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Transcriptional and Post-transcriptional Effects of Nerve Growth Factor on Expression of the Three Neurofilament Subunits in PC-12 Cells*

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Nerve growth factor (NGF) is known to increase the levels of neurofilament proteins in PC-12 pheochromocytoma cells. In this report, we show that the three neurofilament subunits, NF-L, NF-M, and NF-H, are not induced coordinately. NF-H accumulated only after longer term NGF treatment than required for NF-L and NF-M. While NGF treatment resulted in 12- and 14-fold increases in NF-L and NF-M mRNA levels, respectively, over a 14-day period, no increase in the level of NF-H mRNA was observed. This indicated that in PC-12 cells, control of NF-H expression by NGF may occur at the post-transcriptional level. NGF appeared to have no effect on the stability of NF-L mRNA, although it increased the stability of NF-M mRNA relative to that in control PC-12 cells. Analysis of the effect of NGF on the transcription of neurofilament genes showed 4- and 5-fold increases in the rates of NF-L and NF-M gene transcription, respectively, and no increase in the rate of NF-H gene transcription. Taken together these results demonstrate that NGF stimulates the expression of individual neurofilament subunits at the transcriptional and/or post-transcriptional levels.

Neurofilaments (NF)[1] are the intermediate filament subclass expressed specifically in neurons. Mammalian NFs are composed of three subunits having apparent molecular masses by SDS-PAGE of 70 kDa (NF-L), 150 kDa (NF-M), and 200 kDa (NF-H) (1). The NF subunits are encoded by unique genes (2–8), with mice having mRNA transcripts of 2.5 and 4.0 kb for NF-L, 3.0 kb for NF-M, and 4.5 kb for NF-H mRNA (5). It is thought that the 4.0-kb NF-L mRNA species arises from the read-through of a tandem polyadenylation signal in the NF-L gene (6) as has been demonstrated to occur during vimentin gene transcription (9).

While other intermediate filament subclasses share similar gene structures, indicative of a common ancestor (10–14), they show little structural homology with NF genes (2, 4, 5, 7, 8). It appears that NF genes diverged at an early stage from the rest of the intermediate filament gene family (2, 4, 5, 7, 8). NF genes do share one important characteristic with other intermediate filament genes, namely, their tissue-specific expression during development (for reviews see Refs. 15, 16). NF proteins can be detected in cells with an identifiably neuronal phenotype at early stages of embryogenesis (17, 18). At the level of NF gene transcription, mRNA for NF-L and NF-M subunits can be detected in developing mouse brains as early as 11 days of gestation (5). NF-H mRNA, on the other hand, was detectable at significant levels only postnatally (5). Postnatal onset of NF-H expression, at the protein level, has also been demonstrated (19–21). However, these results remain controversial in light of some evidence for prenatal expression of NF-H in central nervous system and peripheral nervous system tissue (18). These discrepancies may be due to differences in methodology, for example the use of antibodies that distinguish between fully phosphorylated and hypophosphorylated variants of NF-H (22–24), or else they may represent variations in the pattern of NF expression in different types of neurons within the brain. A better understanding of how the expression of NF genes is regulated might allow for a reconciliation of these disparate observations.

The PC-12 cell line, which is derived from a rat pheochromocytoma, provides a relatively simple, homogeneous system for studying various aspects of neuronal differentiation (25–27). When cultured in growth medium supplemented with nerve growth factor (NGF), these cells acquire a phenotype resembling that of peripheral neurons (27), while cells maintained in medium lacking NGF remain undifferentiated. Previous studies have demonstrated that PC-12 cells express the NF triplet (28–31, 52) and that treatment of these cells with NGF stimulates the biosynthesis and phosphorylation of NF subunits, as well as increasing the metabolic stability of the subunits (28, 29, 31). NGF-treated PC-12 cells express relatively large amounts of NF-L and NF-M but low levels of NF-H (29–31) and appear to represent an interesting model system for studying the regulation of differential NF gene expression. The present communication examines the effect of NGF treatment on NF subunit expression in PC-12 cells, both at the protein and transcriptional levels. The results show that NGF increases both the transcriptional activity

