Altered Gene Expression in Neurons during Programmed Cell Death: Identification of c-*jun* as Necessary for Neuronal Apoptosis

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Abstract. We have examined the hypothesis that neuronal programmed cell death requires a genetic program; we used a model wherein rat sympathetic neurons maintained in vitro are deprived of NGF and subsequently undergo apoptosis. To evaluate gene expression potentially necessary for this process, we used a PCR-based technique and in situ hybridization; patterns of general gene repression and selective gene induction were identified in NGF-deprived neurons. A temporal cascade of induced genes included "immediate early genes," which were remarkable in that their induction occurred hours after the initial stimulus of NGF removal and the synthesis of some required ongoing protein synthesis. The cascade also included the cell cycle gene c-*myb* and the genes encoding the extracellular matrix proteases transin and collagenase. Concurrent in situ hybridization and nuclear staining revealed that while c-*jun* was induced in most neurons, c-*fos* induction was restricted to neurons undergoing chromatin condensation, a hallmark of apoptosis. To evaluate the functional role of the proteins encoded by these genes, neutralizing antibodies were injected into neurons. Antibodies specific for either c-Jun or the Fos family (c-Fos, Fos B, Fra-1, and Fra-2) protected NGF-deprived neurons from apoptosis, whereas antibodies specific for Jun B, Jun D, or three nonimmune antibody preparations had no protective effect. Because these induced genes encode proteins ranging from a transcription factor necessary for death to proteases likely involved in tissue remodeling concurrent with death, these data may outline a genetic program responsible for neuronal programmed cell death.

MATURATION of the nervous system involves a sculpting process wherein approximately half of all neurons born during neurogenesis die, typically around the time of their functional contact with target tissue. Neurons are thought to compete for sufficient amounts of trophic factor support from target tissues, those that are less successful receive inadequate quantities and die (Oppenheim, 1991). An example of this naturally occurring, or programmed cell death (PCD)[1], takes place in the rat superior cervical ganglia (SCG) in the perinatal period during which approximately 40% of the neurons die (Wright et al., 1983). This process can be recapitulated in vitro by isolating the neurons from fetal rats, maintaining them in the presence of their natural neurotrophic factor, NGF, for 6 d and then removing the NGF. Over the subsequent 48 h, the vast majority of the neurons die in a process that manifests the hallmarks of apoptosis (Arends and Wyllie, 1991). An initial period of ∼12 h without morphologic change is followed by an interval wherein the cell soma shrink, the plasma membranes bleb, and the chromatin condenses, all without marked changes in mitochondria or evidence of osmotic shock (Martin et al., 1988). By 19 h after NGF deprivation, genomic DNA is being degraded into nucleosomal fragments (Deckwerth and Johnson, 1993; Edwards et al., 1991). Neuronal death as assessed by staining with vital dyes begins ∼24 h after deprivation and is largely complete by 48 h. While apoptosis appears a fundamental mechanism during nervous system maturation, the contribution of this form of cell death to neuropathology is currently just beginning to be explored. The recent observations that nucleoso-

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1. *Abbreviations used in this paper:* IEG, immediate early gene; NFM, *neurofilament-M*; PCD, programmed cell death; rhl, *rat-hairy-like*; RT, reverse transcription; SCG, superior cervical ganglia; TOH, tyrosine hydroxylase.

mal DNA fragmentation accompanies neuronal death in models of ischemia (MacManus et al., 1993, 1994; Linnik et al., 1993) and that neurons maintained in vitro can be induced to undergo apoptosis by certain drugs (cytosine arabinoside, [Deckwerth and Johnson, 1993; Martin et al., 1990]), cytokines (leukemia inhibitory factor and ciliary neuron trophic factor, [Kessler et al., 1993]) or the Alzheimers amyloid β-protein (Loo et al., 1993), are suggestive that neuronal apoptosis may be recapitulated aberrantly in the mature nervous system, leading to inappropriate neuronal loss.

Studies with RNA and protein synthesis inhibitors have demonstrated that neuronal PCD in vitro (Martin et al., 1988) and in vivo (Oppenheim et al., 1990), as well as in certain other models of PCD (reviewed in Freeman et al., 1993; Ellis et al., 1991), is dependent on macromolecular synthesis. This has led to the hypothesis that PCD is the result of the activation of a genetic program that culminates in the synthesis of proximate "killer" proteins, or "thanatins" (Johnson et al., 1989). To elucidate the timing of the synthesis of these putative thanatins in the in vitro model described here, we and others have examined the ability of cycloheximide added at various times after NGF deprivation to block neuronal death. In the typical model where neurons are plated on a collagen substratum, 50% of the neurons are no longer salvageable by cycloheximide at ∼17 h after deprivation (Deckwerth and Johnson, 1993; Edwards et al., 1991; Tanaka and Koike, 1992), suggesting that the proteins necessary and sufficient for death in 50% of the cells have been synthesized by this time.

To evaluate this process further, we screened "suspect" genes to identify those that are induced in dying neurons and, therefore, are candidates for mediating neuronal death. Although this primary neuron model system has many positive attributes, including involvement of physiologically appropriate cell death in a well-characterized, highly reproducible, in vitro paradigm, the primary culture aspect of its nature limits the numbers of cells and, thereby, quantities of RNA that are available for analyses. Therefore, we used a quantitative and extremely sensitive PCR-based technique for this screening. Here, we report (a) the identification of a temporal cascade of gene induction and repression that accompanies neuronal death; and (b) the identification of gene products necessary for apoptosis. The genes implicated as necessary include c-jun, which was induced after NGF deprivation, and the Fos family in general, certain members of which were induced after NGF deprivation, i.e., c-fos and fos B, while others were repressed, i.e., fra-1 and fra-2. Much of this work has been reported in abstract form (Estus, S., and E. M. Johnson, Jr. 1992. Soc. Neurosci. Abstr. 18:51; Estus, S., R. S. Freeman, and E. M. Johnson, Jr. 1993. Soc. Neurosci. Abstr. 19:634).

