Early Changes in the Synthesis of Nuclear and Cytoplasmic Proteins Are Induced by Nerve Growth Factor in Differentiating Rat PC12 Cells

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Abstract. Differentiation of rat pheochromocytoma PC12 cells into neuron-like cells was induced by nerve growth factor (NGF) and changes in the apparent rate of synthesis of cellular proteins were analyzed. Attention was particularly focused on the first few hours of exposure to NGF before significant neurite outgrowth was detectable. Cultures were pulse-labeled for 1-h periods with [^{35}S]methionine and proteins were extracted from various subcellular fractions and analyzed by one-dimensional gradient and two-dimensional equilibrium and nonequilibrium gel electrophoresis. The results showed that although the general level of protein synthesis remained constant, by 8 h NGF increased the apparent rate of synthesis of ~11 cytoplasmic and 5 nuclear proteins. For several of these proteins, the effect was apparently NGF-specific, since no induction was observed in dibutyryl cAMP-treated cells. Of interest was the following observation: of the five nuclear proteins, NGF increased the synthesis of two proteins with M_rs of 56,000 [doublet] and 50,000 D that were associated with a biochemically and morphologically defined nuclear matrix fraction. A cytoplasmic protein, with an M_r of 92,000 D (pI 4.8) appeared to be induced de novo by NGF. NGF also decreased the rate of synthesis of several cytoplasmic and nuclear proteins of low molecular mass (<40,000 D). Since only 1-h pulses of [^{35}S]methionine were used, and since experiments with actinomycin D showed that most of these NGF-induced early changes in rates of synthesis included a transcription-dependent step, it seems likely that early effects of NGF include activation of specific genes.

THE PC12 clonal cell line derived from a transplantable rat pheochromocytoma (15) exhibits several characteristics of adrenal medullary chromaffin cells and responds to nerve growth factor (NGF)[1] by expressing many of the phenotypic properties of sympathetic neurons, including neurite outgrowth (15, 16, 57). Although the molecular mechanism by which NGF acts in PC12 cells as well as in normal neurons remains largely unknown, pharmacological data and in vitro transcription assays suggested that several parameters of the NGF-induced differentiation of PC12 cells involve transcriptional events, i.e., induction of neurite outgrowth (3), increased synthesis of NGF-inducible large external glycoprotein (43), and increases in ornithine decarboxylase (OrnDCase) mRNA (9), in *fos* mRNA (14), and in acetylcholinesterase activity (17). In addition, ongoing translation is necessary for the neurite outgrowth response of PC12 cells (16; Tiercy, J.-M., and E. M. Shooter, unpublished data) as well as of other cell types, such as chick sensory neurons (46).

However, a number of studies on PC12 cells, using one- and two-dimensional gel electrophoresis techniques, have led to the conclusion that NGF-stimulated neurite outgrowth does not involve qualitative changes in the synthesis and accumulation of the most abundant cellular proteins. Several quantitative changes have been described, including increased levels of the NGF-inducible large external protein (43), an 80-kD protein (44), a 55–56-kD protein (11), the neurofilament proteins and vimentin (36), a 25-kD glycoprotein immunologically cross-reactive with Thy-1.1 antigen (53), and the tau (7) and MAP1 proteins (7, 18), the latter two occurring concommitantly with increases in microtubule mass and neurite extension (7). The rate of synthesis of a significant fraction of total cellular proteins was found to be modulated similarly by NGF and by two different cAMP analogs, the major cytoskeletal proteins remaining unaffected (12). NGF also markedly increased the release into the medium of a 70-kD and a 30-kD protein (11). The synthesis of a 34-kD single-strand DNA binding protein was inhibited by NGF (2). Among the early effects of NGF are transient synthesis of proto-oncogene *fos* proteins (5, 31), an increase in OrnDCase (9, 22) and tyrosine hydroxylase (19) activities, and stimulation of the phosphorylation of a variety of proteins (18, 23, 34, 62).

Although the PC12 protein phosphorylation studies were

1. *Abbreviations used in this paper*: dbcAMP, dibutyryl cAMP; NEPHGE, nonequilibrium pH gradient gel electrophoresis; NGF, nerve growth factor; OrnDCase, ornithine decarboxylase; RSB, buffer containing 10 mM NaCl, 10 mM Tris-HCl, pH 7.4, and 1.5 mM $MgCl_2$; TNM sucrose, buffer containing 25 mM NaCl, 5 mM $MgCl_2$, 10 mM triethanolamine, pH 7.4, and 250 mM sucrose.

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performed at very early times, i.e., within minutes after addition of NGF, none of the analyses described above considered possible NGF-induced changes in rates of protein synthesis during the first few hours of NGF treatment, nor did they examine the subcellular localization of the induced proteins, except for the Thy-1-cross-reactive glycoprotein (57). It should also be pointed out that most of the previous studies on NGF-induced proteins describe changes in steady-state amounts, because of the use of the cumulative labeling method. In this paper, we present a study on changes in rates of protein synthesis during NGF- and dibutyrl cAMP (dbcAMP)-induced differentiation of PC12 cells, focusing attention on the first 8 h of NGF treatment. One- and two-dimensional gel electrophoretic analyses of [^{35}S]methionine pulse-labeled proteins extracted from PC12 cultures (±NGF) after subcellular fractionation showed that the rates of synthesis of several cytoplasmic and nuclear proteins were increased within the first few hours after NGF treatment and that the time courses of these increases were not all the same. Subnuclear fractionation studies furthermore revealed that two of these NGF-induced proteins were preferentially associated with the nuclear matrix fraction. Finally, the increased resolution of two-dimensional gel electrophoresis allowed us to detect the NGF-induced de novo synthesis of one cytoplasmic protein with an apparent M_r of 92,000 D and a pI of 4.8. We also present evidence that most of these early changes in rates of synthesis include a transcription-dependent step and that induction of several of these proteins was not observed in cultures treated with dbcAMP.

Materials and Methods

Cell Culture

Rat pheochromocytoma PC12 cells were grown in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 5% horse serum (Gibco) and 10% supplemented calf serum (HyClone Laboratories, Logan, UT) in a water-saturated atmosphere of 88% air/12% CO_2. The cultures used for each experiment were prepared by replating growing PC12 cells at 2–3 × 10^4 cells/cm^2 on poly-D-lysine-coated (Sigma Chemical Co., St. Louis, MO) 35-mm or 80-mm tissue culture dishes (Falcon Labware, Oxnard, CA). After 2–4 d, either 100 ng/ml βNGF (referred to as NGF) or 1 mM dbcAMP (Sigma Chemical Co.) were added to the cultures (time 0) in medium supplemented with serum. Medium plus serum alone was added to control cultures. NGF was prepared from submandibular glands of adult male Swiss-Webster mice by the method of Smith et al. (55).

Labeling and Extraction of Total Proteins

PC12 cultures (in 35-mm dishes) were labeled for 1-h periods at different times between 0 and 7 d after addition of NGF with 25 μCi/ml of [^{35}S]methionine (~1,000 Ci/mmol; New England Nuclear, Boston, MA) in methionine-free medium (Irvine Scientific, Santa Ana, CA). When required, NGF was present in the labeling medium. Labeling was terminated by washing the cultures twice with cold (4°C) Ca^{++} and Mg^{++}-free PBS (137 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4.12\ H_2O$, 1.5 mM KH_2PO_4). Total proteins were extracted by adding 100 μl/dish (two dishes per experimental point) of 1% SDS/1 mM NaH_2PO_4, pH 8.5 (30). After several passages through a 27 × ½-gauge needle, lysates were stored at −20°C. Aliquots (10–50 μl) to be analyzed by SDS-PAGE were mixed (2:1, vol/vol) with threefold-concentrated protein sample buffer (final concentration, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.0625 M Tris-HCl, pH 6.7, 0.003% bromophenol blue) (32). The TCA-precipitable radioactivity of the samples was measured on 5–10-μl aliquots that were spotted on 2.4-cm diameter GF/C filters (Whatman, Inc., Clifton, NJ), incubated in 10% TCA for 15 min on ice, washed five times with ethanol, dried, and counted in 2 ml scintillation fluid (Aquasol; Amersham Corp., Arlington Heights, IL).

