THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1992 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 267, No. 26, Issue of September 15, pp. 18278-18283, 1992
Printed in U.S.A.

Expression and Involvement of c-*fos* and c-*jun* Protooncogenes in Programmed Cell Death Induced by Growth Factor Deprivation in Lymphoid Cell Lines*

(Received for publication, April 15, 1992)

Francesco Colotta, Nadia Polentarutti, Marina Sironi, and Alberto Mantovani

From the Istituto di Ricerche Farmacologiche "Mario Negri," via Eritrea 62, 20157 Milano, Italy

Cell death induced by growth factor withdrawal is a programed event in which gene transcription and translation are required. Thus, it is likely that genes encoding for transcriptional factors can play an important role in this process. We have tested this hypothesis by analyzing c-*fos* and c-*jun* protooncogene expression and involvement in lymphoid cells deprived of growth factors. Interleukin (IL)-6- and IL-2-dependent mouse cell lines undergo programed cell death after growth factor deprivation. Northern blot analysis shows that c-*fos* and c-*jun* protooncogenes are rapidly induced (within 60 min) after growth factor deprivation in IL-6- and IL-2-dependent mouse cells. Induction is transient, being undetectable at 120 min after deprivation. Induction of these protooncogenes is at the transcriptional level, as demonstrated by actinomycin D and nuclear run-off experiments. Antisense oligonucleotides directed against c-*fos* and c-*jun* mRNAs consistently reduced the expression of these genes in treated cells. This reduction was associated with increased survival of growth factor-deprived lymphoid cells, thus suggesting that the expression of c-*fos* and c-*jun* protooncogenes may represent an important early event in the activation of the genetic program of cell death.

A considerable proportion of cell death that is physiologically relevant occurs as an active process of self-destruction termed "apoptosis." Apoptosis has been described as a programed, as opposed to accidental, death of cells (1).

The program of cell death can be initiated by a number of external signals, such as glucocorticoid (2), irradiation (3), or withdrawal of growth factors (4–9). Since the program of cell death can be, at least partially, prevented by the presence of RNA or protein synthesis inhibitors (2, 3, 10), it has been postulated that gene transcription and translation are required in this phenomenon.

Although gene sequences have been described to be expressed in different cell types undergoing programed cell death, including blastocystes (11), *C. elegans* (12), hepatocytes (13, 14), hormone-dependent tissues (15, 16), and glucocorticoid-treated lymphoma cells and thymocytes (17, 18), the molecular basis of the activation of the genetic program of death is largely obscure. Most recently, expression of the bcl-2 gene has been shown to protect B cells from apoptosis (19, 20) and also to delay the onset of cell death in interleukin (IL)[1]-3- and IL-4- (but not in IL-2- and IL-6-) dependent hemopoietic cell lines (21). By contrast, interestingly, forced expression of the p53 gene induced death by apoptosis in a murine cell line in the absence of appropriate differentiation or growth signals (9).

Since the program of cell death is an active process requiring gene induction, we reasoned that genes coding for transcriptional factors could be induced in the very early phases of the apoptotic process. To verify this hypothesis, we chose as a model of apoptosis cell death induced in growth factor-dependent cell lines by the withdrawal of the relevant factor, thus avoiding the use of agents, such as glucocorticoids or irradiation, that can induce gene expression not necessarily related to cell death. Since we (22)[2] and others (23–26) have recently described that protooncogenes c-*fos* and c-*jun*, which code for transcriptional factors, are expressed in cells to which a signal of external damage has been delivered, we wondered whether these agents can be induced and may play a role in the phenomenon of programed cell death induced by growth factor deprivation.

Our results indicate that c-*fos* and c-*jun* protooncogenes (but not c-*myc*) are induced as early as 60 min after growth factor deprivation in IL-2- and IL-6-dependent cell lines. The treatment of cells with antisense oligonucleotides directed against c-*fos* and c-*jun* protooncogenes protects cells from cell death induced by growth factor deprivation, indicating a regulatory role of these sequences in the activation of the genetic program of cell death in lymphoid cells.