Materials and Methods

Mouse NGF was prepared by the method of Bocchini and Angeletti (1969). Radiochemicals were purchased from Amersham Corp. (Arlington Heights, IL). Unless otherwise stated, remaining chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

Primary cultures of sympathetic neurons were prepared from SCG of embryonic day 21 rats, as described previously (Martin et al., 1992), except

that the nonneuronal cells were minimized by incubating the dissociated ganglia for 3 h on plastic tissue culture dishes before plating onto either collagen-coated 60-mm dishes (∼25,000 cells/dish) or poly-L-ornithine and laminin-coated (Collaborative Biomedical Products, Bedford, MA) chamber slides (∼2,000 cells/well). From the point of dissociation, the neurons were maintained in culture medium consisting of 90% MEM (Gibco BRL, Gaithersburg, MD), 10% FCS (Hyclone Laboratories, Logan, UT), 50 ng/ml NGF, 20 μM uridine, and 20 μM fluorodeoxyuridine; after 5–7 d in culture, the proportion of nonneuronal cells, i.e., fibroblasts and Schwann cells, was ∼2%. Neurons were deprived of NGF by replacing the NGF-containing medium with the same medium, except that a polyclonal goat anti-NGF antiserum was substituted for NGF. In experiments involving the inhibition of protein synthesis, neurons were treated with cycloheximide at a concentration of 1 μg/ml.

cDNA Preparation

Primary cultures (∼25,000 neurons/dish) were maintained in the presence of NGF for 6 d and then deprived of NGF for the indicated intervals. Poly-A⁺ RNA was isolated by using an oligo-dT-cellulose mRNA purification kit (QuickPrep Micro kit; Pharmacia Fine Chemicals, Piscataway, NJ) and concentrated by coprecipitation with glycogen, all as directed by the manufacturer. We used Poly-A⁺, presumably functional, RNA for these studies to avoid the possible amplification of cDNAs corresponding to nonfunctional RNA fragments in the subsequent PCR step. Half of the mRNA was converted to cDNA by reverse transcription (RT) with Moloney murine leukemia virus reverse transcriptase (Superscript; Life Technologies Inc., Grand Island, NY) with random hexamers (16 μM) as primers. The 30-μl reaction contained 50 mM Tris (pH 8.3), 40 mM KCl, 6 mM $MgCl_2$, 1 mM dithiothreitol, 500 μM each dATP, dTTP, dCTP, and dGTP, and 20 U RNasin (Promega Corp., Madison, WI). After 10 min at 20°C, the samples were incubated for 50 min at 42°C, and the reaction was then terminated by adding 70 μl water and heating to 94°C for 5 min. Equivalent results were obtained when the other half of the mRNA was reverse transcribed with avian myeloblastosis virus reverse transcriptase (Promega).

PCR Analysis

For PCR amplification of specific cDNAs, stock reactions (50 μl) were prepared on ice and contained 50 μM dCTP, 100 μM each dGTP, dATP, and dTTP, 15 μCi α-[³²P]-dCTP (3,000 Ci/mmol), 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris (pH 9.0), 0.1% Triton X-100, 1 μM each primer, 1 U Taq polymerase, and 1% of the cDNA synthesized in the RT reaction. Primer sequences are available upon request. The stock solutions were separated into three equal aliquots that were covered with a drop of mineral oil and subjected to various numbers of PCR cycles to determine the minimum number of cycles necessary to detect the PCR product. The typical reaction conditions were 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C. With few exceptions, the results depicted here represent 16–20 cycles of amplification; the data for S100β, transin, and collagenase were obtained with 24 cycles, while that of mkp-1 required 28 cycles. The number of PCR cycles required to generate detectable cDNA product does not necessarily correlate well with the abundance of the original mRNA; while the efficiency of RT-PCR is typically quite consistent for a given RNA and primer pair, RT efficiency can vary between RNAs because of variabilities in RNA secondary structure and between primers because of amplification efficiency. Therefore, the data presented should be taken to be indicative of changes in the abundance of mRNAs between samples and only a rough indicator of absolute mRNA levels. After amplification, the cDNAs were separated by electrophoresis on 12% polyacrylamide gels, visualized by autoradiography of the dried gels, and quantified with a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). The identity of the cDNA amplified by each primer pair was confirmed typically by either direct sequencing (fmol DNA Sequencing System; Promega) or by subcloning the amplified cDNAs into pBluescript (Stratagene, La Jolla, CA) and then sequencing the inserts. Parallel experiments examining PC12 cells treated with NGF alone or NGF and cycloheximide confirmed that the induction of each immediate early and related gene was detectable.

In Situ Hybridization

Cells were maintained on poly-L-ornithine/laminin-coated chamber slides, deprived of NGF for the indicated intervals, fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, and processed for in situ hybridization (Wanaka et al., 1990), except that proteinase K treatment was omitted. Slides were hybridized at 55°C for 16–18 h with ³⁵S-labeled RNA

probes (500,000 cpm/slide) corresponding to rat c-*jun* or c-*fos*. DNA templates for the c-*jun* and c-*fos* riboprobes were provided by S. Carroll and J. Milbrandt (Dept. of Pathology, Washington University School of Medicine, St. Louis, MO). Antisense and sense riboprobes were synthesized by using T7 and T3 RNA polymerases (Stratagene) and α-[^{35}S]UTP. Sense probes served as specificity controls. After treatment with ribonuclease and high stringency washes, slides were processed for emulsion autoradiography for 11–14 d. After development of the emulsion, the cells were stained for 10 min with Hoechst 33,258 (1 μg/ml) (Molecular Probes, Inc., Eugene, OR) in water, followed by a 10-min water rinse. Slides were viewed with phase-contrast, dark-field, and fluorescence microscopy.

Antibody Microinjection

Neurons, maintained for 3–4 d in vitro, were transferred to NGF-containing (50 ng/ml) L-15 medium. Approximately 25 neurons per dish were then injected (Graessmann and Graessmann, 1983) with affinity-purified polyclonal antibodies. The concentrations of these antibodies were: anti-Fos family, 5.0 mg/ml; anti-c-Jun, 8.0 mg/ml; anti-Jun B, 8.5 mg/ml; anti-Jun D, 5.9 mg/ml; anti-Jun family, 10 mg/ml; each of three separate nonimmune IgG preparations was 10 mg/ml. Antibodies specific for individual Jun family members were those used previously (Kovary and Bravo, 1991*a*, 1991*b*, 1992), while Jun and Fos family antibodies were prepared freshly; the specificity and neutralizing ability of the freshly prepared antibodies was validated as described previously (Kovary and Bravo, 1991*b*). After the microinjections, the neurons were returned to standard NGF-containing medium for 2 h and then either continued with NGF or deprived of NGF. Approximately 40 h later, the neurons were fixed with 4% paraformaldehyde, permeabilized for 10 min with 0.05% NP-40 and 2% BSA in PBS, incubated with 2% BSA in PBS for 1 h, and then with a goat anti-rabbit IgG labeled with the fluor cy-3 (Chemicon International, Inc., Temecula, CA) in 2% BSA in PBS overnight. Since the injected antibodies were raised in rabbits, this cy-3-conjugated antibody allowed us to identify injected neurons. After washing in PBS and then staining with Hoechst 33,258 for 5 min, injected neurons were scored for chromatin integrity by an observer lacking knowledge of their treatments. Approximately 25% of the injected cells did not regain membrane integrity after the injection and died rapidly; these dead cells did not influence the results obtained at the time of scoring because the injected antibodies rapidly dissipated from the dead cells, precluding their subsequent labeling by the fluorescent antibody.