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[1] The abbreviations used are: NF, neurofilament; ACT-D, actinomycin D; kb, kilobase; kDa, kilodalton; NGF, nerve growth factor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; NF-L, NF-M, and NF-H, low (68 kDa), middle (145 kDa), and high (200 kDa) molecular weight neurofilament subunits, respectively; S.S.C., standard saline-citrate solution.

and mRNA levels for NF-L and NF-M, but not for NF-H. Apparently, the genes coding for the three NF subunits are not expressed coordinately.

MATERIALS AND METHODS

PC-12 Cell Culture—PC-12 cells were obtained from Dr. L. A. Greene (New York University Medical School) and were maintained according to published procedures (27) as described previously (31). Cells were harvested by trypsinization, washed free of trypsin, and plated at densities of 5×10^6 cells/150-mm dish. Cultures were maintained in either complete medium consisting of 85% Dulbecco's modified Eagle's medium, 10% heat-inactivated horse serum (Gibco), 5% fetal bovine serum (Gibco), and antibiotics, or complete medium plus 150 ng/ml 7S NGF (Dr. R. Stach, State University of New York, Syracuse). In some experiments actinomycin D (ACTD, Boehringer Mannheim, Canada) was added to the medium at 5 μg/ml final concentration for periods up to 25 h.

Immunoblot Analysis of NF Proteins from PC-12 Cell Cytoskeletons—PC-12 cells were maintained in complete medium or complete medium plus NGF for up to 14 days. At 1, 4, 8, and 14 days, cells were harvested and cytoskeletons extracted and solubilized as described (31). Protein concentrations were determined by a dye-binding assay (32) and equal amounts of protein (5 μg) from the solubilized cytoskeletons were fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (33) using an 8% separating gel. The resolved proteins were then transferred electrophoretically (34) to a nitrocellulose membrane (0.1 μM pore size; pH 79, Schleicher & Schuell) for 3 h at 1 A and 25 V. The resultant nitrocellulose membrane blot was probed with a mixture of affinity-purified antibodies against the three NF subunits and antibody-binding bands were visualized by ^{125}I-protein A binding (Du Pont-New England Nuclear, Canada, 10 μCi/μg) following autoradiography after exposure for 1–4 days to XAR 5 x-ray film (Eastman Kodak) as previously described (35).

Northern Blot Analysis of NF mRNA from PC-12 Cells—PC-12 cells were grown either in complete medium or for 1, 4, or 14 days in NGF-containing medium. At the appropriate times, plates were washed 3 times with ice-cold phosphate-buffered saline, cells harvested by scraping with a rubber policeman, and total RNA extracted and purified in 6 M guanidine hydrochloride as previously described (36). The mRNA content of each sample was estimated by use of a [^{3}H]poly(U) binding assay (37). RNA samples, containing 400 ng of poly(A)$^+$ RNA, were fractionated by electrophoresis on a 1% formaldehyde-agarose gel (38) and were transferred to a nylon membrane (Biotrans; ICN, Canada) by capillary transfer according to manufacturer's instructions. The Northern blots were probed with a 0.3-kb mouse cDNA to NF-L (5), a 0.6-kb mouse cDNA to NF-M (5), a 1.2-kb fragment of the mouse NF-H gene (5), or a 1.7-kb mouse cDNA to NF-H. Probes were labeled by the random primer labeling technique (39, 40) to specific activities of $\sim 10^8$ cpm/mg. Blots were hybridized by incubation at 65 °C for 2 h in a solution consisting of 1 M NaCl, 1% SDS, 10% dextran sulfate (P-L Pharmacia, Canada), 100 μg/ml sheared, denatured salmon sperm DNA and the appropriate denatured probe. Washes were performed to stringencies of 0.1 × SSC at 65 °C, except when blots were hybridized with the genomic NF-H probe which were washed to stringencies of 0.5 × SSC at 65 °C. The damp blots were wrapped in clear plastic wrap and exposed at −70 °C to x-ray film (note above) with intensifying screens (Cronex Lightning Plus; Du Pont) (41) for up to 7 days. Blots were subsequently deprobed prior to the next round of hybridization, by incubation for 30 min at 65 °C in a solution consisting of 96% deionized formamide, 10 mM Tris-HCl, pH 7.5, and 10 mM EDTA, followed by a brief rinse with 1 × SSC.