EXPERIMENTAL PROCEDURES

Cell Culture—Mouse myeloma cell lines 7TD1 and B9 were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (HyClone Laboratories, Sterile Systems, Logan, UT), 1.5 mM glutamine, 0.24 mM asparagine, 0.55 mM arginine, 50 μM 2-mercaptoethanol, 0.1 mM hypoxanthine, 16 μM thymidine, and, as source of IL-6, a conditioned medium from human endothelial cells (10% final dilution). The mouse CTLL cell line was cultivated in RPMI 1640 medium with 10% fetal calf serum, 20 mM Hepes, 50 μM 2-mercaptoethanol, and 200 units/ml IL-2 (Glaxo, Geneva). Cells were routinely cultivated in 72-cm^2 plastic flasks (Falcon Labware, Lincoln Park, NJ) at 37 °C in 5% CO_2 in air. All tissue culture reagents were from GIBCO and were tested for endotoxin contamination by *Limulus* amebocyte lysate assay (Microbiological Associates, Walkersville, MD) with a sensitivity in our hands of 0.1–0.2 ng/ml of *Escherichia coli* Westphal lipopolysaccharide and found to be negative.

* This work was supported by the Consiglio Nazionale delle Ricerche, p.f. BTBS, and ACRO. The contribution of the Italian Association for Cancer Research is gratefully acknowledged. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: IL, interleukin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid.

[2] F. Colotta, N. Polentarutti, M. Sironi, and A. Mantovani, unpublished data.

Growth Factor Deprivation Experiments—Cells were allowed to grow in complete medium supplemented with IL-6 or IL-2 until reaching a concentration of 1–1.2 × 10^6/ml; after 4 days, cells were pooled, distributed in 50-ml plastic conical tubes (Falcon Labware), and centrifuged at 440 × *g* for 10 min. Cells were then resuspended either in the same medium (control) for in medium deprived of the relevant growth factor (IL-6 or 7TD1 and B9 cells and IL-2 for CTLL cells). Then cells were plated at a concentration of 2 × 10^5/ml in 96-well plates (150 μl/well, Falcon Labware) and evaluated for viability at 24 and 48 h as detailed below. After the appropriate incubation time, 1 volume of trypan blue (0.4%, GIBCO) was added to 5 volumes of cells. After incubation at room temperature for 5 min, cells were counted in a hemocytometer. All counts were done in triplicate after coding samples.

Estimation of Apoptotic Cells—Cytocentrifuged cells were fixed in methanol, stained with May-Grünwald-Giemsa (Merck), and examined by oil-immersion light microscopy at a final magnification of × 1000. All samples were coded, and at least 500 cells/slide were examined. Apoptotic cells were identified according to the following criteria (1): condensed and fragmented nuclei, blebbing of plasma membranes, and decrease in cell size.

DNA Fragmentation—Fragmented and intact DNAs were evaluated as described (2). Cells (5 × 10^5) were centrifuged in Eppendorf tubes at 13,000 × *g* for 2 min; washed with cold phosphate-buffered saline, and lysed with 10 mM Tris (pH 7.5), 1 mM EDTA, and 0.2% Triton X-100 (Merck). After incubation on ice for 15 min, low and high molecular weight DNAs were separated by centrifugation at 13,000 × *g* at 4 °C for 20 min. Centrifugation-resistant low molecular weight DNA in the supernatant was precipitated with 12.5% trichloroacetic acid (Merck) for 18 h, whereas pellets were added with 300 μl of cold 12.5% trichloroacetic acid. Samples were then centrifuged at 13,000 × *g* at 4 °C for 5 min, and DNAs in the precipitates were extracted with 30 μl of 5 mM NaOH and 30 μl of 1 M perchloric acid (Merck) at 70 °C for 20 min. Then 120 μl of diphenylamine reagent (27) were added, and samples were incubated at 37 °C for 18 h. 120 μl from each sample were then transferred to flat-bottomed 96-well plates (Falcon Labware), and absorbance at 600 nm was measured on an automated plate reader (Titertek Multiskan, Flow ICN, Milano, Italy). Fragmentation was calculated as percentage of total DNA (supernatant and pellet) recovered as low molecular weight DNA in the supernatant.