Labeling of the Cells, Isolation of PC12 Nuclear and Cytoplasmic Fractions, and Extraction of Proteins

After 7 h, control, NGF- (100 ng/ml), and dbcAMP- (1 mM) treated PC12 cultures (grown on 80-mm dishes) were pulse-labeled for 1 h with 20 μCi/ml of [^{35}S]methionine. When required, NGF or dbcAMP were present in the labeling medium. In parallel cultures (±NGF or ±dbcAMP), 2 μg/ml actinomycin D (Calbiochem-Behring Corp., La Jolla, CA) were present from 0 to 8 h. At the end of the labeling time, cultures were washed twice with cold PBS and cells were lysed with 2 ml/dish of RSB (10 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1.5 mM $MgCl_2$) (24) containing 1% Triton X-100 and 0.5 mM phenylmethylsulfonyl fluoride (PMSF; Sigma Chemical Co.). The lysates were passed five times through a 22 × 1½-gauge needle, incubated on ice for 10 min, and centrifuged for 5 min at 1,000 *g* (JA-21 rotor; Beckman Instruments, Fullerton, CA). The supernatant was referred to as the cytoplasmic fraction. The first nuclear pellet was washed with the same buffer (nuclear wash 1) and then in RSB alone (nuclear wash 2). In some experiments, nuclei were centrifuged (10 min at 1,500 *g*) through a 0.88-M sucrose cushion. The final pellet was referred to as the nuclear fraction. It was noted that an extensive syringing of the PC12 cell lysates led to significant breakage of nuclei. Cytoplasmic proteins were precipitated for 1 h at 4°C with 20% TCA and the resulting pellets washed twice with a solution containing 70% acetone (vol/vol), 20% ethanol (vol/vol), 10 mM Tris-HCl, pH 7.4, and 0.0001% bromophenol blue (35), lyophilized, and resuspended in the protein sample buffer. Nuclei were lyophilized and resuspended in protein sample buffer.

Subfractionation of Nuclei and Extraction of Proteins

Nuclei were fractionated by a modification of the procedure of Berezney and Coffey (1). Purified nuclei were gently resuspended in TNM sucrose (25 mM NaCl, 5 mM $MgCl_2$, 10 mM triethanolamine, pH 7.4, 250 mM sucrose) at 2–4 × 10^6 nuclei/ml. DNase I (20 μg/ml; RNase-free) and 0.5 mM PMSF were added and the mixture incubated for 30 min on ice. Digested nuclei were pelleted by centrifugation for 10 min at 1,500 *g*. The supernatant was termed DNase I extract. The nuclei were then incubated for 30 min on ice in 1 ml high salt buffer (2 M NaCl, 10 mM Tris-HCl, pH 7.4, 0.2 mM $MgCl_2$) in the presence of 0.5 mM PMSF. The resulting nuclear structures, referred to as the nuclear matrix fraction, were sedimented for 15 min at 4,000 *g*, dried under vacuum, and resuspended in 120 μl of protein sample buffer. The supernatant was referred to as the high salt extract. Proteins were extracted from the DNase I and the high salt extracts either with 20% TCA, as described above, or by precipitation with 8 vol of acetone, then lyophilized and resuspended in 100 and 200 μl, respectively, of protein sample buffer. For radioactivity measurements (Table II) and photomicroscopy (Fig. 3), the nuclear matrix pellet was gently resuspended in TNM sucrose.

When proteins were to be analyzed by two-dimensional gel electrophoresis, the resulting dried pellets were resuspended in lysis buffer (9 M urea, 2% wt/vol NP-40, 5% β-mercaptoethanol, 2% ampholines, pH 3.5–10 [LKB Instruments, Gaithersburg, MD]) (47).

Gel Electrophoresis in One and Two Dimensions

Proteins were analyzed either in 10% or in linear polyacrylamide gradient (7.5–15% acrylamide) slab gels (24 × 18 × 0.15 cm) in the discontinuous SDS-containing buffer system as described by Laemmli (32). Both the stacking (5% acrylamide) and separating gels contained 1 mM Na_2-EDTA and the upper buffer contained 14 mM β-mercaptoethanol (35). Electrophoresis was at 24 mA per gel until the bromophenol tracking dye had migrated 20 cm. After staining with 0.075% Coomassie Brilliant Blue (Sigma Chemical Co.) and destaining, gels were dried and covered with x-ray film (X AR-5, Kodak) for 1–20 d at −70°C. Standard proteins (50 μg/lane; Sigma Chemical Co.) were: myosin, M_r 205,000 D; β-galactosidase, M_r 116,000 D; phosphorylase b, M_r 97,400 D; bovine albumin, M_r 66,000 D; ovalbumin, M_r 45,000 D; and carbonic anhydrase, M_r 29,000 D. Analysis of labeled proteins by two-dimensional gel electrophoresis with isoelectric focusing in the first dimension was performed as described (47). Isoelectric focusing gels contained 4% of pH 3.5–10 ampholines and 2% of pH 4–6 ampholines; electrophoresis was for 8,000 V·h. Two-dimensional gel electrophoresis using nonequilibrium pH gradient electrophoresis (NEPHGE) in the first dimension was performed as described (48). The first dimension gels contained 6% of pH 3.5–10 ampholines and electrophoresis was for 2,500 V·h. The SDS-polyacrylamide gels for the second dimension contained 10% acrylamide. The gels were stained with Coomassie Blue, destained, fluorographed, and covered with x-ray film using an intensifying screen

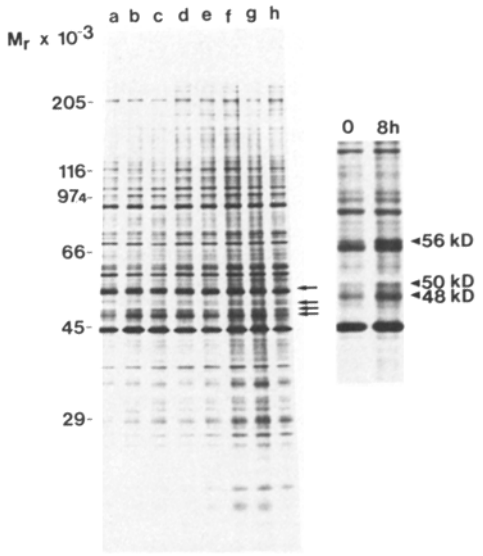

Figure 1. Rates of protein synthesis in total PC12 cell extracts at different times after addition of NGF. PC12 cultures were pulse-labeled for 1-h periods with [^{35}S]methionine at 0 (lane *a*), 3 (lane *b*), 8 (lane *c*), 16 (lane *d*), 24 (lane *e*), 48 (lane *f*), and 72 h (lane *g*), and 7 d (lane *h*) after addition of 100 ng/ml NGF. Total proteins were extracted and aliquots containing the same amounts of protein, as judged by Coomassie Blue staining and corresponding to ~7.5 × 10^4 cpm/lane were analyzed in a 7.5–15%-gradient gel. Slightly more protein was loaded on lanes *f* and *g*. The figure shows the autoradiograph of the dried gel (9-d exposure). Arrows indicate the NGF-induced proteins with apparent M_rs of 56,000, 51,500, 50,000, and 48,000 D. The insert shows an enlarged section of an autoradiograph of a gel showing the control (0 h) and after 8 h of exposure to NGF (8 h).

(Merry-X-ray, San Francisco, CA). pH gradients were measured on parallel gels cut into 5-mm slices as described (47).

Results

Analysis of [^{35}S]Methionine Pulse-labeled Total Proteins

Changes in protein synthesis that occur in PC12 cells in response to NGF were determined by pulse-labeling monolayer PC12 cultures with [^{35}S]methionine for 1-h periods at different times between 0 and 7 d after addition of NGF. Attention was focused on the first 24 h, since neurites could be detected in some cells as early as 8–12 h, and by 24 h about 5% of the cells had neurites (not shown; 20, 21), the neurites being defined as neuritic processes longer than one cell body diameter and displaying a growth cone at their tip. Under the conditions used in this study, a maximum of ~80% of the cells were bearing neurites by 7 d in accordance with previous studies (15, 20, 21). Total labeled proteins were extracted at 0, 3, 8, 16, 24, 48, and 72 h, and 7 d, and aliquots containing the same amount of protein were analyzed by one-dimensional SDS-PAGE; proteins were revealed by Coomassie Blue staining and by autoradiography (Fig. 1). Under these conditions, ~100 individual bands were routinely resolved. The staining patterns (not shown) of the proteins extracted at any time between 0 and 7 d were similar in control and NGF-treated cultures. This result was expected in view of previous experiments that indicated no qualitative and very few quantitative changes in PC12 proteins (12, 43, 44). However, the autoradiographs (Fig. 1) revealed that NGF increased the incorporation of [^{35}S]methionine into four bands with apparent M_rs of 56,000, 51,500, 50,000, and 48,000 D, whereas the labeling of the other proteins remained virtually unchanged. It is not possible to tell from these one-dimensional analyses whether the bands contain more than one protein. However, for ease of description, these are referred to as the 56-, 51.5-, 50- and 48-kD proteins, respectively, without implying that they represent single proteins. Results presented later indeed show that the 56-kD protein contains two proteins with slightly different isoelectric points. The time course of induction of these proteins was different. Increased synthesis of the 56-, 50-, and 48-kD proteins was already detected 3 h after addition of NGF (Fig. 1, lane *b*). NGF-induced synthesis of the 56-kD protein reached a maximum at ~8 h (lane *c* and insert), remained at this level until at least 24 h (lane *e*), and then decreased until it reached, by 72 h (lane *g*), a level even slightly lower than that observed in naive PC12 cells. Increased synthesis of the 50- and 48-kD proteins was also maximal at ~8 h (lane *c* and insert) but remained at this level for at least 3 d (lane *g*). Induction of the synthesis of the 51.5-kD protein was first detectable by 8 h (lane *c*) and increased continuously until up to at least 7 d (lane *h*). The same pattern of increased protein synthesis was observed after 8 h of NGF treatment when PC12 cells were grown on uncoated dishes (not shown).