Nuclear Run-off Assays—Nuclei from cells (1×10^7) maintained in control medium or in NGF-supplemented medium for 8 days were isolated and nuclear run-off transcription assays were performed as previously described (42). Linearized plasmid DNAs containing appropriate inserts were denatured by incubating in 0.3 M NaOH, at 70 °C for 60 min. The denatured DNA was neutralized by addition of ammonium acetate to 1 M and was bound to the nitrocellulose membrane using a slot-blot filtration manifold (Bethesda Research Laboratories-GIBCO). The wells of the apparatus were washed 3 times with 1 M NH_4OAc then the membrane was removed, dried, and baked *in vacuo* at 80 °C for 2 h. Hybridizations, using 10^7 cpm/ml of input RNA, as well as subsequent washing steps were performed as previously described (42). The plasmids used in this study include a 2.7-kb cDNA insert to mouse NF-L (5), a 0.6-kb cDNA to mouse NF-M (5), and a 1.7-kb cDNA to mouse NF-H, all in pUC 8 as well as a cDNA to actin in pBR322 (pam91, Ref. 44). Controls consisted of pUC 18, and pBR322 DNA, without inserts.

Northern Blot Analysis of mRNA from Actinomycin D-treated PC-12 Cells—PC-12 cells were grown in complete medium or medium supplemented with NGF for 5–6 days. At that time ACTD at a final concentration of 5 μg/ml was added to the medium. At 0, 5, 10, 15, and 25 h after ACTD administration RNA was isolated from the cells and subjected to Northern analysis. Hybridizations with cDNA probes to each NF subunit were performed sequentially on the same Northern blot as described above. Hybridization signals on autoradiographs were quantified using a laser beam densitometer (LKB).

RESULTS

The effect of NGF on the levels of NF proteins in the cytoskeletal fraction of PC-12 cells was examined by Western blot analysis. The results in Fig. 1 show a dramatic increase in the levels of NF-L and NF-M in PC-12 cells after 4 days of treatment with NGF. Although NF-L and NF-M are only barely visible in the control extract, there is a basal level of immunoreacting material in cells grown in the absence of NGF (see for example Ref. 31). While the increase in NF-L and NF-M levels occurred simultaneously, the level of NF-H increased much more slowly. The latter subunit was barely detectable after 4 days of exposure to NGF but showed a steady increase thereafter. By 14 days of NGF treatment, NF-H immunoreactivity reached significant levels. The NF-H band appeared to be somewhat diffuse, likely reflecting the presence of NF-H in a variety of phosphorylation states (31, 45) since the polyclonal antibodies used in this study recognized both fully phosphorylated and hypophosphorylated variants of the NF subunits (31).

In light of the magnitude of the NGF effect on the levels of NF subunits in PC-12 cells, we determined whether NGF also affected the levels of NF mRNA. The results of Northern analyses are shown in Fig. 2. In agreement with observations by others (46), Fig. 2*A* shows an NGF-stimulated increase in