DNA Electrophoresis—Cells (5 × 10^5) were centrifuged in Eppendorf tubes at 13,000 × *g* for 2 min, washed in phosphate-buffered saline, and lysed in 0.5 ml of hypotonic buffer (10 mM Tris, 1 mM EDTA, 0.2% Triton X-100) for 15 min on ice. Lysates were then centrifuged at 13,000 × *g* at 4 °C for 20 min. Pellets of high molecular weight DNA were added with 0.4 ml of 10 mM EDTA, 50 mM Tris, 0.5% Sarkosyl (Sigma), 0.5 μg/ml proteinase K (Boehringer Mannheim) and incubated at 48 °C for 18 h, whereas centrifugation-resistant, low molecular weight DNA was incubated with RNase A (20 μg/ml, Boehringer Mannheim) at 37 °C for 1 h. Low and high molecular weight DNAs were then extracted with phenol/chloroform, precipitated with 0.5 M NaCl and 1 volume of propanol, resuspended in water, added with loading buffer (2.5% Ficoll, 0.025% bromphenol blue, 0.025% xylene cyanol), heated at 75 °C for 5 min, and electrophoresed in 1% agarose containing 1 μg/ml bromide (Sigma) at 50 V in 40 mM Tris acetate. DNA was visualized by UV examination and photographed with a Polaroid camera. Size of DNA has been compared with a standard ladder (Boehringer Mannheim).

Northern Blot Analysis—Total RNA was isolated by the guanidine isothiocyanate method (28), with minor modifications (29). Ten micrograms of total RNA were analyzed by electrophoresis through 1% agarose-formaldehyde gels, followed by Northern blot transfer to GeneScreen Plus sheets (Du Pont-New England Nuclear). The plasmids containing a murine c-*fos* genomic sequence, a c-*jun* cDNA, and a c-*myc* cDNA were nick-translated with [α-^{32}P]dCTP (3000 Ci/mmol, Amersham International, Buckinghamshire, United Kingdom). Membranes were pretreated and hybridized in 50% formamide (Merck) with 10% dextran sulfate (Sigma), washed twice with 2 × SSC (1 × SSC: 0.15 mol/liter sodium chloride, 0.015 mol/liter sodium citrate) and 1% sodium dodecyl sulfate (Merck) at 60 °C for 30 min, and finally washed twice with 0.1 × SSC at room temperature for 30 min. Membranes were exposed for 12–24 h at −80 °C with intensifying screens. RNA transfer to membranes was checked by UV irradiation, as shown in each figure.

Run-off—Nuclei were isolated 45 min after resuspension of cells in medium with or without growth factors (see above). To isolate nuclei, 40 × 10^6 cells were washed twice with ice-cold Hanks' balanced salt solution (GIBCO) with Ca^{2+} and Mg^{2+} and then resuspended in 0.5 ml of lysis buffer (10 mmol/liter Tris-HCl (pH 7.4), 3 mmol/liter $MgCl_2$, 10 mmol/liter NaCl, 0.5% Nonidet P-40). After 5 min of incubation on wet ice, tubes were centrifuged at 440 × *g* at 4 °C, and cells were resuspended in 250 μl of ice-cold freezing buffer (Tris-HCl (pH 8.3), 40% glycerol, 5 mmol/liter $MgCl_2$, 0.1 mmol/liter EDTA (pH 8)). Then 60 μl of 5 × run-offer buffer (25 mmol/liter Tris-HCl (pH 8), 12.5 mmol/liter $MgCl_2$, 750 mmol/liter KCl, 1.25 mmol/liter each of dGTP, dCTP, and dATP) and 100 μCi of [α-^{32}P]UTP (6000 Ci/mmol, Amersham International) were added to 230 μl of nuclei suspension and incubated at 30 °C for 30 min. Elongated transcripts were then isolated using the guanidine/cesium procedure (28), with 50 μg of yeast tRNA added as carrier. The RNA pellet was resuspended in 180 μl of ice-cold TNE (0.5 mmol/liter Tris-HCl (pH 8), 1.5 mol/liter NaCl) and denaturated by adding 20 μl of 2 N NaOH on ice for 10 min. The solution was neutralized by the addition of Hepes (pH 7.2) (0.48 mmol/liter final concentration). RNA was then precipitated adding 880 μl of ethanol; the pellet was resuspended in 100 μl of hybridization solution (10 mmol/liter TES, 2% sodium dodecyl sulfate, 10 mmol/liter EDTA, 300 mmol/liter NaCl), and radioactivity was checked with a β-counter. The RNA solution was hybridized at 65 °C for 48 h to DNA immobilized on nitrocellulose filters. Filters were then washed with several changes of 0.2 × SSC at 65 °C for 30 min and incubated at 37 °C in 0.2 × SSC with 1 μg/ml RNase A for 30 min. Filters were then exposed for autoradiography as described above. For immobilization of DNA on filters, plasmids (5 μg) were denaturated with 0.3 mmol/liter NaOH at 60 °C for 30 min, neutralized with ammonium acetate (4 mol/liter final concentration), and spotted onto nitrocellulose filters (Schleicher & Schuell) using a slot-blot apparatus. Plasmids used contained c-*fos*, c-*jun*, and c-*myc* sequences and, as control, a mouse α-actin cDNA. As a negative hybridization control, an equal amount of pBR322 was transferred to membranes.