An alternative but less likely explanation for the NGF-induced increase in the labeling of these specific proteins may be a decrease in their turnover rate occurring during the 1-h labeling period. Total proteins of control and NGF-treated cultures were thus labeled with [^{35}S]methionine from 7 to 8 h and analyzed by SDS-PAGE at the end of the pulse and also after a 5-h chase in cold medium. The results showed a similar difference in the labeling of the 56-, 50-, and 48-kD proteins between control and NGF-treated cells after the 5-h chase (not shown), as compared with that observed at the end of the labeling period (Fig. 1, insert). If decreased turnover were solely responsible for the increased labeling of these three proteins after a 1-h pulse, the effect would have been amplified after the cold chase. Increased rates of translation are thus essentially responsible for the increased incorporation of [^{35}S]methionine in the NGF-induced proteins.

Intracellular Localization of NGF-induced Proteins

To analyze the intracellular distribution of NGF-induced early proteins, PC12 cells incubated with and without NGF were pulse-labeled with [^{35}S]methionine from 7 to 8 h, and nuclear and cytoplasmic fractions were isolated by use of 1% Triton X-100 in hypotonic buffer (RSB; 24). We have tested different lysis buffers and found that the Coomassie Blue staining patterns of PC12 nuclear and cytoplasmic proteins were almost indistinguishable, whether the cell fractionation was performed in RSB containing either 0.5–1.0% Triton X-100 or 0.5–1.0% NP-40, or in TNM sucrose containing either detergent at the same concentrations. However, the ratio of radioactivity in nuclear proteins relative to that in cytoplasmic proteins (after a 1-h pulse) was slightly lower with the hypotonic buffer compared with the isotonic buffer,

Table I. Proteins Induced in PC12 Cells by Short-term Exposure to NGF (8 h)

Analysis of cell fraction	Molecular mass × 10^{-3}											
1 d												
Total				56			51.5	50	48	(38)		
Cytoplasmic*	123‡§		75‡				51.5	50‡	48	38‡		
Nuclear*				56‡§				50‡§	48			
Nuclear, high salt				(56)					(48)			
Nuclear, matrix				56				50	48			
2 d												
Cytoplasmic, equilibrium		92	75		55			50		38		25
Cytoplasmic, nonequilibrium					54‡§(d)				48‡	38‡§(d)	30‡	
Nuclear, nonequilibrium*						53‡§						
Nuclear, high salt, equilibrium											35	
Nuclear, matrix, equilibrium				56(d)				50				

* In these experiments the effect of dbcAMP and of actinomycin D were also tested.
‡ NGF-dependent increase in labeling abolished by actinomycin D.
§ Labeling not affected by dbcAMP.
(d) Refers to the presence of two proteins of equal molecular mass but different isoelectric points.

Table II. Distribution of DNA, RNA, and Protein in the Different Subcellular Fractions

Cell fraction	DNA*	RNA*	Protein*
Cytoplasmic	2.3	77.9	87.6
Nuclear wash 1	0.8	1.5	2.4
Nuclear wash 2	0.6	0.9	0.4
DNase I extract	1.0	0.5	0.7
High salt extract	92.5	18.8	5.4
Nuclear matrix	2.8	0.4	3.5
Total	100‡	100‡	100‡

PC12 cultures were labeled for 44 h with 0.5 μCi/ml [^{3}H]methyl-thymidine or 0.5 μCi/ml [^{3}H]uridine or 2 μCi/ml [^{35}S]methionine, respectively, in normal medium plus serum. Cultures (~5 × 10^6 cells/dish) were fractionated as described in Materials and Methods and the amount of TCA-precipitable radioactivity in each subcellular fraction was measured on 50-μl aliquots in triplicate. The total radioactivity obtained from the sum of values for each fraction was within 4% of the total radioactivity measured immediately at the beginning of the cell fractionation procedure.
* Percent of total radioactivity.
‡ The 100% values were 840,800 cpm for [^{3}H]dT, 1,065,500 cpm for [^{3}H]uridine, and 747,450 cpm for [^{35}S]methionine.

resulting perhaps from a better purification of nuclei. The distribution of DNA in these fractions (Table II, see below) also indicated that nuclei breakage was minimal. This is consistent with the observation of Schechter and Bothwell (54), who reported the isolation of nuclei with minimal cytoskeletal contamination by using 0.5–1% Triton X-100 in PBS and mechanical shearing.

Fig. 2, *A* and *B* show the results of a typical experiment where aliquots of [^{35}S]methionine-labeled cytoplasmic and nuclear proteins were analyzed on 7.5–15% gradient gels. Autoradiography of the cytoplasmic extracts (Fig. 2 *A*, lane *c*) revealed that the NGF-induced increase in the apparent rate of synthesis of the 51.5-, 50-, and 48-kD proteins observed on whole cell extracts was also apparent in cytoplasmic fractions (Table I). As was noted in whole cell extracts, induction of the 51.5-kD protein was barely detectable by 8 h (see also the time course in Fig. 1). These analyses also revealed an NGF-induced increase in the apparent rate of synthesis of three other cytoplasmic proteins migrating with M_rs of 123,000, 75,000, and 38,000 D (Fig. 2 *A*, lane *c*; Table I). In some experiments the NGF-induced increase in 38-kD protein synthesis was also detected in total cell lysates, i.e., without enrichment in cytoplasmic proteins. Analysis of the nuclear extracts (Fig. 2 *B*) showed that the increased rate of synthesis of the 50- and 48-kD proteins (lane *c*) brought about by NGF was also detected in this fraction. On the other hand, the increase in the labeling of the 56-kD protein was only observed in the nuclear fraction. Thus, as evidenced by these analyses, NGF increases the rate of synthesis of four cytoplasmic proteins (123, 75, 51.5, and 38 kD), of one nuclear protein (56 kD), and of two other proteins that appear in both cytoplasmic and nuclear extracts (50 and 48 kD). The distribution of these last two proteins may well reflect their heterogeneity.

NGF-induced Synthesis of Early Protein Is Apparently Transcription-dependent

The same experimental design was also used to determine whether the early increase in the synthesis of specific proteins in response to NGF required ongoing RNA synthesis. PC12 cultures were treated for 8 h with and without NGF in the presence or absence of 2 μg/ml actinomycin D. Control experiments were performed by pulse-labeling PC12 cells (±NGF) from 7 to 8 h with [^{3}H]uridine in the absence or presence of different concentrations of actinomycin D and then measuring the incorporation of TCA-precipitable radioactivity. It was observed that 2 μg/ml actinomycin D (added at time 0) was sufficient to block RNA synthesis by 94 ± 2%. Most importantly, the same extent of inhibition was already measured after the first hour of incubation in actinomycin D.