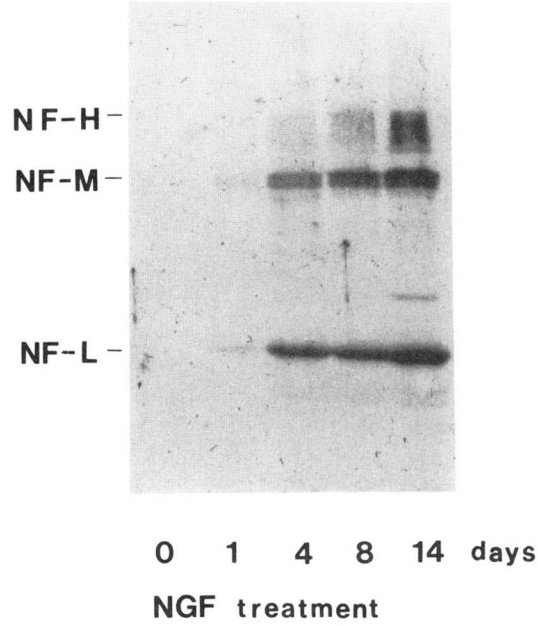

FIG. 1. **Western blot analysis of PC-12 cytoskeleton-associated NF proteins in PC-12 cells.** Cytoskeleton proteins of control PC-12 cells (0 day) or PC-12 cells grown in NGF-containing medium for 1, 4, 8, or 14 days were resolved by SDS-PAGE and transferred to a nitrocellulose filter. The resultant Western blot was probed with a mixture of antibodies specific for each of the NF triplet proteins. Antibody-antigen complexes were visualized by incubation with ^{125}I-protein A and subsequent autoradiography (4-day exposure). Positions of rat brain NF proteins used as standards (*NF-L*, *NF-M*, and *NF-H*) are indicated at the left.