Results

### RT-PCR Quantitation of Changes in Gene Expression in NGF-deprived Neurons

To assess changes in mRNA levels in neuronal cultures undergoing apoptosis, dissociated SCG neurons (~25,000 neurons per time point) were maintained on a collagen substratum and then deprived of NGF for various intervals. For each interval, Poly-A$^+$ RNA was isolated, converted to cDNA, and portions of the cDNA were subjected to PCR. We validated this RT-PCR assay in initial studies by showing that the PCR product yields were linear with respect to input RNA and that the technique could be used to detect an expected gene induction in SCG neuronal cultures, i.e., HSP70 after a heat shock (data not shown). To determine a baseline of gene expression in the cultures during the NGF deprivation time course, we examined the expression patterns of general cellular markers: cyclophilin, expressed constitutively in all cell types; neurofilament-M, tyrosine hydroxylase and neuron-specific enolase, mRNAs unique to neurons, and S100β, a marker for Schwann cells, one of the nonneuronal cell types in the cultures. In initial experiments, the amount of RNA used in each RT-PCR reaction was held constant. Under these conditions, the neuronal markers decreased over time and, as the proportion of mRNA derived from nonneuronal cells increased, the levels of S100β increased, causing S100β to appear to be induced (data not shown). Subsequently, we found that when the RT-PCR technique was

normalized such that each RT-PCR sample used a constant proportion, e.g., 0.5%, of the RNA isolated from each culture, the resultant PCR data indicated that the mRNAs encoding the neuronal markers decreased over the duration of NGF withdrawal while that encoding S100β remained constant (Fig. 1 *A*). While the parallel behavior of the cyclophilin and neuronal marker mRNAs reflected the purity of the largely neuronal cultures, the magnitude of their decrease before death was unexpected and may reflect decreased RNA stability before neuronal death (see Discussion).

These expression patterns were then compared with those of candidate "death genes" that were studied because they are induced in other PCD models (reviewed in Freeman et al., 1993), see also (Woronicz et al., 1994; Liu et al., 1994; Discussion). Except for c-*fos*, the genes either manifested expression patterns similar to those of the neuronal markers (Fig. 1 *B*) or were not detected, i.e., *NGFI-B* (*nur 77*); a robust *NGFI-B* induction was detectable in control experiments (data not shown). In marked contrast to the decreasing expression of other genes, c-*fos* was induced sharply 15 h after NGF deprivation (Fig. 1 *C*). In pursuit of this observation, we identified several other immediate early genes (IEGs) that were also induced, including c-*jun*, *MKP-1*, *fos-B*, *jun-B*, rat-hairy-like (*rhl* or *HES-1*), and *NGFI-A*. This induction was selective because the pattern associated with neuronal marker genes was observed for IEGs such as *fra-1*, *NFkappaB* (*relA*) (Fig. 1 *C*), *RP-8* (Fig. 1 *B*), as well as *fra-2* and *RP-2* (data not shown); other IEGs such as *KROX-20* were not detected, similar to *NGFI-B*, although robust signals were observed in control experiments (data not shown).

To assess indirectly whether the transcription factors encoded by these induced genes (Angel and Karin, 1991; Cochran, 1993) may be functional, we examined genes that these factors are known to induce. The overexpression of c-Jun increases c-*myb* (Nicolaides et al., 1992), which encodes a transcription regulator that is elevated during late G$_1$ and linked with the G$_1$-to-S-phase cell cycle transition (Gewirtz et al., 1989); c-*myb* was induced in dying neurons with a pattern similar to c-*jun* (Fig. 1 *C*). In addition, two members of the collagenase extracellular matrix protease family, *transin* and *collagenase*, are induced by c-Fos/c-Jun complexes (McDonnell et al., 1990) and, indeed, were induced in the neuronal cultures, peaking at 20 h (Fig. 1 *C*). When considered en masse, these expression patterns delineated a temporal cascade of three waves of gene induction (Fig. 1 *D*), wherein c-*jun*, *MKP-1*, and c-*myb* were induced early, followed by several genes that were induced nearly simultaneously, e.g., c-*fos*, *fos B*, *jun B*, and *NGF-IA*, and then *transin* and *collagenase*.