Pulse-labeling with [^{35}S]methionine was performed from 7 to 8 h, and the cultures were then separated into nuclear and cytoplasmic fractions. Aliquots containing the same amounts of protein, as judged by Coomassie Blue staining (Fig. 2, *A* and *B*, lanes *a* and *b*) were analyzed on gradient gels. The autoradiographs (Fig. 2) showed that actinomycin D decreased the overall rate of protein synthesis in both control and NGF-treated cultures. This result was anticipated from the 30–50% decrease in the incorporation of TCA-precipitable [^{35}S]methionine in the cytoplasmic fractions (±NGF) and the 66–80% decrease in the nuclear fractions (±NGF) (not shown). However, the decreased rate in overall protein synthesis was slightly less marked in NGF-treated

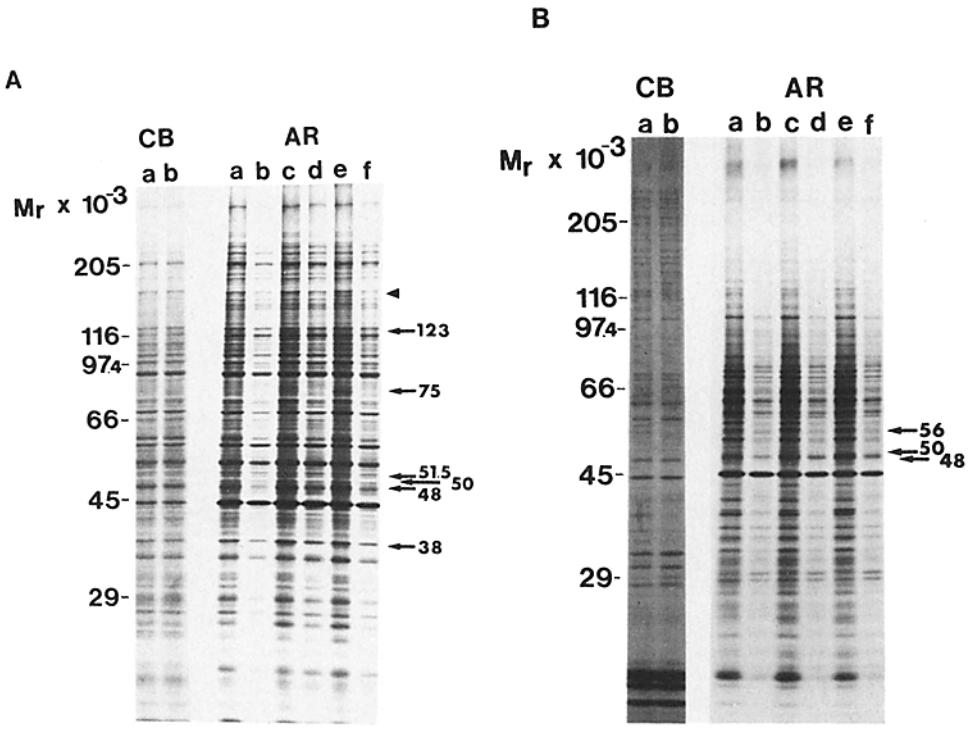

Figure 2. Analysis of [^{35}S]methionine pulse-labeled cytoplasmic (*A*) and nuclear (*B*) proteins in control, NGF-, and dbcAMP-treated PC12 cultures in the presence or absence of actinomycin D. PC12 cultures were pulse-labeled with [^{35}S]methionine from 7 to 8 h in the absence (lanes *a* and *b*) or presence of 100 ng/ml NGF (lanes *c* and *d*) or in the presence of 1 mM dbcAMP (lanes *e* and *f*) added at time 0. In one set of the control (lane *b*), NGF- (lane *d*), and dbcAMP-treated (lane *f*) cultures, 2 μg/ml actinomycin D was added at time 0. Proteins were extracted from cytoplasmic (*A*) and nuclear (*B*) fractions as described in Materials and Methods, and aliquots containing the same amounts of proteins were analyzed in 7.5–15%-gradient gels. The figure shows the Coomassie Blue (CB) staining patterns of cytoplasmic (*A*, lanes *a* and *b*) and nuclear (*B*, lanes *a* and *b*) proteins of the controls and the controls plus actinomycin D, respectively. The patterns of the other lanes were identical. The corresponding autoradiographs (AR) of the dried gels are shown (*A*, 5-d exposure; *B*, 10-d exposure). Cytoplasmic proteins in lanes *a*, *c*, and *e* and *b*, *d*, and *f* contained ~10 × 10^4 and 6 × 10^4 cpm, respectively. Nuclear proteins in lanes *a*, *c*, and *e* and *b*, *d*, and *f* contained 7 × 10^4 and 1.5 × 10^4 cpm, respectively. Arrows indicate NGF-induced proteins; arrowhead indicates dbcAMP-induced 170-kD protein.

cytoplasmic fractions (~30%), compared with untreated controls (40–50%), perhaps reflecting an NGF-induced increase in mRNA stability. NGF-increased synthesis of the cytoplasmic 123-, 75-, 50-, 48-, and 38-kD proteins (Fig. 2 *A*, lanes *b* and *d*) was markedly decreased by actinomycin D. The effect of the inhibitor was particularly striking for the 123-, 75-, and 38-kD proteins that became undetectable (Fig. 2 *A*, lane *d*). The cytoplasmic 48-kD protein was still synthesized at a slightly higher level in NGF-treated cultures as compared with untreated controls. The NGF-increased labeling of nuclear 56- and 50-kD proteins was also completely abolished in actinomycin D–treated cultures (Fig. 2 *B*, lanes *b* and *d*). These results suggested that NGF-induced increase in the synthesis of these early proteins required ongoing transcription. It is not yet known whether this transcription-dependent step is the synthesis of the pre-mRNAs coding for these induced proteins or the synthesis of other RNA species required, either directly or via their protein products, for posttranscriptional modifications (splicing, transport) or translation of these mRNAs.

dbcAMP Does Not Induce the Synthesis of the 123-, 56-, and 50-kD Proteins

In the above experiment, parallel cultures were also treated for 8 h with 1 mM dbcAMP in the presence or absence of 2 μg/ml actinomycin D, added at time 0. It has been reported that dbcAMP induces a rapid but transient neurite outgrowth in PC12 cells (16, 21). Under our experimental conditions, a maximum of ~30% of the cells bore neurites after 3–4 d of dbcAMP treatment and this number remained the same until at least 7 d. The cultures (±dbcAMP) were labeled with [^{35}S]methionine from 7 to 8 h, and separated into nuclear and cytoplasmic fractions that were analyzed by SDS-PAGE as described above.

The autoradiographs in Fig. 2, *A* and *B* (lanes *e*) show that dbcAMP did not increase the apparent rates of synthesis of the 123- and 50-kD proteins, nor of the nuclei-associated 56-kD protein. On the other hand, dbcAMP increased the synthesis of the 48- and 38-kD proteins to about the same extent as NGF, and the synthesis of the 51.5-kD protein to a lesser extent. It also increased the rate of synthesis of the 75-kD protein, but here the effect of dbcAMP was significantly more noticeable than that of NGF (Fig. 2 *A*, lane *e*). In addition, dbcAMP specifically increased the incorporation of [^{35}S]methionine into a protein of ~170 kD (arrowhead in Fig. 2 *A*, lane *e*). Where rates of synthesis were increased by dbcAMP it was also in a transcription-dependent manner, i.e., it was abolished by actinomycin D (Fig. 2 *A*, lane *f*).

Intranuclear Localization of NGF-induced Proteins

To further analyze the distribution within the nuclei of the 56-, 50-, and 48-kD proteins whose synthesis was increased by NGF, the following fractionation procedure was performed. PC12 cultures (±NGF) were pulse-labeled with [^{35}S]methionine from 7 to 8 h and nuclei isolated as described above. The purified nuclei were then incubated in the presence of 20 μg/ml DNase I (RNase-free), which resulted in a DNase I extract. The nuclei were further treated with 2 M NaCl (1) resulting in a high salt extract, or chromatin fraction (4, 10), and in a pellet of residual nuclear structures referred to as nuclear matrix.