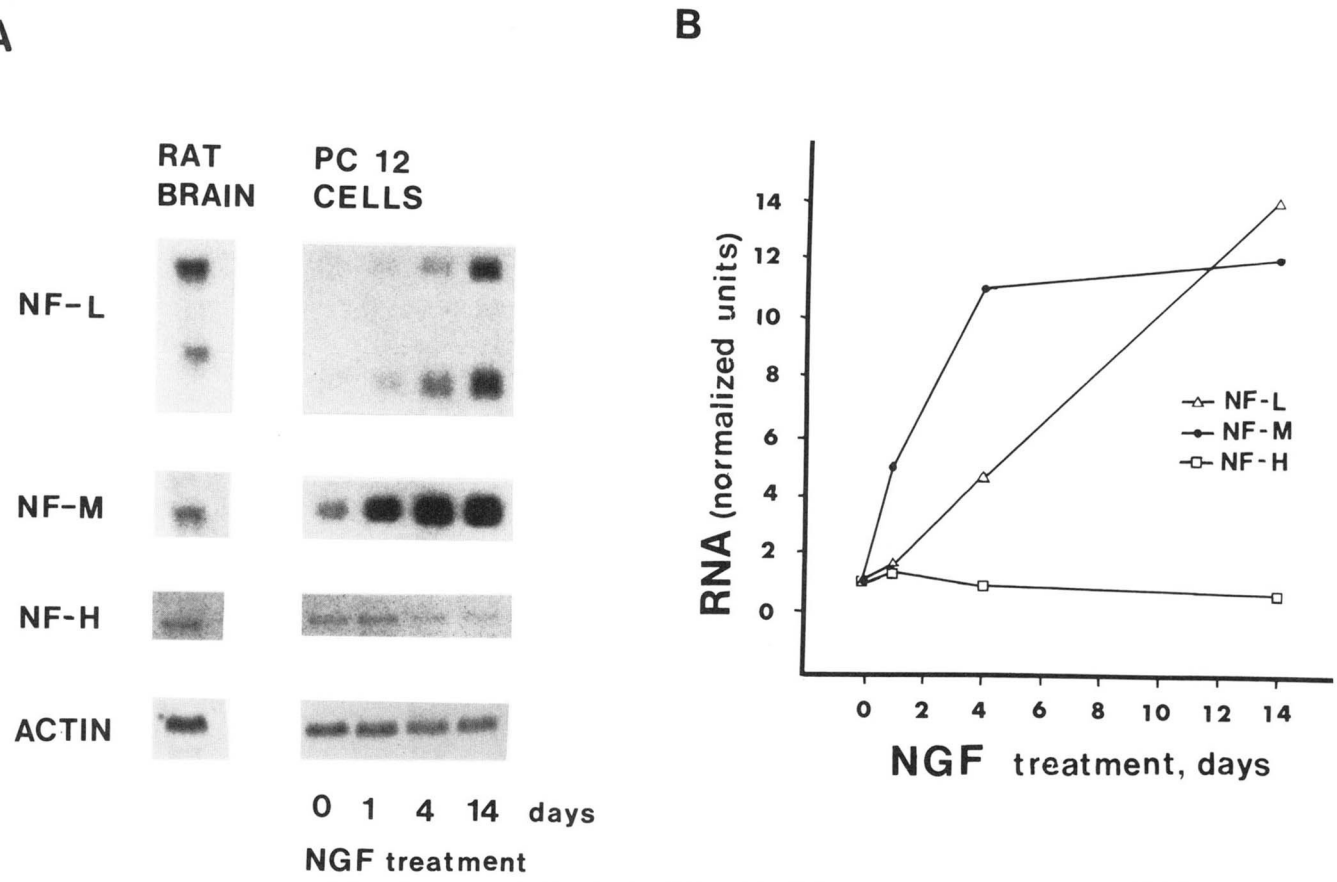

FIG. 2. **Northern blot analysis of NF subunit mRNA levels in PC-12 cells.** *A*, RNA (400 ng of mRNA) from control PC-12 cells (0 day) or cells maintained for 1, 4, or 14 days in NGF, as well as total RNA (20 μg) from 15-day-old rat brain, were fractionated on a formaldehyde-agarose gel and transferred to a nylon membrane. The resultant Northern blot was successively hybridized with ^{32}P-labeled probes to mouse NF-L, NF-M, NF-H, and actin. Filters were exposed to x-ray film for 1–6 days. *B*, relative changes in NF subunit mRNA levels with time of exposure to NGF were quantified by laser densitometry and normalized with respect to actin mRNA levels, which remained almost constant over the 14-day period. NF-L mRNA levels represent the sum of both mRNA variants.

the level of NF-L mRNA. Similarly, NGF treatment also resulted in increased levels of NF-M mRNA. However, NF-H mRNA levels did not increase in response to NGF-treatment. The NGF effects were quantified by densitometry (Fig. 2*B*). After 14 days of treatment with NGF, total NF-L mRNA levels (*i.e.* the sum of both the 2.5- and 4.0-kb species) had increased 14-fold and NF-M mRNA 12-fold over control levels. The time course of NF-L and NF-M mRNA induction differed in that while NF-L mRNA levels increased almost linearly over 14 days, NF-M mRNA increased much more rapidly so that by 4 days of NGF treatment almost 90% of the maximal level had been reached. The level of NF-H mRNA did not increase and may have declined slightly after 14 days of NGF treatment. These results indicated that NGF had a different effect on the expression of NF-H as compared to the two smaller subunits. Both mRNA and protein levels increased in parallel with NGF treatment with NF-L and NF-M whereas the increase in NF-H was not accompanied by an increase in the corresponding mRNA.

To better assess the effect that NGF exerts on the levels of various NF mRNAs in PC-12 cells, we made use of the nuclear run-off assay to measure the transcriptional activity of the NF genes in control and NGF-treated cells. The results in Fig. 3 show that NGF treatment resulted in increased transcription of the NF-L and NF-M genes but little or no increase in the transcription rate of NF-H. Densitometry of the autoradiograph showed approximately 4- and 5-fold increases in NF-L and NF-M gene expression, respectively (data not shown). Actin gene transcription represented a control for a gene that does not respond greatly to NGF treatment (47). As can be seen in Fig. 3, actin gene transcription was increased only slightly, about 1.5–2 times over control levels in response to NGF. The specificity of the assay was tested by checking for spurious hybridization to plasmid vectors. As can be seen in Fig. 3, no such hybridization could be detected.