The IEG induction during apoptosis was remarkably delayed for genes of this class (reviewed in Angel and Karin, 1991; Cochran, 1993), beginning hours after the initial stimulus of NGF removal. This suggested that these genes were not induced in the typical IEG manner, i.e., in direct response to external perturbation, but rather as a consequence of intracellular events. To evaluate this possibility further, we examined whether the induction of the IEGs was dependent on ongoing protein synthesis; at the time of NGF removal, cycloheximide was added at a concentration of 1 μg/ml, which blocked virtually all protein synthesis (Martin et al., 1988). mRNA was isolated 15 h later and analyzed for the expression of the prototypic IEGs, c-*jun* and c-*fos* (Fig. 2). Treatment with cycloheximide caused an induction
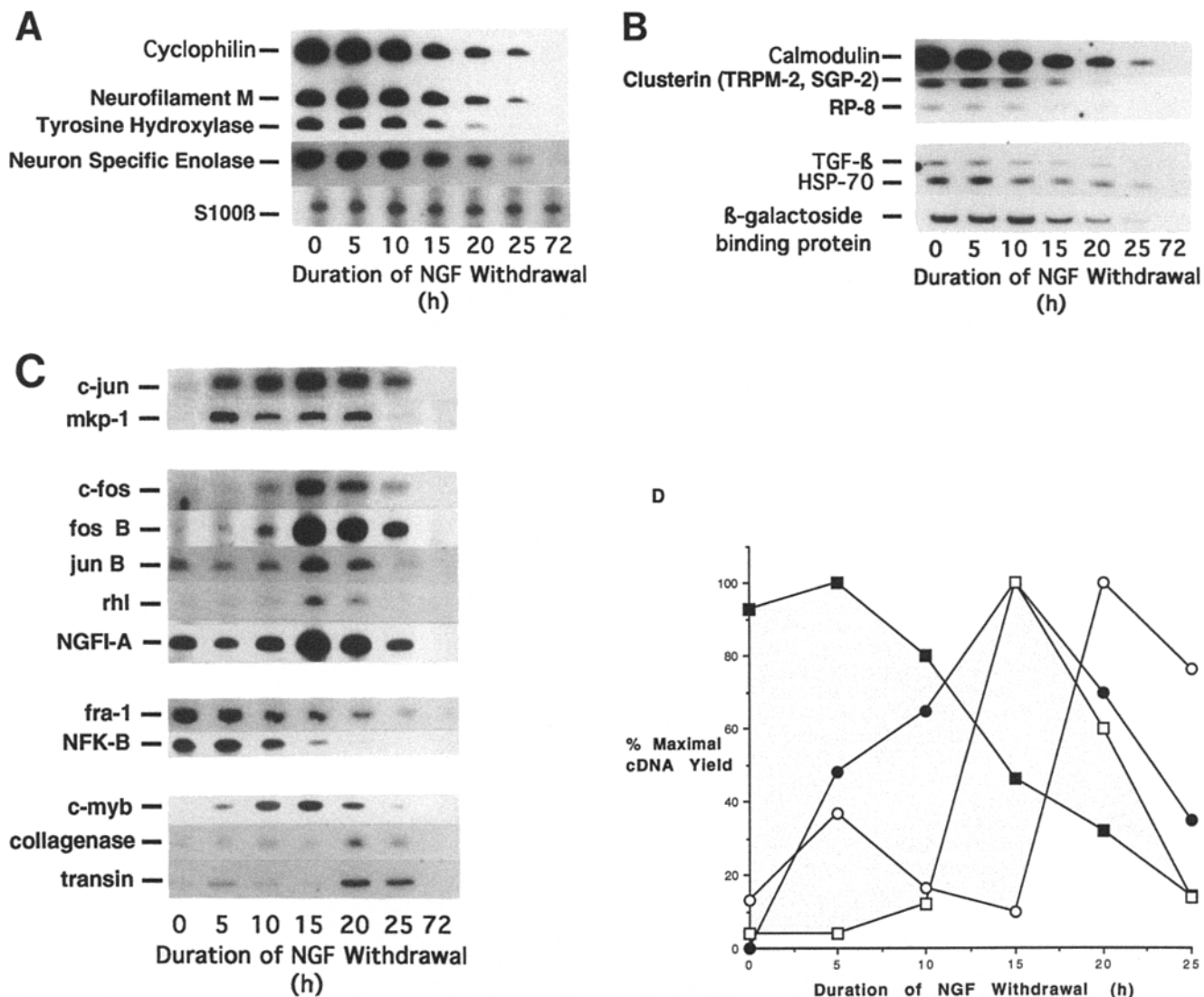
Figure 1. mRNA expression in SCG cultures undergoing NGF deprivation–induced apoptosis. (*A*) Cellular marker genes; (*B*) genes induced during PCD in other model systems; (*C*) immediate early and related genes; (*D*) waves of gene activation and repression. To assess changes in mRNA levels, primary cultures (∼25,000 neurons/dish) were maintained with NGF for 6 d and then deprived of NGF for the indicated intervals; poly-A+ RNA was isolated, half converted en masse to cDNA, and then 1% of the resultant cDNA was analyzed in each PCR sample. The data presented are from a single preparation of neuronal cultures. Each gene induction was confirmed in at least two independent neuronal preparations. The depicted gene inductions were not observed in response to medium change per se. -■-, *Neuron-specific enolase*; -●-, *c-jun*; -□-, *c-fos*; -○-, *transin*.

of each gene even in the presence of NGF (Fig. 2, lanes *3* and *5*), a characteristic of IEGs that has been reported before in other cell types, although typically in instances involving much shorter cycloheximide treatments (Greenberg et al., 1986). Simultaneous NGF deprivation and cycloheximide treatment had an additive effect on c-*jun* induction, suggesting that c-*jun* was induced by virtue of posttranslational events. In contrast, simultaneous NGF deprivation and cycloheximide treatment resulted in no c-*fos* induction. We interpreted this observation as a suggestion that the induction of c-*fos* after NGF withdrawal required ongoing protein synthesis. We were surprised that NGF deprivation blocked the induction of c-*fos* by cycloheximide; the mechanism whereby cycloheximide treatment induces c-*fos* is unclear, but was ap-

parently either not present or inhibited in NGF-deprived neurons. Hence, c-*jun* induction was in a typical "immediate" fashion, whereas the c-*fos* induction was dependent on ongoing protein synthesis, a highly atypical finding that suggested that the induction of this gene resulted from intracellular events that lay well downstream of the external perturbation of NGF withdrawal.

In Situ Analyses of Gene Expression and Chromatin Condensation

The decrease in expression of each gene except S100β at 72 h after deprivation strongly suggested that these genes were expressed in neurons and not in the small number of fibroblasts and Schwann cells contaminating the cultures.
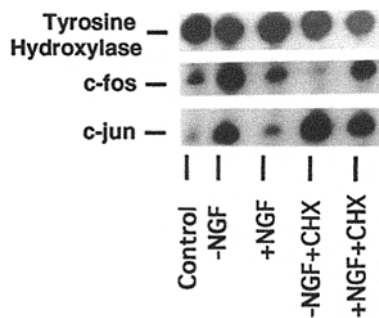
Figure 2. c-jun is induced via posttranslational mechanisms, whereas c-fos induction requires protein synthesis. After neuronal cultures were deprived of NGF for 15 h in the presence or absence of cycloheximide (1 μg/ml), mRNA was isolated and analyzed for changes in gene expression by using RT-PCR. The tNGF sample represents a medium change control. c-jun and c-fos were each induced by treatment with cycloheximide in the presence of NGF or by NGF deprivation. When neurons were subjected to both NGF deprivation and cycloheximide, c-jun was induced in an additive fashion, whereas the induction of c-fos was abrogated. These results were replicated in two separate neuronal preparations.

However, to confirm this and to assess the heterogeneity of neuronal expression, we performed in situ hybridization analyses for two representative IEG, c-jun and c-fos. Because the collagen substratum is not stable during in situ hybridization conditions, neurons were maintained on poly-L-ornithine/laminin, which slightly decreased the rate of neuronal death (unpublished observations). To visualize chromatin, neurons were stained with Hoechst 33,258. In NGF-deprived cultures, c-jun was induced in most neurons at 8, 15, and 24 h (Fig. 3, A and B). Consistent with the RT-PCR results, little c-jun or c-fos expression was detected in NGF-maintained cultures (Fig. 3, C and D). In contrast to the widespread c-jun induction, the induction of c-fos was sharply restricted to a subset of neurons undergoing NGF withdrawal (Fig. 3, E–L). Moreover, the chromatin of c-fos–positive neurons typically manifested a punctate or condensed globular appearance (Fig. 3, H and K); by phase-contrast microscopy, the morphology of these neurons was altered in a manner suggestive of cellular shrinkage and/or withdrawal from the extracellular matrix (Fig. 3, I and L). Thus, c-jun was expressed for extended periods in neurons undergoing NGF deprivation, while c-fos was induced transiently in neurons just before or during chromatin condensation.