As shown in Table II, the PC12 nuclear matrix contained ~3.5% of total cell protein. Although the measurements were based on [^{35}S]methionine cumulative labeling experi-

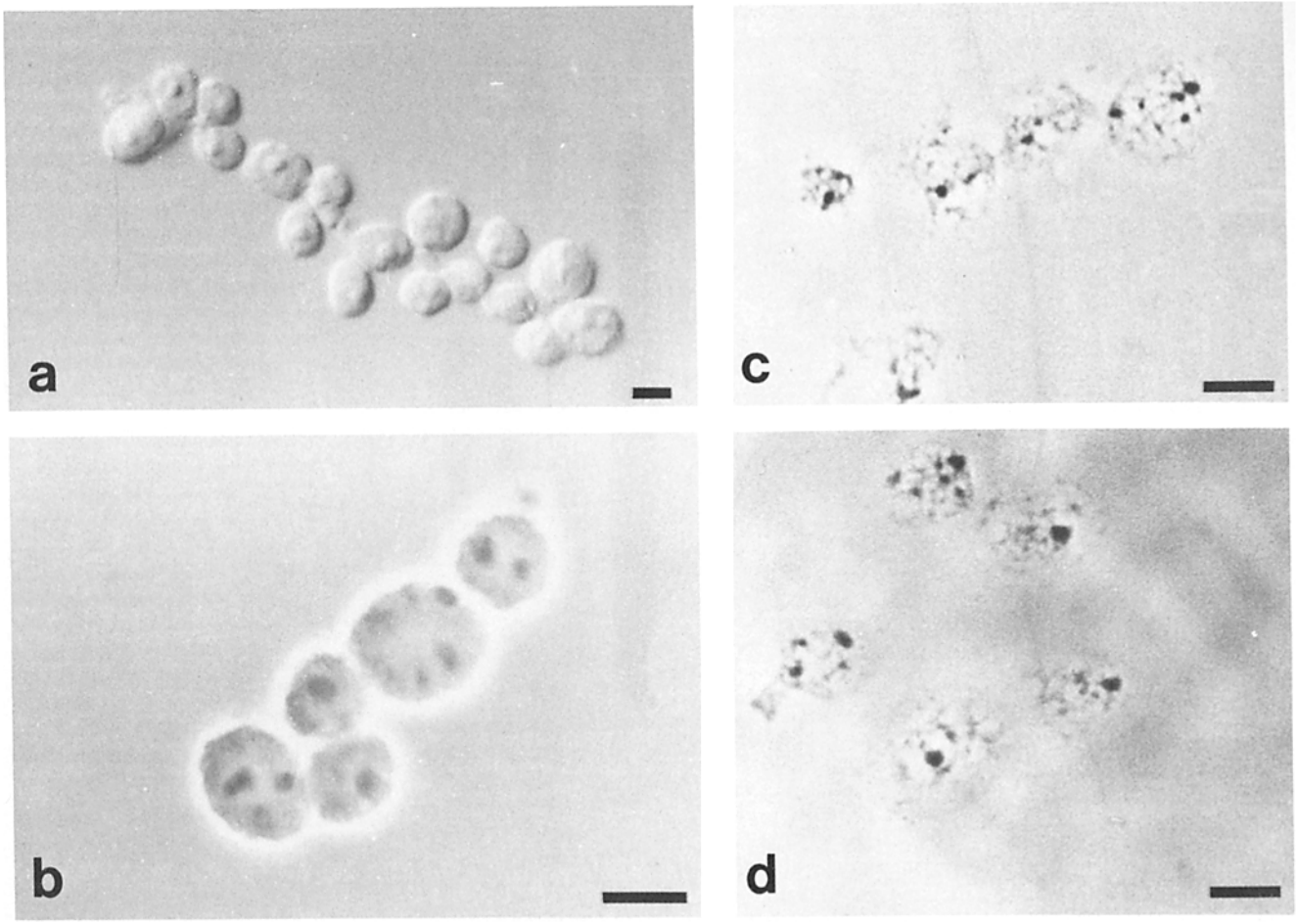

Figure 3. Morphology of unfixed PC12 nuclei and nuclear matrix structures. Purified nuclei and nuclear matrix fraction were prepared from PC12 cells as described under Materials and Methods and photographed, using a Zeiss inverted microscope. *a* and *b* show Nomarski interference and phase-contrast micrographs of nuclei, respectively. *c* and *d* show phase-contrast micrographs of a nuclear matrix preparation. Bar, 20 μm.

ments and should be taken as estimates, the figure is comparable to the results published by others, using similar (but not identical) cell fractionation procedures (10, 52, 56). The DNase I and high salt treatments removed most of the DNA and RNA from nuclei (Table II), leaving a nuclear matrix fraction containing ~3% of nuclear DNA, ~2% of nuclear RNA, and one-third of the nuclear proteins (Table II). Under our conditions, nearly 30% of newly synthesized RNA (1-h pulse with [^{3}H]uridine) copurified with the nuclear matrix (not shown).

As observed by high magnification Nomarski interference and phase-contrast light microscopy, the purified nuclei (Fig. 3 *a* and *b*, respectively) appeared essentially devoid of detectable cytoplasmic filaments, at least at the level of resolution allowed by the technique. The nuclear matrix preparation under phase-contrast light microscopy (Fig. 3, *c* and *d*) appeared as less dense, slightly smaller structures, as compared with intact nuclei, with nucleoli still visible, and with the characteristic fibrillogranular network appearance described for the nuclear matrix of other mammalian cells (1, 29, 56).

When isolated PC12 nuclei were treated with double detergent (0.5% NP-40/0.5% deoxycholate) (4) in TNM sucrose buffer, no change in the nuclear protein staining pattern could be detected; nor did a DNase I/RNase A treatment of the nuclear matrix fraction result in a different protein staining pattern (not shown). This is in accordance with previous observations that the presence of the characteristic matrix proteins does not depend on association with the bulk of nuclear RNA (41) and that heterogeneous nuclear RNA is not an integral component of the nuclear matrix because it can be digested with RNase without altering the matrix architecture (39, 40, 45, 59).

The distribution of TCA-precipitable [^{35}S]methionine (representing newly synthesized proteins) in the various subcellular fractions is shown in Table III. Between 7 and 8% of total radioactivity was recovered in the nuclear fraction from control cells and those treated with NGF for 8 h. More specifically, it was found that in untreated controls 30 ± 0.5% of the nuclear radioactivity was associated with the nuclear matrix structures, whereas in the NGF-treated cultures it amounted to 39 ± 2%, as measured in three independent experiments. In contrast, the slight increase in TCA-precipitable radioactivity observed after NGF treatment in most of the other subcellular fractions (Table III) was not consistently observed in several independent experiments and might be due to small variations in cell number/dish either before or after washing the cultures at the end of the radioactive pulse.

Aliquots of each subnuclear fraction (±NGF) pulse-

Table III. Distribution of [35S]Methionine Pulse-labeled PC12 Proteins in the Different Subcellular Fractions

Fraction	NGF	cpm × 10^{-4}*	Percent‡
Cytoplasmic	−	2,542	89.5
	+	2,884	90.5
Nuclear wash 1	−	63	2.2
	+	66	2.1
Nuclear wash 2	−	11	0.4
	+	12	0.4
DNAse I extract	−	21	0.7
	+	14	0.4
High salt extract	−	154	5.4
	+	131	4.1
Nuclear matrix	−	50	1.8
	+	78	2.5
Total	−	2,841	100
	+	3,185	100

Control and NGF-treated cultures (two dishes per experimental point) were pulse-labeled with [35S]methionine from 7 to 8 h. Cells were fractionated as described in Materials and Methods, and TCA-precipitable radioactivity was measured in each subcellular fraction.
* Total cpm per fraction.
‡ Percent of total cpm.

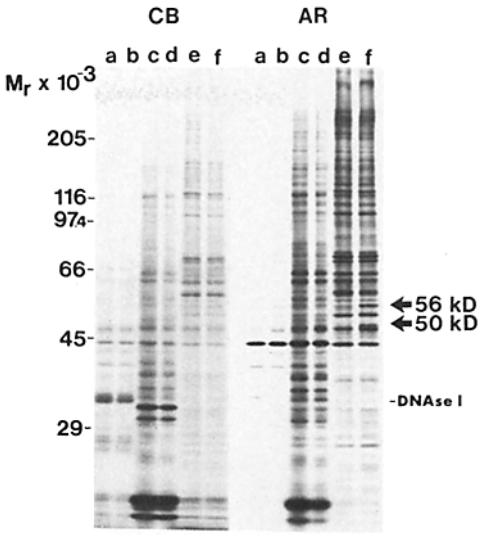

Figure 4. Intranuclear localization of early NGF-induced proteins. PC12 cultures were labeled with [35S]methionine from 7 to 8 h after addition of NGF and nuclei were purified from the untreated (lanes *a*, *c*, and *e*) and treated (lanes *b*, *d*, and *f*) cultures. Nuclei (3–6 × 10^6 nuclei per experimental point) were fractionated as described in Materials and Methods into DNase I extracts (lanes *a* and *b*), high salt extracts (lanes *c* and *d*), and nuclear matrix fractions (lanes *e* and *f*). Samples containing the same amounts of proteins within each subnuclear fraction (i.e., control and +NGF) were analyzed in a 7.5–15%-gradient gel. Lanes *a* and *b*, protein samples corresponding to 4.5 × 10^5 cells (10^4 cpm); lanes *c* and *d*, 3 × 10^5 cells (4.8 × 10^4 cpm); lanes *e* and *f*, 1.2 × 10^5 cells (1.2 × 10^4 cpm). The figure shows the Coomassie Blue staining pattern (CB) and the corresponding autoradiograph (AR, 9-d exposure). Arrows indicate the two nuclear matrix-associated NGF-induced proteins.