To examine the question of whether NGF increases steady state levels of NF-L and NF-M mRNA by also increasing the stability of these transcripts, PC-12 cells grown in control or NGF-supplemented medium were exposed to ACTD, a potent inhibitor of mRNA synthesis. The RNA from cells maintained for various times up to 25 h in the presence of ACTD was subjected to Northern blot analysis and the results are shown in Fig. 4. For the purpose of the analysis, total NF-L mRNA levels were taken to be the sum of both NF-L mRNA species (2.5 and 4.0 kb). Despite the differences in the absolute amounts of NF-L mRNA between PC-12 cells treated with NGF and control cells (Fig. 4*A*), densitometric analysis (Fig. 4*B*) showed little if any difference in the relative half-lives of total NF-L mRNA. In contrast to total NF-L mRNA, the half-life of NF-M mRNA in PC-12 cells was increased significantly by NGF treatment (Fig. 4*C*), from about 10 h in control cells to 17 h in NGF-treated cells. The faint signal

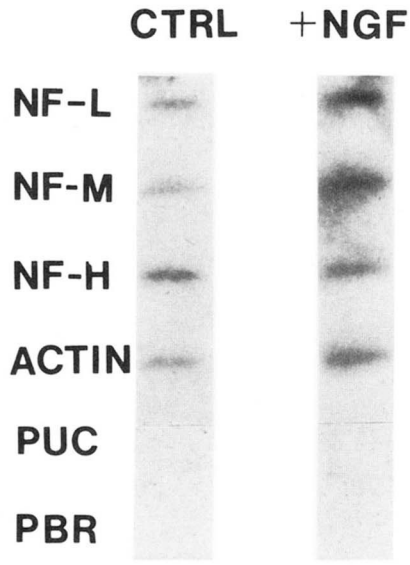

FIG. 3. **Analysis of NF subunit gene transcription in control or NGF-treated PC-12 cells.** Plasmid DNAs containing cDNA inserts for each of the NF genes or actin, as well as pUC18 and pBR322 DNA were bound to nitrocellulose filters and hybridized to ^{32}P-labeled mRNA run-off transcripts (1×10^7 cpm/ml) isolated from nuclei of control cells or cells maintained in NGF as described under "Materials and Methods." Filters were exposed to x-ray film for 2 days.

observed for NF-H mRNA in either control or NGF-treated PC-12 cells (Fig. 4*A*) precluded densitometric analysis, but there appeared to be little difference in its relative stability. Since all hybridizations were performed on the same Northern blot, the effect of NGF on the stability of NF-M mRNA appeared specific and limited to the mRNA for this subunit only. Thus, it appears that NGF acts to increase NF-L mRNA levels in PC-12 cells primarily by increasing the transcription rate of the NF-L gene. In the case of NF-M mRNA the increased steady state levels appear to be due to increases both in the transcription rate of the NF-M gene and the stability of NF-M mRNA. Since steady state NF-H mRNA levels in PC-12 cells do not appear to respond to NGF administration, the increase in NF-H subunit expression may be due to a post-transcriptional or translational effect.

DISCUSSION

We and others have shown that PC-12 cells show a time-dependent increase in NF content when treated with NGF (28–31, 58). Increases in NF-L and NF-M occurred relatively quickly and in parallel becoming detectable after 1 day in NGF and approaching maximum levels after 4 days. In this report we show that NF-H biosynthesis does not respond similarly to NGF. The increase in NF-H content was much slower as readily detectable amounts were only seen after 14 days of treatment with NGF. This differential expression of NF-L and NF-M as compared to NF-H in PC-12 cells has also been observed in developing rat brain and other *in vivo* systems (19–21).

Previous work has demonstrated that NGF treatment of PC-12 cells results in increased levels of NF-L mRNA and that this increase is due to increased transcription of the gene (46). We have confirmed and extended these findings by showing that a similar increase in the level of NF-M mRNA with NGF treatment was also due to increased transcription. Thus, the NF-L and NF-M genes behave similarly in their response to NGF, both at the transcriptional level and at the level of expression. However, while NGF treatment does not

