, total [^{3}H]RNA was isolated from PC12 cells labeled with 1 mCi/ml [^{3}H]uridine for 3 h in complete media containing 50 ng/ml NGF. One group of replicate plates (~5×10^5 cells per dish) was rinsed 6 times in serum-free media containing 50 ng/ml NGF and was chased with complete media containing NGF, 5 mM uridine and 5 mM cytidine for 13–22 h (open circles). The other group was rinsed 6 times in serum-free media and was chased in complete media containing 5 mM uridine and 5 mM cytidine (open squares). [^{3}H]RNA cpms per hybridization were 25–50% of the hybridizations in panel A. The results are the average of duplicate filters. Decay curves were fitted with R values of 0.97 (open circles) and 0.98 (open squares) with $T_{1/2}$=3.9 h (+NGF) and 3.7 h (−NGF).

in comparison to cells treated with NGF alone. Our data suggest that the CHX-resistant pathways that regulate VGF gene transcription play a significant role in VGF mRNA regulation, as demonstrated by the transcriptional induction of the VGF gene and the superinduction in VGF mRNA levels detected in the presence of NGF + CHX. As expected, induction of VGF mRNA levels by NGF could be completely inhibited if PC12 cells were simultaneously treated with NGF and the RNA polymerase inhibitor actinomycin D (Act-D) (Fig. 2, panel B). Estimates of mRNA half-lives have been obtained previously by blocking new mRNA synthesis using Act-D, and measuring the levels of specific mRNAs after increasing lengths of Act-D treatment [15]. Measurement of the level of VGF mRNA in naive PC12 cells treated with Act-D for 0–48 h is shown in Fig. 2, panel C. In these cells, VGF mRNA appeared to turn over very slowly, with a half-life of >48 h. This estimate of half-life was obtained by determining the absolute levels of VGF mRNA using RNase protection analysis, and plotting these values on a logarithmic scale against treatment time (as in the pulse chase analysis in Fig. 3). Note that the half-life of VGF mRNA was found to be similar in naive PC12 cells (Fig. 2, panel C) and cells simultaneously treated with Act-D + NGF (Fig. 2, panel B). Since NGF treatment of PC12 cells leads to a rapid, transient increase in VGF gene expression, and the transcription rate returns toward relatively low basal levels after ~3 h of treatment (see Fig. 1), we measured the decrease in VGF mRNA levels in cells treated for 8–32 h with NGF as an approximation of the half-life. Cultures were treated with NGF for 3 h, after which Act-D was added to half of the cultures and NGF treatment alone was continued in the other half. Total RNAs were harvested after 8, 24 and 32 h of treatment, and VGF mRNA levels were determined by RNase protection assay (Fig. 2, panel D). Note that VGF mRNA levels remained elevated after 24 and 32 h of treatment with NGF + Act-D (Fig. 2, panel D, lanes 5 and 7) compared to treatment with NGF alone (Fig. 2, panel D, lanes 6 and 8). The VGF mRNA half-life, estimated from the analysis in Fig. 2, panel D, was found to be ~6–7 h in NGF-treated PC12 cells, and ~11–12 h in PC12 cells treated with both NGF and Act-D, compared to >48 h in naive PC12 cells treated with Act-D alone, suggesting that NGF treatment decreases rather than increases VGF mRNA stability. Since VGF half-life is the same in cells treated with Act-D prior to NGF, or with Act-D alone, it appears that the NGF-induced decrease in VGF mRNA stability requires new RNA synthesis. It is possible that the degradative enzymes which recognize the consensus AUUUA destabilization sequence [16] in the 3'-untranslated region of rat VGF mRNA [8] may be involved in VGF mRNA turnover and may themselves be rapidly induced by NGF.

3.3. *Estimation of VGF mRNA half-life by pulse/chase analysis*

Since Act-D treatment of PC12 cells increased the estimated half-life of VGF mRNA in comparison to untreated cultures, a more accurate estimate of the half-life of VGF mRNA in NGF-treated PC12 cells was obtained using [^{3}H]uridine pulse/chase analysis. [^{3}H]uridine incorporation into VGF mRNA in un-

treated PC12 cells was very low (Fig. 3, panel A), most likely as a result of a low basal rate of VGF transcription (see Fig. 1) and relatively low levels of VGF mRNA in naive PC12 cells. Treatment of PC12 cells with NGF for 3 h led to a substantial increase in [^{3}H]uridine incorporation into VGF cytoplasmic mRNA (Fig. 3, panel A). Labeled cells were then rinsed extensively with RPMI, and incubated in complete medium containing NGF and excess unlabeled uridine and cytidine. Levels of ^{3}H-labeled cytoplasmic VGF mRNA remained elevated during the first 6 h of the chase (Fig. 3, panel A), most likely because the intracellular pool of [^{3}H]UTP takes several h to decrease in size, as has previously been discussed [11]. After a 24 h chase, the level of ^{3}H-labeled VGF mRNA was found to have substantially decreased (Fig. 3, panel A). If the level of VGF mRNA decreased by first order kinetics between 6 and 24 h of the chase period, an mRNA half-life of 4 h would be estimated. Addition of Act-D to the chase appeared to slightly reduce the level of ^{3}H-labeled VGF cytoplasmic mRNA and to prolong the mRNA half-life to 10 h (Fig. 3, panel A), a value which is very similar to that derived from the analysis of Fig. 2, panel D. To further examine the effect of NGF on VGF mRNA stability, VGF mRNA half-life was determined in PC12 cells labeled with [^{3}H]uridine for 3 h in the presence of NGF, and chased for 13–22 h in either the presence or absence of NGF (Fig. 3, panel B). The protocol employed here to wash out NGF has been shown previously to remove sufficient NGF to allow reinduction of both VGF [3] and immediate early genes [2] in fully differentiated neuronal PC12 cells that are deprived of and re-exposed to NGF. Parallel decay curves were obtained regardless of whether PC12 cells were chased in the presence or absence of NGF, and the half-life of VGF mRNA in PC12 cells labeled with [^{3}H]uridine in the presence of NGF was estimated to be ~4 h. Therefore, exposure of PC12 cells to NGF for 3–28 h did not increase VGF mRNA stability, but rather markedly reduced VGF mRNA half-life to ~4 h. We conclude that NGF rapidly induces VGF gene expression primarily by stimulating VGF gene transcription, through both CHX-sensitive and CHX-resistant regulatory pathways. Consequently, transcriptional induction of the immediate early and VGF genes by NGF may result from the activation of both shared and unique signal transduction pathways.

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