Oligonucleotides—We designed 18-mers corresponding to the sense or antisense sequences flanking the translation initiation region of the murine mRNAs for c-*fos* and c-*jun*. Oligonucleotides were as follows: c-*fos* sense, 5′-TTCTCGGGTTTCAACGCC-3′, c-*fos* antisense; 5′-GGCGTTGAAACCCGAGAA-3′; c-*jun* sense; 5′-ACTGCAAAGATGGAAACG-3′; and c-*jun* antisense, 5′-CGTTTCCATCTTTGCAGT-3′. Oligonucleotides were synthesized with the phosphoramidite method using a Beckman Model 200A synthesizer. Oligonucleotides were precipitated three to four times with absolute ethanol and then resuspended in culture medium.

Cells to be treated with oligonucleotides were resuspended as detailed above in growth factor-deprived medium and plated in triplicate at 2 × 10^5/ml in 96-well plates (150 μl/well). Oligonucleotides were added at a final concentration of 40 μg/ml at time 0. After 24 and 48 h, 5 μg/ml was further added to each well. Viable cells were then evaluated after 48 h of culture as detailed above, always in triplicate and after coding samples. At least 100 cells were counted per well.

Reverse-transcribed Polymerase Chain Reaction Analysis of c-fos and c-jun Transcripts—Cells treated with sense or antisense oligonucleotides as detailed above were examined for c-*fos* and c-*jun* transcripts by a semiquantitative reverse-transcribed polymerase chain reaction-based approach as described recently by Golay *et al.* (30). Briefly, after treatment with sense or antisense oligonucleotides, 3 × 10^4 cells were transferred in Eppendorf tubes, washed with saline, and resuspended in 150 μl of guanidine isothiocyanate solution (4.2 M) with 0.5% sodium lauryl sarcosinate, 25 mM sodium citrate (pH 7), 100 mM 2-mercaptoethanol, and 1 μg *E. coli* RNA. Then 4.5 μl of 2 M sodium acetate (pH 4), 180 μl of water-saturated phenol, and 35 μl of chloroform were added. After incubation on ice for 10 min, samples were centrifuged for 10 min at 4 °C. The water phase was transferred to another tube and precipitated with ethanol. Pellets were then dried and resuspended in 3 μl of water. Then each sample was added with 17 μl of a master mixture with reverse transcriptase buffer (5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3)), 2.5 μM random hexamers, 1 mM each deoxynucleotide triphosphate, 1 unit/μl RNase inhibitor, and 2.5 units/μl molonex murine leukemia virus reverse transcriptase (Perkin-Elmer Cetus Instruments). Samples were incubated for 10 min at 25 °C and then at 42 °C for 45 min. Then each sample was amplified in 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, 0.2 M each deoxynucleotide triphosphate, and 2.5 units/100 μl Taq DNA polymerase (Perkin-Elmer Cetus Instruments), and a 5 ng/ml concentration of each specific primer. Primers were synthesized as described above and were as follows: c-*fos* forward and backward, 5′-GGTCATCGGGGATCTTGC-3′ and 5′-

ATGGGCTCTCCTGTCAAC-3′, respectively; c-*jun* forward and backward, 5′-ACTGGGAAGCGTGTTCTG-3′ and 5′-ACTGCAAAGATGGAAACG-3′, respectively; β-actin forward and backward 5′-CCTTCCTGGGCATGGAGTCCTG-3′ and 5′-GGAGCAATGATCTTGATCTTC-3′, respectively. Amplification was carried out in an automated thermal cycler (Perkin-Elmer Cetus Instruments) at 95 °C for 1.5 min, at 55 °C for 1.5 min, and at 72 °C for 1.5 min. Amplification was stopped at 15 cycles, *i.e.* during the exponential phase of the amplification as assessed in preliminary experiments. To measure the efficiency of the extraction of RNA and of reverse transcription, 2 μl of the same reverse transcriptase reaction were amplified with β-actin-specific primers as an internal control. Polymerase chain reaction products were run on an agarose gel, blotted onto nitrocellulose filters, and then hybridized with the appropriate plasmid probes labeled with [α-^{32}P]dCTP as detailed above.