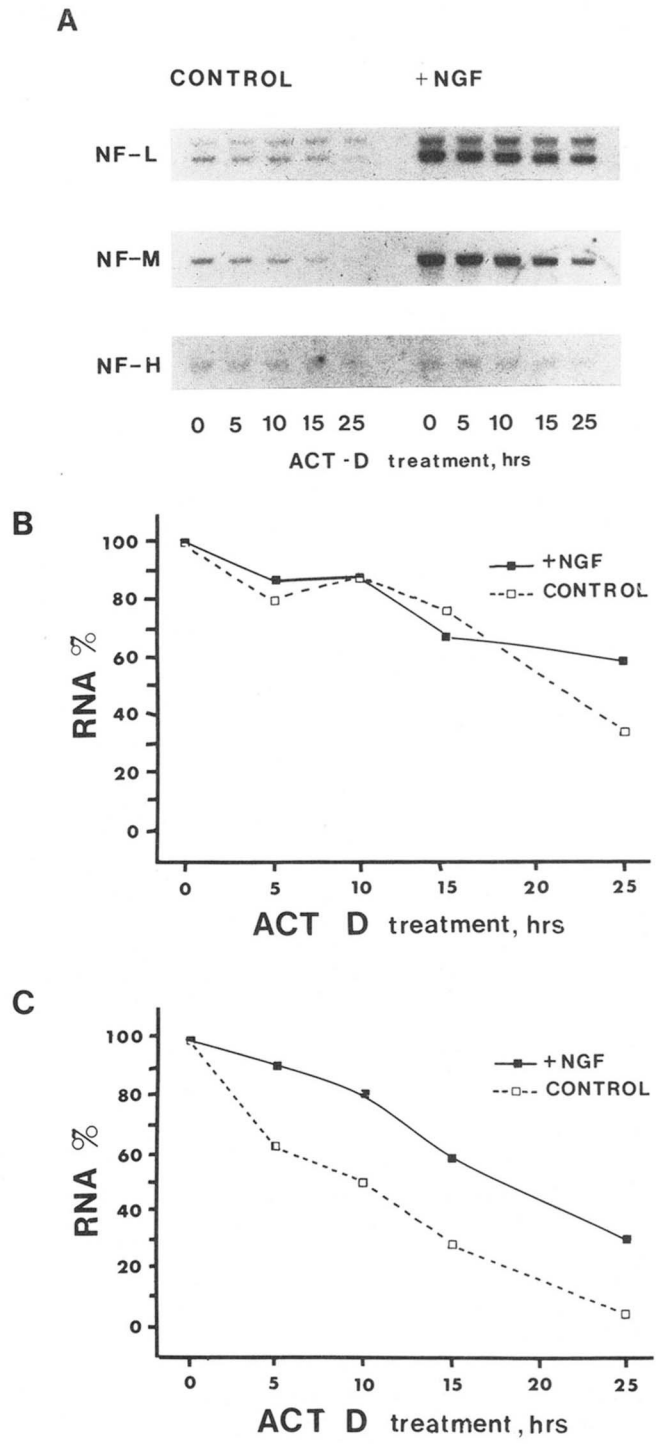

FIG. 4. **Relative stability of NF subunit mRNA in control or NGF-treated PC-12 cells.** *A*, PC-12 cells grown in the absence or presence of NGF were exposed to ACTD (5 μg/ml) for 0, 5, 10, 15, or 25 h. At these times RNA was purified and subjected to Northern analysis as previously described. Northern blots were hybridized with ^{32}P-labeled cDNA probes to NF subunits. Filters were exposed to film for 1–5 days. *B*, decreases in the levels of NF-L mRNA in cells grown in the absence or presence of NGF with time of exposure to ACTD were quantified by laser densitometry. The relative amounts of mRNA at each time point represent the sum of both NF-L species and are expressed as a percentage of mRNA levels at 0 time (100%). *C*, analysis performed as in *B* for NF-M mRNA.

modulate the stability of NF-L mRNA to a significant degree, it does appear to stabilize NF-M mRNA. This may account for the more rapid rise in NF-M mRNA levels after admin-

istration of NGF.