labeled with [35S]methionine from 7 to 8 h were analyzed on 7.5–15%-gradient gels. The result of a typical experiment is shown in Fig. 4. Again, the protein staining patterns of the different subnuclear fractions were identical whether or not the cells were exposed to NGF for 8 h. The DNase I treatment released a few proteins, the major band comigrating with actin. Most of the histone core proteins were released with the 2-M NaCl extraction (Fig. 4, CB, lanes *c* and *d*), as well as most of the low molecular mass proteins in the range of 15–40 kD (1, 56). The nuclear matrix protein fraction (Fig. 4, CB, lanes *e* and *f*) was specifically enriched with a number of bands migrating with apparent M_rs from 49,000 to 170,000 D. The most prominent proteins in the 60,000–70,000-D molecular mass range probably correspond to the lamin proteins from the dense lamina underlining the nuclear envelope (1, 6, 13, 56). The quantitative distribution of proteins within the three subnuclear fractions, as estimated by staining with Coomassie Blue (see legend to Fig. 4), correlated well with the estimation of protein content either by [35S]methionine cumulative labeling (Table II), or by colorimetric analysis. The results showed a DNase I extract/high salt extract/nuclear matrix fraction ratio of protein content of 0.3:3.0:1.0 (not shown).

Although histone H1 was completely removed after the high salt treatment, and was thus undetectable in the nuclear matrix fraction, some histone core proteins were present in this latter fraction even though digestion of DNA was almost complete. Similar observations were made during the isolation of mouse 3T3 nuclear matrix (4). As suggested by Long et al. (40), this may result from specific binding between histones and non-histone proteins. This hypothesis is consistent with our observation that in PC12 cells treatment of the nuclear matrix with both DNase I and RNase A did not completely remove traces of histone core proteins.

The autoradiograph (Fig. 4) did not reveal many changes in rates of protein synthesis in any of the three subnuclear fractions, as expected from the overall pattern of nuclear proteins (see Fig. 2 *B*, lanes *a* and *c*). The 48-kD protein was found in both the high salt extract and in the nuclear matrix fraction, but its rate of synthesis was only increased in the latter fraction. (Fig. 4, lanes *c–f*). The 50-kD protein was found exclusively in the nuclear matrix fraction extracted from 8 h NGF-treated cultures and was barely detectable in the controls (Fig. 4, lanes *e* and *f*). The 56-kD protein was highly enriched in the nuclear matrix fractions and its apparent rate of synthesis was increased in NGF-treated cultures by a factor of three, as judged by densitometer tracings of the autoradiograph (Fig. 4, lanes *e* and *f*). Traces of the 56-kD protein were also detected in the DNase I and high salt extracts; however, in both instances no increase could be observed upon NGF treatment (Table I).

Two-dimensional Gel Electrophoretic Profiles of [35S]Methionine Pulse-labeled Proteins Extracted from Cytoplasmic Fractions, High Salt Extracts, and Nuclear Matrix Fractions

The possibility of isolating biochemically and morphologically distinct subnuclear fractions from PC12 cells prompted us to analyze the [35S]methionine pulse-labeled high salt extract and nuclear matrix as well as cytoplasmic fractions by two-dimensional gel electrophoresis, the proteins being revealed by Coomassie Blue staining and fluorography. Although previous studies performed on whole cell extracts did

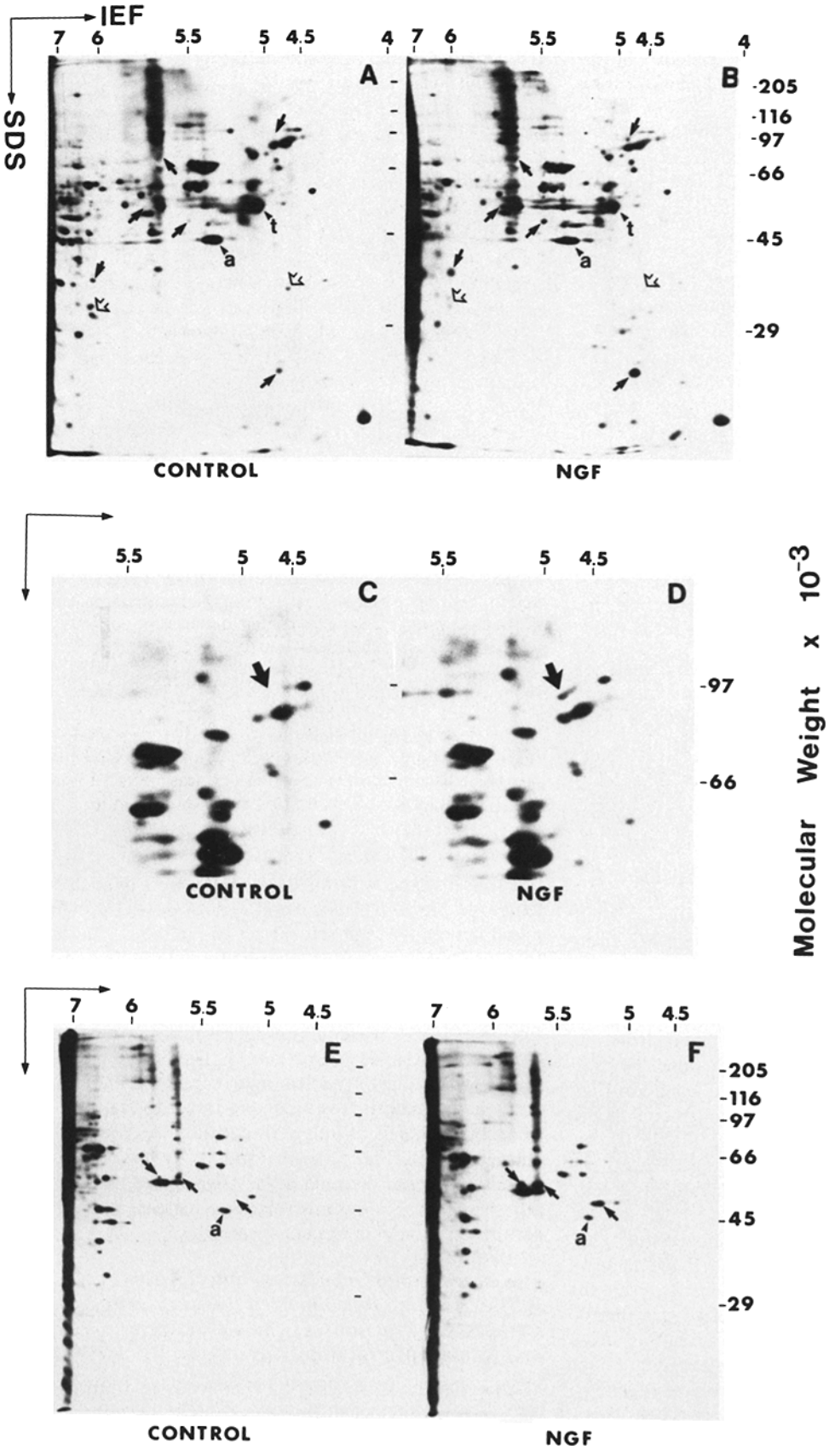

Figure 5. Two-dimensional gel electrophoretic profiles of [^{35}S]methionine pulse-labeled proteins extracted from the cytoplasmic fractions, high salt extracts, and nuclear matrix fractions. PC12 cultures were incubated for 8 h in the absence (*A*, *C*, and *E*) or presence (*B*, *D*, and *F*) of NGF and pulse-labeled with 50 μCi/ml [^{35}S]methionine from 7 to 8 h. Cell fractionation and two-dimensional gel electrophoresis were carried out as described in Materials and Methods. (*A* and *B*) Cytoplasmic proteins (600,000 cpm/gel; 4-d exposure); (*C* and *D*) cytoplasmic proteins, enlarged fluorograph showing the induction of 92-kD protein (450,000 cpm/gel; 4-d exposure); (*E* and *F*) nuclear matrix proteins (90,000 cpm/gel; 11-d exposure). Arrows indicate proteins that were found to be reproducibly (n = 5) induced after 8 h in NGF and open arrows point to the proteins whose labeling was decreased by NGF. The proteins actin and tubulin are marked *a* and *t*, respectively.

not reveal qualitative changes in rates of protein synthesis after NGF treatment (12, 43), it was hoped that isolation of a particular subcellular fraction would allow detection of minor proteins that would otherwise remain masked by the high complexity of total protein extracts.