RESULTS

Various growth factor-dependent cell lines undergo cell death (apoptosis) after growth factor deprivation. In a first series of experiments, we examined whether two IL-6-dependent cell lines (7TD1 and B9) undergo programed cell death after IL-6 withdrawal. After removal of IL-6, 7TD1 and B9 cells progressively died, with ~50% viable cells after 48 h (Fig. 1). Cell death appears to fulfill the criteria used to define the apoptotic process: cells showed a typical morphology (condensed and fragmented nuclei, blebbing of plasma membrane, and decrease in cell size) (Fig. 1); a consistent proportion of DNA was recovered as fragmented low molecular weight DNA (Table I); and electrophoresis analysis demonstrated the characteristic pattern of fragmentation in multiples of ~200 base pairs (Fig. 2).

Having shown that IL-6 deprivation causes 7TD1 and B9 cells to die with the characteristics of apoptosis, these cell lines were used for the analysis of the expression of specific genes during cell death induced by growth factor withdrawal. In addition to 7TD1 and B9 cells, we also used the murine IL-2-dependent leukemia CTLL cell line, for which growth factor deprivation is well-characterized as a "classical" model of cell death by apoptosis (4), as confirmed by us in preliminary experiments (data not shown).

As a first approach to study the possible involvement of specific genes in the early phases of programed cell death, we examined by Northern blot analysis the expression of c-*fos* and c-*jun* in growth factor-deprived cells 30–120 min after withdrawal. Since it is well known that these protooncogenes are expressed in actively proliferating cells, to minimize baseline levels of the expression of c-*fos* and c-*jun*, total RNA was extracted from cells rendered virtually quiescent by cultivating them for 4 days in medium with IL-6 at a relatively high concentration (1–1.2 × 10^6/ml). Cells were then centrifuged and resuspended (10^6 cells/ml) either in medium lacking IL-

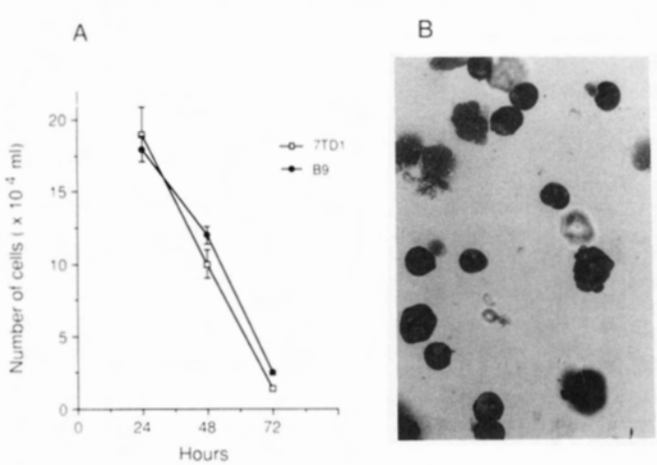

FIG. 1. **Viability of 7TD1 and B9 cells after growth factor deprivation.** *A*, survival of IL-6-deprived cells. 7TD1 and B9 cells were resuspended in IL-6-deprived culture medium at 5 × 10^5/ml in 96-well plates (150 μl/well), and viability was assessed at various times by trypan blue dye exclusion. Data are the mean ± S.D. of three experiments. In each experiment, all counts were done in triplicate. *B*, morphological characteristics of IL-6-deprived 7TD1 cells. Cells were cytocentrifuged after 24 h of culture in IL-6-deprived medium, stained with May-Grünwald-Giemsa, and then examined at a final magnification of × 1000. The percentages of cells showing morphological characteristics of apoptosis are reported in Table I.

TABLE I

7TD1 cells showing morphological characteristics of apoptosis and percent DNA fragmentation after IL-6 deprivation

Cells were resuspended in medium with or without IL-6 as detailed under "Experimental Procedures" and then at various intervals of time evaluated for cells showing the morphological characteristics of apoptosis in cytocentrifuged preparations and for DNA fragmentation. Similar results have been obtained with B9 cells. Data reported here are mean ± S.D. of triplicates from a representative experiment out of three in which we obtained similar results.