In other systems, stabilization of mRNA has been shown to be of importance in the superinduction of c-fos mRNA by inhibitors of protein synthesis, although the mechanism is unknown (48). Glucocorticoid regulation of growth hormone mRNA levels may also depend on mRNA stabilization, in this case through the induction of a stabilizing protein as well as by an increased extent of polyadenylation of mRNA (49). This latter mechanism has been demonstrated to increase the stability of viral mRNA (60). A ~50 nucleotide long AU-rich sequence in the 3′-noncoding region has been shown to confer instability to various mammalian mRNAs (61). As well, the regulation of β-tubulin mRNA stability has been linked to a ~100 nucleotide long translated region at the 5′ end of the mRNA (62). The mechanism for the stabilization of NF-M mRNA by NGF in PC-12 cells is not known, nor is it clear why this effect is specific for NF-M mRNA and not for mRNA of the other two subunits. Since NF-M protein is transiently expressed in a subpopulation of chick neuron precursors (50), it does not seem that co-expression or co-regulation of NF-L and NF-M is obligatory.

NGF did not increase NF-H mRNA levels over the period examined (14 days), nor did NGF increase transcription of the NF-H gene. These findings emphasize the distinct nature of both the NF-H subunit and the NF-H gene. Perhaps the difference in the regulation of the NF-H expression reflects the unique properties of NF-H in the axon. It has been shown, for example, that the appearance of NF-H in the axon results in an 8-fold decrease in the rate of the slow component of axonal transport (20). NF-H has also been demonstrated to be a component of the cross-bridging structures found in the axon (20, 51–54). These and other observations have led to the hypothesis that NF-H may serve to stabilize the axonal cytoskeleton (20, 54). If this is the case, then expression of NF-H during early stages of neuronal growth and development may prove detrimental to axonal growth and/or plasticity.

Although NGF treatment of PC-12 cells did not result in increased levels of NF-H mRNA, a slow but significant increase in NF-H immunoreactivity was observed over 14 days of NGF treatment. This may reflect control of NF-H expression in PC-12 cells at the post-transcriptional level. Such a mechanism has been demonstrated to exist for controlling the expression of a keratin subtype in hyperproliferative skin cells (55). Alternatively, NGF may act post-translationally to increase the metabolic stability of the NF-H polypeptide in PC-12 cells, as has been shown for NF-L and NF-M subunits (31). Since a correlation has been shown between the extent of NF subunit phosphorylation and resistance to degradation (56), NGF may render NF-H more stable by increasing its phosphorylation level (31). In this regard, it should be noted that the NF-H immunoreactivity observed on Western blots after prolonged NGF treatment consists of multiple bands, indicating a multiplicity of phosphorylation states (31).

PC-12 cells express fairly low levels of NF-H when compared, for example, to cultured peripheral neurons derived from neonatal rat (30). PC-12 cells have also been shown to express other intermediate filament proteins, such as vimentin (29), cytokeratins (57), and a novel 57-kDA intermediate filament protein that is also NGF-inducible (58). It is not clear whether these examples indicate that NF expression in PC-12 cells is abnormal, as previously suggested (29, 30), but it is interesting to note that the delayed expression of NF-H after NGF treatment is similar to the *in vivo* developmental pattern of NF subunit expression (19–21). Neurons comprise a diverse set of cells, which, unlike PC-12 cells, may be exposed to many external cues *in vivo*. NGF alone may not be sufficient to induce substantial levels of NF-H in PC-12 cells. Other factors in the extracellular environment, including cell-cell interactions, may also have to be considered in this regard.

It is clear from these studies and previous ones (31) that NGF-induced differentiation is a complex process, having both transcription-dependent and independent phases (59). Transcription-dependent processes can be divided into short term responses, taking place over a few minutes or hours, and long term responses, occurring after many hours or even days (27). It is the long term NGF-induced responses that are associated with the acquisition of the neuronal phenotype in PC-12 cells (27). However, it is presently not known what cis- or trans-acting factors, in concert with NGF, may be involved in the regulation of this developmental program. *In vitro* experiments designed to analyze the regulatory domains of the NF subunit genes may provide the means to address this question directly.

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