Comparison of the cytoplasmic fractions (which contained ~90% of total TCA-precipitable radioactivity, Table III) from control and induced PC12 cultures are shown in Fig. 5, *A* and *B*, respectively. The three major radioactive spots that were equally labeled in both control and NGF-treated cytoplasmic fractions corresponded to actin (43 kD, pI 5.2–5.4), tubulin (55 kD, pI 5.1), and to a heterogenous spot of ~72 kD (pI 5.3). These proteins were also the major spots among the few detectable by Coomassie Blue staining (not shown). The fluorographs not only confirmed that NGF increased the labeling of proteins with apparent M_rs of 75,000 (pI 5.6), 50,000 (pI 5.4), and 38,000 D (pI 6.0), as already noted in the one-dimensional analysis, but they also revealed two other proteins whose labeling was increased by NGF, i.e., the 55,000- (pI 5.7) and 25,000-D (pI 4.7) proteins. It was also noted that NGF markedly decreased the labeling of three low molecular mass proteins of 32, 34, and 36 kD (open arrows in Fig. 5, *A* and *B*). As observed in all of five separate fluorographs, NGF also induced the appearance of a new protein with an M_r of 92,000 D (pI 4.8; Fig. 5, *A*, *B*, *C*, and *D*), which appears to be synthesized de novo since it was undetectable in controls (even after overexposure of the films). Induction of the 92-kD protein was completely blocked when cultures had been treated with 2 μg/ml actinomycin D (not shown). The two-dimensional profiles of proteins from the high salt extracts were similar to those of chromatin fractions extracted from other mammalian cells (4, 10) (data not shown). The high salt extracts were enriched in acidic proteins with M_rs of <40,000 D, as expected for a non-histone chromosomal protein pattern. The profiles did not differ significantly between control and NGF-treated cultures with the exception that NGF increased the labeling of a 35-kD protein (pI 5) and markedly decreased the labeling of a 39-kD protein (pI 5.3).

The NGF-induced changes in rates of protein synthesis observed in the nuclear matrix fraction, which retained ~2.5% of newly synthesized total proteins (Table III) are shown in Fig. 5, *E* and *F*. Although low amounts of radioactive tubulin could be detected in the high salt extracts, virtually no tubulin was observed in the nuclear matrix fractions. As shown in Fig. 5 *F*, 8-h treated cultures exhibited a marked increase in the rate of synthesis of a 50-kD protein (pI 5.3) and of two 56-kD proteins with pIs of 5.7 and 5.8, respectively. The fact that the NGF-induced 50-kD protein was exclusively present in the nuclear matrix fraction is a good indication that it corresponds to the NGF-induced 50-kD protein detected by one-dimensional gel electrophoresis (Fig. 4, lane *f*).

Analysis of [^{35}S]Methionine Pulse-labeled Cytoplasmic and Nuclear Proteins by NEPHGE

To obtain a better resolution in the pH 6–9 range, aliquots of [^{35}S]methionine pulse-labeled cytoplasmic proteins were analyzed in the nonequilibrium system. Control and NGF-treated cultures were pulse-labeled with [^{35}S]methionine from 7 to 8 h either in the absence or presence of 2 μg/ml actinomycin D added at time 0. Cytoplasmic fractions were isolated as described above and aliquots containing the same amounts of proteins (as judged by analysis of parallel samples on one-dimensional gels) were analyzed. The results of a typical experiment are shown in Fig. 6. As expected from the results described above (Fig. 2 *A*, lanes *a* and *b*; Fig. 5, *A* and *B*), the patterns of labeled cytoplasmic proteins were almost identical in control and NGF-treated cultures. However, these analyses showed (Fig. 6 *B*) that NGF dramatically increased the rate of synthesis of proteins with M_rs of 30,000 (pI 7.2), 38,000 (doublet, pI 6.6–6.8), 48,000 (pI 5.3), and 54,000 D (doublet, pI 5.6–5.8); the 30, 38 (pI 6.6), and 54-kD proteins being detectable in control cultures only after overexposure of the film. Induction of these six proteins was completely abolished in NGF-treated cultures under conditions where nearly complete inhibition of RNA synthesis was achieved (Fig. 6 *d*). This is in contrast to the lack of effect of actinomycin D on the rate of synthesis of several other proteins, i.e., actin or a triplet of proteins with an M_r of 58,000. The nonequilibrium method thus identified two of the cytoplasmic proteins seen in the equilibrium analyses, the 38,000- and 54,000-D molecular mass species, and showed that they are doublets, while also identifying two other proteins induced by NGF (Table I). No increase in the rate of synthesis of the cytoplasmic 38- and 54-kD proteins was observed in dbcAMP-treated cells (not shown). Taken together, the one- and two-dimensional analyses show that NGF reproducibly increases the rates of synthesis of ~11 cytoplasmic proteins and induces de novo synthesis of one other cytoplasmic protein within 8 h of exposure (Table I).

Analysis of [^{35}S]methionine pulse-labeled nuclear proteins in the nonequilibrium system did not reveal any significant changes, with the single exception of a 53-kD protein (pI 6.3) whose rate of synthesis was induced by more than 10-fold by 8 h after addition of NGF. The induction was transcription-dependent and did not occur when cells were treated with dbcAMP (not shown). The combined analyses reveal that NGF increases the rates of synthesis of five nuclei-associated proteins within 8 h of exposure (Table I).

Discussion

In this work, changes in the rates of protein synthesis were studied in PC12 cultures during the early steps of NGF-induced differentiation. As expected from previous studies (11, 12, 43, 44) no overall increase in protein synthesis was observed at either early or later times after induction by NGF (Fig. 1). Similarly, Huff et al. (25) did not find any significant increase in the overall rate of PC12 protein synthesis by 48 h after treatment with NGF. Also, Yamada and Wessells (61) showed that NGF-promoted axon elongation in chick dorsal root ganglia in vitro was not accompanied by a major increase in overall protein synthesis.

In view of the previous report (20) that total protein content (per DNA unit) slightly increased after NGF-treatment, it is possible that NGF may act at a posttranslational level, by decreasing the overall protein turnover rate. We therefore suggest that the slight accumulation of tubulin over a time course of 7 d after induction of NGF (7), may be the result of an NGF-induced decrease in the turnover of tubulin. Although relatively few data are yet available on protein stability in the nervous system, it is interesting to note that the protein breakdown rate in the immature rat brain is about twice as high as it is in the adult (33).