## Intracellular Microinjections of Neutralizing Antibodies

To examine the critical question of whether members of the Jun or Fos protein families are functionally necessary for neuronal apoptosis, we microinjected neurons with antibodies that recognize either the entire Jun family (c-Jun, Jun B, and Jun D), the entire Fos family (c-Fos, Fos B, Fra-1, and Fra-2), or individual family members (Kovary and Bravo, 1991a, 1991b, 1992). These affinity-purified rabbit polyclonal antibodies have been characterized extensively. Their specificity was documented in immunoprecipitation studies examining cells at various stages of the cell cycle, as well as proteins synthesized in reticulocyte lysates in vitro (Kovary

and Bravo, 1991a, 1991b, 1992). The antibodies specific for individual family members were prepared by first passing the antibodies over columns containing other family members and then absorbing the effluent onto a column containing the protein of interest, from which the final antibody samples were eluted. The neutralizing ability of each antibody was evaluated by microinjecting the antibodies into serum-starved, quiescent fibroblasts that were then induced to reenter the cell cycle by serum stimulation (Kovary and Bravo, 1991b). Antibodies directed against the Jun or Fos family or any individual Jun family member block cell cycle reentry; antibodies against individual Fos family members are only effective in combination (Kovary and Bravo, 1991b). For the studies reported here, neurons were microinjected with the antibodies, allowed to recover for several hours, and were then either maintained with NGF (to assess antibody toxicity) or deprived of NGF for ∼40 h (to assess effects on apoptosis). Neurons were then fixed, stained with a fluorescent antibody against rabbit IgG (to identify injected neurons) and with Hoechst 33,258 (to label chromatin), and scored for chromatin integrity by an observer lacking knowledge of their treatments. NGF-deprived neurons injected with nonimmune control IgG preparations underwent apoptosis as usual; soma and neurites degenerated and chromatin was typically condensed or nonexistent (Fig. 4). In contrast, NGF-deprived neurons that were injected with antibodies against the Jun or Fos family were largely protected from apoptosis; neurons manifested a plump soma, intact neurites, and normal chromatin (Fig. 4, Table I). Since these results strongly suggested that at least one member of each family was necessary for death, we examined antibodies specific for individual family members. Unequivocal results were obtained with antibodies specific for Jun family members. Antibodies against c-Jun, but not Jun B or Jun D, were nearly as effective as the Jun family antibody in blocking death (Table I), suggesting that the Jun family antibody acted primarily through neutralizing c-Jun. In summary, although alternative techniques will be necessary to assess individual Fos family members adequately, the results obtained with the Fos family antibody strongly suggest that at least one
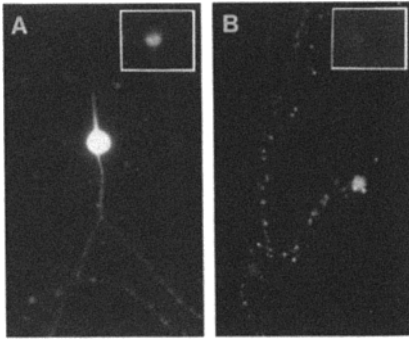
Table I. Neutralizing Antibodies Protect Neurons from NGF Deprivation

Antibody specificity	Number of cells scored	Chromatin Integrity (Percentage of cells scored)		
		Uniform	Condensed	Undetectable
Jun Family	204	78	10	11
Fos Family	271	76	6	18
c-Jun	71	59	23	18
Jun B	68	12	4	84
Jun D	80	1	4	96
Nonimmune IgG	279	6	11	83

"Uniform" refers to chromatin that appeared homogenously distributed within the nucleus, "condensed" refers to chromatin that was clearly punctate or globular, and "undetectable" refers to cells wherein the chromatin was digested and no longer detected. Three different nonspecific IgG preparations were tested at concentrations (10 mg/ml) equal to or greater than each of the specific antibody preparations and did not block apoptosis. Accumulated data from 3–11 independent experiments are presented; each neuron that was identified as injected was scored. None of the antibodies were toxic to neurons maintained in the presence of NGF.

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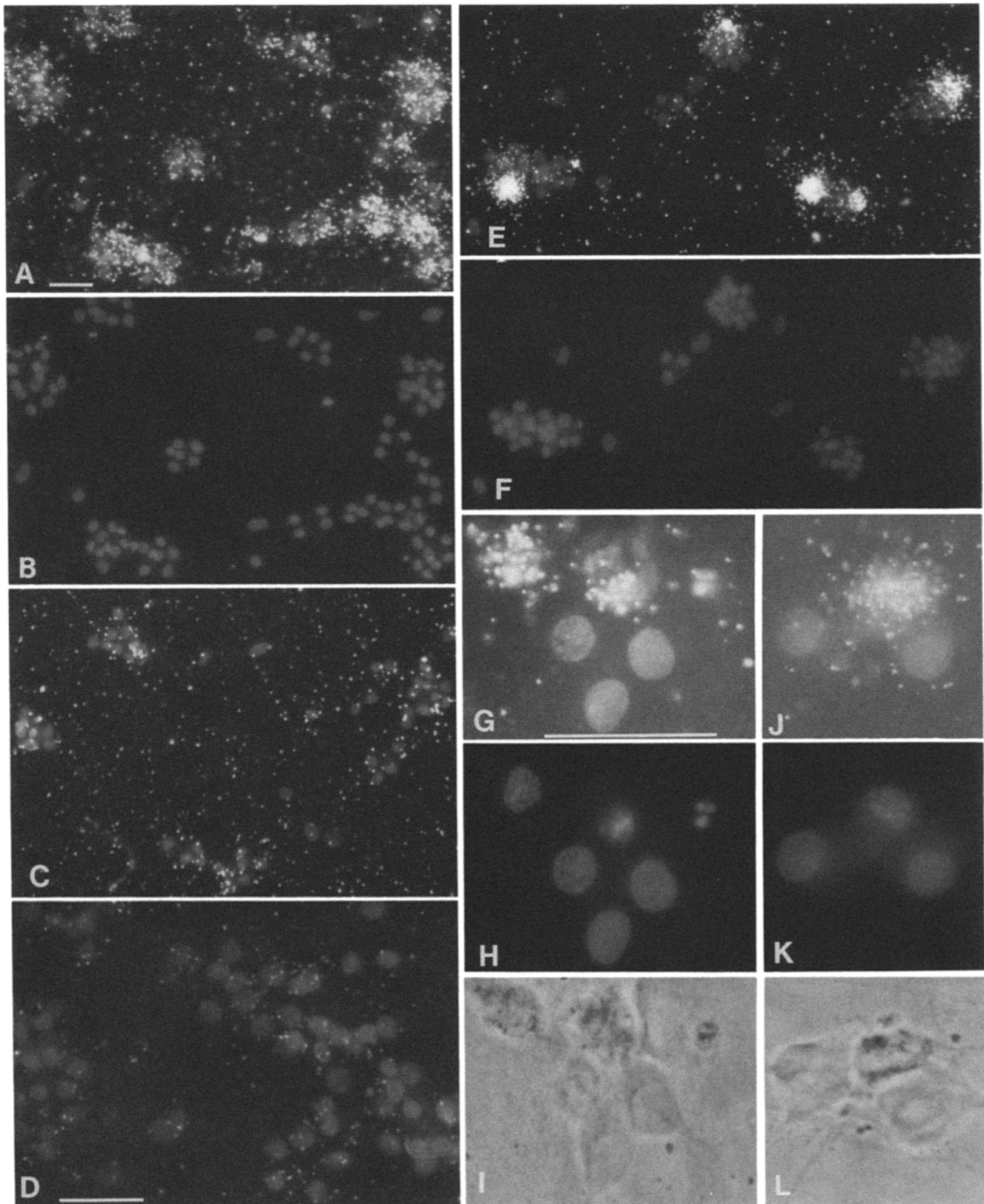
Figure 3. c-*jun* is induced in most neurons, whereas c-*fos* is restricted to neurons with apoptotic chromatin. Neuronal cultures were analyzed for c-*jun* (*A–C*) or c-*fos* (*D–L*) expression by performing in situ hybridization and for chromatin integrity by staining with Hoechst 33,258. Cultures were maintained with NGF (*C* and *D*) or deprived of NGF for 24 h (*A, B,* and *E–L*). The depicted images represent dark-field and fluorescence (*A, C, D, E, G,* and *J*), fluorescence (*B, F, H,* and *K*), or phase-contrast (*I* and *L*) microscopy. Multiple images of the same neurons are depicted in *A* and *B; E* and *F; G, H,* and *I;* and *J, K,* and *L.* Sense cRNA probes did not label above background. These results were replicated in at least three separate neuronal preparations. c-*jun* was also induced at 8 and 15 h after NGF deprivation (data not shown). Bar, 50 μm; *A–C, E,* and *F* were at the same magnification; *G–L* were at a fourfold greater magnification.

Figure 4. Microinjected neutralizing antibodies protect neurons from apoptosis. The depicted neurons were microinjected with a rabbit polyclonal affinity-purified antibody against the c-Jun family (*A*) or an equal amount of nonimmune control antibody prepared similarly (*B*). After 40 h of NGF deprivation, the neurons were fixed and labeled with a fluorescent-labeled secondary antibody against rabbit IgG (to detect injected neurons) and Hoechst 33,258 (to reveal chromatin integrity; see insets).

member of this protein family was necessary for death. Moreover, these results indicate that one gene induced after NGF deprivation, c-*jun*, was necessary for neuronal apoptosis.

Discussion

This manuscript contains several primary results. First, NGF deprivation leads to wholesale decreases in neuronal RNA expression. Second, counter to this generalized RNA decrease, several genes were induced in temporal waves. Third, the protein products of certain of the modulated genes appeared necessary for apoptosis to proceed; these proteins included c-Jun specifically and member(s) of the Fos family. Last, the successful use of RT-PCR to quantify a great many genes in exceedingly small amounts of RNA represents a validation for this technique as a screening process. For example, in the work reported here, cDNA sufficient for 200 PCR analyses was generated with the RNA isolated from ∼25,000 neurons; this quantity of RNA (∼0.5 μg of total RNA) is equivalent to that isolated from the SCG of four rat fetuses. Since typical ribonuclease protection or Northern blot assays require more RNA than this for each analysis, the examination of the many genes referred to here would involve such a great number of rats that this study would have been impractical without the use of RT-PCR.

Patterns of Gene Expression during Neuronal Apoptosis

Patterns of both gene repression and induction have been identified in SCG neurons undergoing NGF deprivation-induced apoptosis. Indeed, the vast majority of genes that were examined, ∼70 mRNAs, as well as rRNA (data not shown), decreased in prevalence after NGF deprivation. The magnitude of this decrease was significant (∼50%) by 15 h after NGF removal, before the neurons were committed to die, and well before the neurons actually began to lose membrane integrity at 24 h. Although a decrease in global RNA synthesis is suggested by studies involving radiolabeled uri-

dine incorporation (Deckwerth and Johnson, 1993), we hypothesize that the global decrease in RNA levels also reflects an active RNA degradative process based on the following reasoning: if the decrease in expression is caused solely by decreased global synthesis, RNAs would be expected to decline with rates that reflect the half-life of each RNA species. However, the vast majority of RNAs declined with similar patterns after NGF deprivation, and since these RNAs are very diverse, e.g., *NF-M* versus *fra-1*, we expect that the RNAs will have variable half-lives. We are presently pursuing this possibility by examining the half-lives of specific mRNAs before and during NGF deprivation.

Counter to this general decrease in gene expression, several temporal waves of gene induction occurred in the hours after NGF deprivation. Transcription factors such as c-*jun* and c-*myb*, as well as the phosphatase *mkp-1*, were induced earliest, followed by the transcription factors c-*fos*, *fos B*, *NGFI-A*, and *rhl* at 15 h after deprivation, and then *transin* and *collagenase* at 20 h. Although the IEGs may induce some of the other genes, i.e., c-*myb*, *transin*, and *collagenase*, the events leading to the induction of IEGs themselves remain to be elucidated. That the IEGs were induced many hours after the initial stimulus of NGF removal was suggestive that the genes were induced in response to intracellular events. Indeed, examination of the effects of cycloheximide on c-*jun* and c-*fos* induction revealed that while the induction of the former was increased with cycloheximide treatment, as would be expected for an IEG, the induction of c-*fos* (and *fos B*, data not shown) was blocked by cycloheximide. This finding was consistent with the possibility that c-*jun* was induced by posttranslational mechanisms (perhaps oxidative stress; see below), whereas c-*fos* was induced as part of a cellular genetic program that required ongoing protein synthesis. In situ analysis revealed that c-*fos* expression was highly restricted to neurons manifesting condensed chromatin. Whether c-*fos* induction was responsible for, or a result of, chromatin condensation requires further study. On the possibility that increased intracellular Ca^{++} activated c-*fos* transcription via increased phosphorylation of the cAMP response element binding protein, a known mediator of Ca^{++}- and cAMP-enhanced c-*fos* transcription (reviewed in Angel and Karin, 1991; Cochran, 1993), we labeled neurons with an antibody specific for this phosphoprotein (Ginty et al., 1993); no increased labeling was observed in NGF-deprived neurons manifesting condensed chromatin, although a robust labeling was observed in parallel samples treated with a cAMP analogue (data not shown).