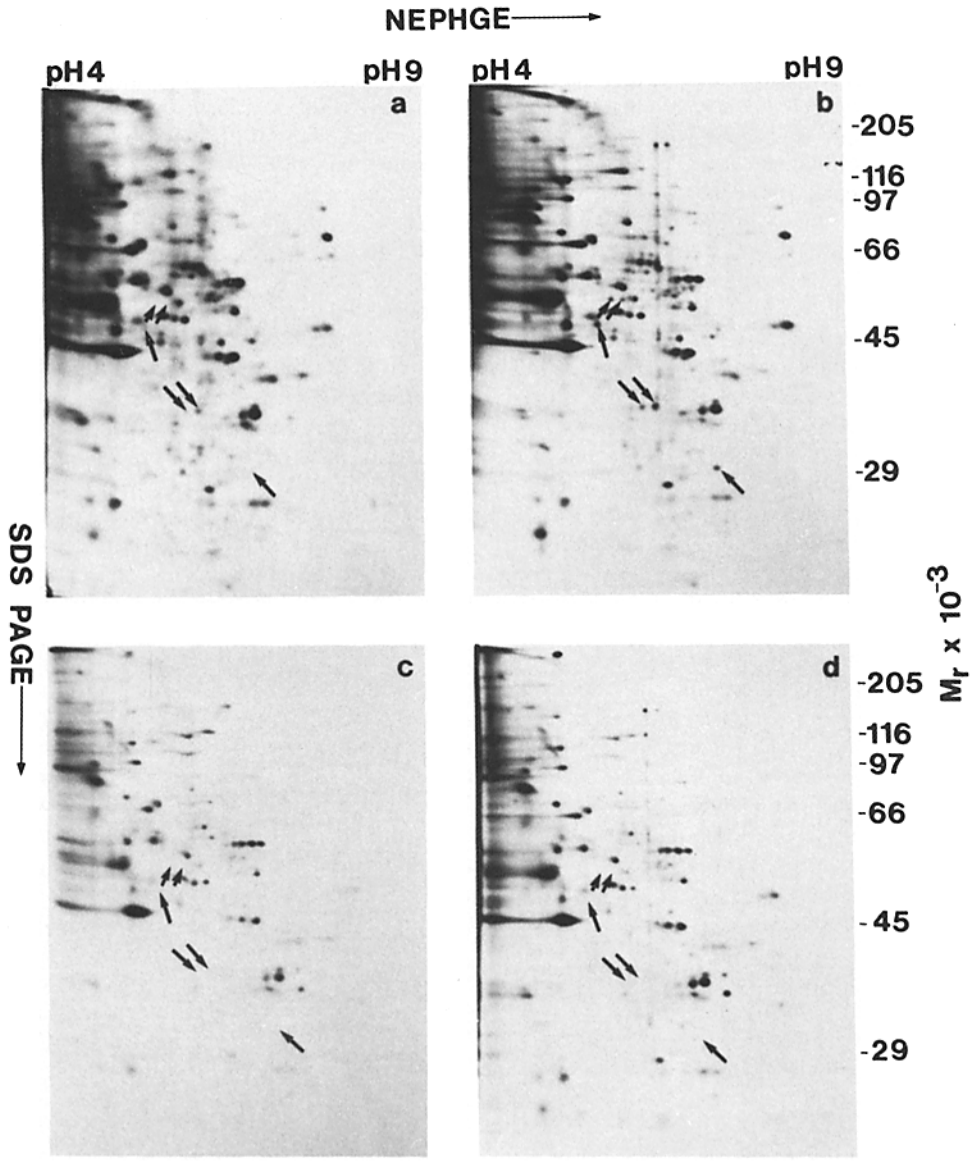

Figure 6. Analysis of [^{35}S]methionine pulse-labeled cytoplasmic proteins by NEPHGE. PC12 cultures were incubated for 8 h in the absence (*a* and *c*) or presence (*b* and *d*) of NGF and pulse-labeled with 50 μCi/ml [^{35}S]methionine from 7 to 8 h. In *c* and *d*, 2 μg/ml actinomycin D were present in the culture medium from 0 to 8 h. Cytoplasmic fractions were isolated as described in Materials and Methods and 30-μl aliquots were analyzed by NEPHGE as described in Materials and Methods. Labeled proteins were revealed by fluorography (4-d exposure). (*a* and *b*) 300,000 cpm/gel; (*c*) 125,000 cpm/gel; (*d*) 172,000 cpm/gel. The arrows indicate proteins that were induced by NGF and whose rates of synthesis were completely inhibited by actinomycin D.

The rates of synthesis of ~11 cytoplasmic and 5 nuclear PC12 cell proteins are increased by NGF (Table I) and the noncoordinate induction of some of these proteins (Fig. 1) suggests different functional roles, i.e., some may be involved in aspects of the differentiation pathway other than neurite outgrowth. The transient induction of the nuclear 56-kD protein by NGF (Fig. 1) was not expected to result in a significant increase in the amount of the protein. Indeed, no Coomassie Blue–stained band comigrating with the 56-kD protein was detectable in controls and in the NGF-treated cultures. However, increased levels of the 51.5-, 50- and 48-kD proteins were anticipated from their time courses of induction (Fig. 1) but, with the exception of the 51.5-kD protein, could not be conclusively detected on the Coomassie Blue–stained gels. This might be due to a faster turnover rate of the 50- and 48-kD proteins, as compared with the bulk part of cellular proteins, or to the fact that the same amounts of the total protein extracts were loaded on each lane, thereby masking increased levels of specific bands by the overall increase in total protein (20). Alternatively, if these NGF-induced proteins mainly accumulate in the neurites, they could be lost selectively upon washing the cultures before protein extraction. Normalization of the proteins loaded on each lane on a DNA basis might be necessary to detect increased levels of these NGF-induced proteins.

We were also able to detect NGF-induced de novo synthesis of a cytoplasmic protein with an M_r of 92,000 D (pI 4.8; Fig. 5, *A*, *B*, *C*, and *D*). The identity of this protein is presently unknown, but it should be noted that Levi et al. (38) have recently isolated a cDNA clone corresponding to an early NGF-induced mRNA encoding a 90-kD protein. It is also clear that NGF significantly decreases the apparent rate of synthesis of several nuclear and cytoplasmic proteins migrating in the 30–40-kD range (Fig. 5). The turndown synthesis of some proteins may be needed in order to proceed to the differentiation pathway, but it might also be directly related to a decrease in the rate of entry of the cells through the cell cycle.

Subnuclear fractionation studies showed that the 56- and 50-kD-proteins (Figs. 4 and 5) were specifically associated

with nuclear matrix structures prepared by successive DNAse I and 2 M NaCl extractions of purified nuclei and containing ~36% of nuclear proteins (Table II). Treatment of PC12 cells with NGF did not alter the polypeptide pattern obtained after DNAse I digestion and high salt extraction of the nuclei (Fig. 4, *e* and *f*), suggesting that the early steps of differentiation in PC12 cells are not accompanied by global structural changes in the organization of chromatin. This was perhaps expected since both control and NGF-treated PC12 cells represent asynchronous populations of cycling cells, at least during the first few days (15, 27). It is of considerable interest that three major NGF-induced proteins are associated with a nuclear matrix fraction, since the latter is now thought to play a role during DNA replication (50) and heterogeneous nuclear RNA transcription, processing, and transport (28, 39). Since NGF does influence part or all of these nuclear metabolic pathways, it is tempting to suggest that NGF-induced nuclear 56- and 50-kD proteins are an important and early step on the differentiation pathway.

The identity of these three proteins remains presently unknown. However, the insolubility in Triton X-100, the partial resistance to high salt extraction, and the apparent relative molecular mass pointed to the possibility that the 56-kD proteins might be vimentin. Although vimentin has been described as a major protein in the nuclear matrix fractions prepared from HeLa cells (56), 3T3 cells (4), BHK cells (6), or canine kidney cells (10), no radioactive spot corresponding to vimentin (with a pI very close to that of tubulin) was detected in the PC12 (±NGF) nuclear matrix preparations. This suggested either that contamination of nuclear preparations with intermediate filaments (37) was minimal, or alternatively, that our PC12 clone does not express detectable amounts of vimentin (49). Using a vimentin polyclonal antibody (kindly provided by Dr. R. O. Hynes, Massachusetts Institute of Technology, Cambridge, MA) (26), we were able to show, by Western blotting, that no immunoreactive protein could be found in the PC12 nuclear fraction in the molecular range expected for vimentin, whereas the antibody reacted with a cytoplasmic 58-kD protein (Tiercy, J.-M., and E. M. Shooter, unpublished data). That the 56-kD proteins described in this paper are not OrnDCase (M_r 55,000 D; reference 42) was inferred from the following arguments: (*a*) the time course of induction differs from the known time course of OrnDCase activity (9, 22); (*b*) the pI of the NGF-induced 56-kD proteins are significantly more basic than the pI of OrnDCase (42); and (*c*) induction of 56-kD proteins was not observed with dbcAMP. It is possible that the NGF-induced 56-kD proteins may be related to the recently described (51) peripherin, a 56-kD protein (pI 5.6) found specifically in the neurons of the peripheral nervous system. We might also consider the possibility that the nuclear matrix-associated 56-kD proteins correspond to c-*fos* or c-*myc* proteins, although the time course of induction by NGF and the inability of dbcAMP to induce the 56-kD proteins are not in favor of this interpretation.

The experiments also showed that the induction of the cytoplasmic 123-, 54-, 38-, and nuclear 56-, 53-, and 50-kD proteins was not observed after treatment with dbcAMP (Fig. 2 and Table I) and thus appeared to be NGF-specific. It is tempting to suggest that the inability of dbcAMP to sustain long term neurite outgrowth may be related to the impairment of the synthesis of a limited number of proteins. It is known in this regard that dbcAMP induces microtubule-associated protein 1 accumulation to a significantly lesser extent than NGF (7). It was striking to note that, although the majority of NGF-induced cytoplasmic proteins was also inducible by dbcAMP, none of the four NGF-induced nuclear proteins tested so far was inducible by dbcAMP. This suggests that some nuclei-associated proteins are required for complete differentiation of PC12 cells by NGF. The experiments also point to the fact that the effects of NGF on these proteins is under transcriptional control (Fig. 2). It thus would be of great interest to test the hypothesis that other agents that induce either unstable neurites or a much lower density of outgrowth, such as fibroblast growth factor (58), extracellular matrix derived from bovine corneal endothelial cells (60), or C6 glioma-conditioned medium (8), or that do not induce neurite outgrowth at all, like epidermal growth factor, are also unable to induce these nuclear proteins and are thus unable to allow PC12 cells to differentiate past a certain limiting step. In any case, the data suggest that early effects of NGF, before the onset of significant neurite outgrowth takes place, include activation of specific genes.

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