That both gene activation and repression may be critical for neuronal death is suggested by consideration of the known biology of the Jun and Fos families. Members of the Jun family form homodimers or heterodimers among themselves, while members of the Jun and Fos families form a variety of heterodimers (reviewed in Angel and Karin, 1991; Cochran, 1993). In the instance of c-*jun* and either *fra-1* or *fra-2*, both of which largely decreased after NGF deprivation, the resulting heterodimer appears antagonistic to c-Jun/c-Fos heterodimers at the *collagenase* promoter (Suzuki et al., 1991); the delayed induction of *collagenase* and *transin* could reflect competition between increasing levels of c-Fos and decreasing levels of Fra-1 or Fra-2. Hence, after NGF deprivation, both increases and decreases in gene expression likely result in an intricate variety of homo- and het-

erodimers among members of the Jun and Fos families. The absence of inhibitory dimerization partners may facilitate a normally "benign" gene, such as c-jun, causing toxicity. Consequently, although the concept of a "death program" is often considered in the context of gene activation, our results lead us to suggest that the decline of certain mRNAs (and subsequently proteins) may be equally important. Indeed, one might view the death program as representative of a differentiation pathway leading to a physiologically appropriate phenotype (dead) during development. As with other differentiation pathways, this death program would involve the upregulation of certain genes, e.g., c-jun, and the downregulation of other mRNAs, e.g., fra-1. Thus, the death program may constitute a combination of increased and decreased gene expression (Fig. 5).

Role of Altered Gene Expression in Apoptosis

In considering which of these gene inductions may be critical for death, we began by comparing the timing of their induction relative to the declining ability of cycloheximide to protect the neurons from death, which, in the system described here, is ~50% reduced at 17 h after NGF deprivation (Deckwerth and Johnson, 1993). On this criterion, the majority of genes were induced within a time frame consistent with their having a critical role in neuronal death, except for transin and collagenase, which were induced at 20 h. Although apparently not critical for death, these extracellular matrix pro-

"Some Up, Some Down" Model for Neuronal PCD
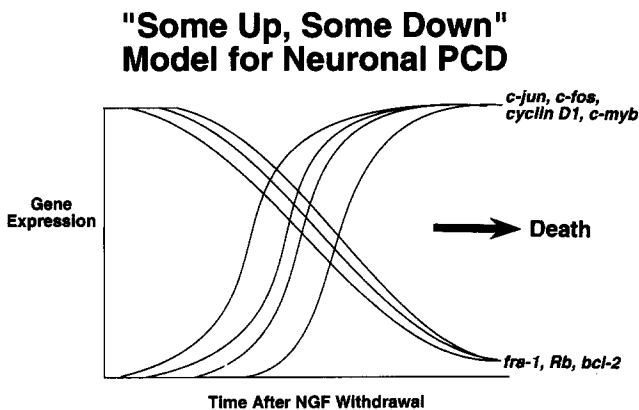
Figure 5. "Some up, some down" (SUiSiDe) model of gene expression leading to neuronal apoptosis. This figure schematically depicts the hypothesis that neuronal apoptosis requires concomitant gene induction and repression. According to this scenario, trophic factor withdrawal leads to the induction of genes that may be necessary for death, e.g., c-jun, c-fos, c-myb, and cyclin D1 (Freeman et al., 1994). This induction becomes sufficient to induce apoptosis when normally inhibitory genes are concurrently repressed. The products of these inhibitory genes may function by hindering the induced gene products directly. For example, Fra-1 binds to and blocks certain actions of c-Jun (Suzuki et al., 1991) and is repressed during apoptosis. Similarly, pRb binds to and appears to antagonize cyclin D1 (Kato et al., 1993; Dowdy et al., 1993; Hinds et al., 1992), and Rb is repressed during neuronal apoptosis (Freeman et al., 1994). Alternatively, the inhibitory genes may act via mechanisms that are presently less clear and, perhaps, less specific, e.g., BCL-2 has been suggested to block apoptosis via an antioxidative mechanism (Hockenbery et al., 1993), and bcl-2 decreases after NGF withdrawal (Greenlund, L. J. S., manuscript in preparation).

teases may facilitate the tissue remodeling that accompanies death.

In considering whether the remaining genes are critical for apoptosis, a possible role is suggested by the function of the protein encoded by one gene, mkp-1. Since MKP-1 inhibits signal transduction through the mitogen-activated protein kinase pathway (Sun et al., 1993) and since some of the actions of NGF may be mediated by this pathway (reviewed in Szeberenyi and Erhardt, 1994), the induction of mkp-1 may serve to raise the threshold required for NGF to reactivate its signal transduction. The possibility that this programmed genetic response serves as an initial commitment point requires further investigation.

The other induced genes are transcription factors, i.e., their only known function is the modulation of gene expression. Considering the possible role of these genes within the context of whether their expression is necessary or sufficient for death, the possibility that their induction per se is sufficient for death is improbable. Each gene that was induced in neurons undergoing apoptosis is also induced in other situations, e.g., c-fos is induced by cAMP. Hence, the increased expression of each of these genes is unlikely to be sufficient in and of itself to induce apoptosis. Alternatively, the expression of these genes may be necessary for death. To begin to evaluate this possibility, we examined whether antibodies that neutralize the Fos or Jun families or specific family members blocked apoptosis; c-Jun and member(s) of the Fos family were implicated as required for apoptosis. This observation was strengthened by the lack of saving ability of antibodies specific for Jun B or Jun D, as well as three separate control nonimmune IgG preparations.

There are at least two scenarios whereby the expression of c-Jun and the Fos family may lead to apoptosis. First, these results support the possibility that neurons are induced to undergo apoptosis because of conflicting growth regulatory signals, i.e., these gene products stimulate the postmitotic neurons to attempt reentry into the cell cycle. Indeed, apoptosis and the cell cycle have been recognized to share morphologic elements for some time (reviewed in Ucker, 1991); the data presented here strengthen the molecular link between these two processes. For example, c-jun, c-fos and fos B were induced during both apoptosis in neurons and also upon cell cycle reentry in fibroblasts; at least c-Jun appears necessary for both processes (Kovary and Bravo, 1991b). Other shared molecular markers include c-myb and cyclin D1 (Freeman et al., 1994); these genes are induced during both PCD and the G1 phase of the cell cycle and the encoded proteins are necessary for cell cycle progression (Nicolaides et al., 1992; Baldin et al., 1993). Moreover, the induction of these cell cycle–associated genes occurs concomitant with decreases in the levels of mRNAs encoding pRb and p53, two suppressors of cell proliferation (Freeman et al., 1994). Although the point(s) where apoptosis and the cell cycle diverge requires clarification, Fra-1 and Fra-2, which can act to inhibit c-Jun and c-Fos (see above), are induced subsequent to c-jun and c-fos during cell cycle reentry (Cohen and Curran, 1988) but not during neuronal apoptosis; their repression during apoptosis may represent the loss of critical regulatory factors. Peripheral support for an association between PCD and the cell cycle may also be derived from the induction of rhl during apoptosis, which might reflect a shift in the neurons from a differentiated postmitotic state towards

a less-differentiated mitotic state; *rhl* is a homologue of the *Drosophila* gene *hairy*, which is itself associated with neurogenesis (Jan and Jan, 1990). To summarize, elements often associated with cell cycle reentry and progression are induced in neurons undergoing NGF deprivation–induced apoptosis. For the postmitotic neurons, these elements may well represent a conflicting growth regulatory signal. By analogy with other systems wherein such signals induce cells to undergo apoptosis, e.g., c-*myc* overexpression in fibroblasts made quiescent by serum deprivation (Askew et al., 1991; Evan et al., 1992), large T antigen expression in postmitotic cells (Al-Ubaidi et al., 1992; Feddersen et al., 1992; Hammang et al., 1993), or p53 expression in transformed cells (Ryan et al., 1993; Shaw et al., 1992; Yonish-Rouach et al., 1993), these conflicting signals may initiate neuronal apoptosis.

A second scenario suggested by integrating the results reported here with those in the references that follow involves oxidative stress. Since BCL-2 appears to block oxidative damage (Hockenbery et al., 1993) and since overexpression of BCL-2 blocks SCG neuronal apoptosis (Garcia et al., 1992), oxidative stress may contribute to apoptosis. Several of the genes that were induced in neurons undergoing apoptosis are also induced by oxidative stress, including c-*jun* (Manome et al., 1993; Devary et al., 1991) and *mkp-1* (Keyse and Emslie, 1992). Hence, the induction of these genes may represent the transduction of an oxidative stress signal to a genetic cascade that ultimately results in neuronal death. This scenario is not mutually exclusive of the "conflicting growth signals" scenario.

It is unclear whether the genetic cascade identified here constitutes a universal mechanism for mammalian PCD, or even whether such a genetic pathway exists. Identification of a single gene product involved in all cases of mammalian PCD, similar to *reaper* in *Drosophila* (White et al., 1994) or *ced-3* or *ced-4* in *Caenorhabditus elegans* (Ellis et al., 1991), would support this possibility, but such a gene has not yet been demonstrated. Clearly, genes that are induced in other mammalian PCD models were not induced in the neuronal model system used here. These genes and model systems included *NGFI-B* in activated thymocytes (Woronicz et al., 1994; Liu et al., 1994), *hsp-70* (Buttyan et al., 1988), *TGF-β* (Kyprianou and Isaacs, 1989), or *TRPM-2* (Buttyan et al., 1989) in testosterone-deprived prostate epithelium, and *RP-2*, *RP-8* (Owens et al., 1991), *calmodulin* (Dowd et al., 1991), and *β-galactoside binding protein* (Goldstone and Lavin, 1991) in glucocorticoid-treated thymocytes. These results reinforce the possibility that different genetic programs are required for apoptosis in different systems. This is particularly applicable to *NGFI-B*, a gene reported necessary for T cell receptor activation–induced apoptosis (Woronicz et al., 1994; Liu et al., 1994). Such inconsistencies have two alternative interpretations. First, assuming that these induced genes are part of the death program and not coincidental, these gene products may function in a cell type–specific manner that brings the various cell types to a point of convergence in a common death process. Second, the possibility exists that there may be many cell type–specific pathways that independently result in cell death. Because of the gross similarity in the morphology of apoptotic cell death among different models, the first scenario seems more likely. One possible explanation for the differences in gene expression

may involve the observation that mitotic cells frequently undergo apoptosis only during the G_1 phase of the cell cycle (Ucker, 1991); since neurons are in a postmitotic, presumably G_0 state, neurons may move into an apoptotic pathway in a different fashion.

Activation of c-*fos* or c-*jun* expression appears to be a common element in many instances of mammalian PCD. For example, c-*fos* is induced in rat prostate epithelium undergoing apoptosis after testosterone deprivation (Buttyan et al., 1988), as well as in several in vivo instances of naturally occurring cell death, including that of the palate epithelium, heart valves, and developing tooth (Smeyne et al., 1993). Both c-*fos* and c-*jun* have been implicated in the PCD of certain lymphoid cell lines (Colotta et al., 1992). c-*jun* is induced in vivo in neurons after ischemia (Dragunow et al., 1993); at least a portion of the accompanying neuronal death appears apoptotic because the characteristic nucleosomal ladder is detected (MacManus et al., 1993, 1994; Linnik et al., 1993) and the size of the infarcted area is reduced by cycloheximide treatment (Linnik et al., 1993). While c-*fos* knockouts manifest some pathology (Wang et al., 1992; Johnson et al., 1992) that may be reflective of abnormal PCD (Smeyne et al., 1993), our observation that the very similar c-*fos* and *fos B* genes were induced in parallel suggests a degree of redundancy and that both may need to be blocked to inhibit neuronal PCD fully. c-*jun* knockout mice die at ∼11.5 d of gestation; it has not been reported whether PCD is abnormal in these mice (Hilberg, 1993; Johnson, 1993). In conclusion, the results presented here, identifying patterns of gene activation and repression during neuronal apoptosis and implicating one of the induced genes, c-*jun*, as necessary for neuronal apoptosis, provide strong support for the hypothesis that mammalian neuronal death results from the active expression of a genetic program; determining the genes that are more proximal to death, the role of gene repression, and the relevance of these results to apoptosis in other cell types requires further investigation.

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