Time	IL-6	Apoptotic cells	Fragmented DNA
h		%	%
8	+	2.0 ± 1.1	5.9 ± 2.0
	−	5.3 ± 1.9	14.8 ± 2.0
24	+	3.1 ± 0.8	4.3 ± 1.8
	−	20.8 ± 2.8	32.3 ± 3.2
40	+	4.2 ± 1.4	5.9 ± 2.1
	−	45 ± 4.8	74.1 ± 6.1

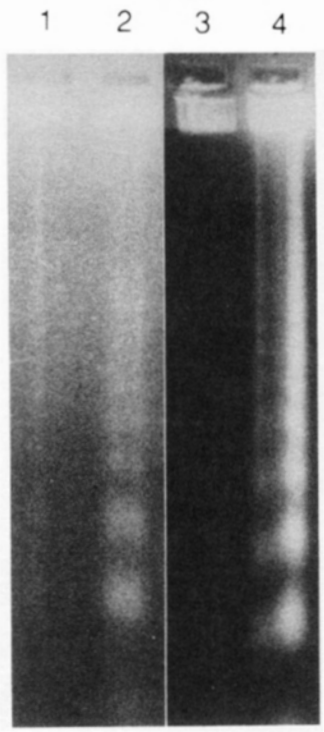

FIG. 2. **Gel electrophoresis analysis of high and low molecular weight DNAs isolated from growth factor-deprived 7TD1 and B9 cells.** High and low molecular weight DNAs were isolated as detailed under "Experimental Procedures" from cells cultivated in IL-6-deprived medium for 40 h and then separated by gel electrophoresis through 1% agarose gel. As shown, low molecular weight DNA (*lanes 2* and *4* for 7TD1 and B9 cells, respectively) migrated as multiples of ~200 base pairs, whereas high molecular weight DNA appeared intact (*lanes 1* and *3*, respectively). Cells cultivated in IL-6-supplemented medium did not show appreciable amounts of low molecular weight DNA (data not shown).

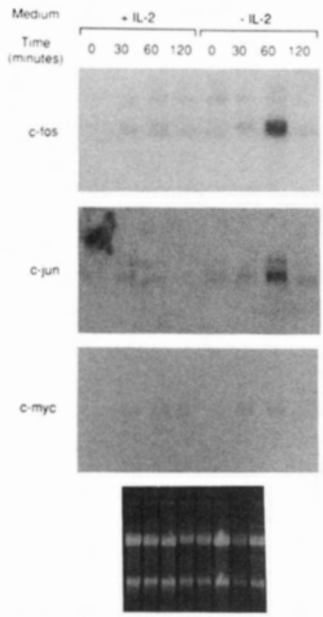

FIG. 3. **Expression of c-*fos*, c-*jun* and c-*myc* protooncogenes in IL-2-deprived CTLL cells.** CTLL cells were cultivated for 4 days in medium supplemented with IL-2 at a concentration of 10^6/ml. Then cells were centrifuged and resuspended either in the same medium containing IL-2 in which they have been cultivated or in medium lacking IL-2. Then total RNA was extracted at various times and examined for gene expression by Northern blot analysis. The same filter had been hybridized to c-*fos*, c-*jun*, and c-*myc* probes. The *bottom panel* shows the ethidium bromide-stained RNAs blotted onto the filter.

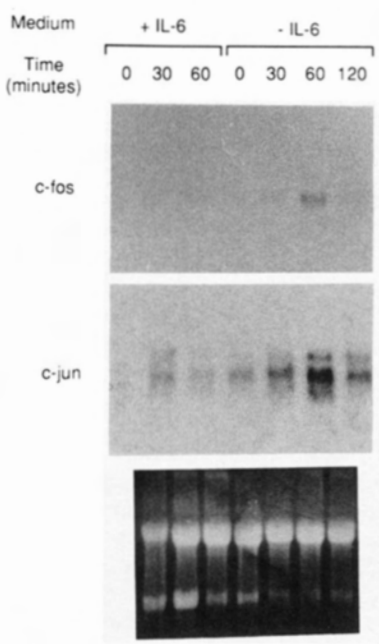

FIG. 4. **Expression of c-*fos* and c-*jun* protooncogenes in 7TD1 cells deprived of IL-6.** Cells were cultivated as described under "Experimental Procedures" and in the legend of Fig. 3. The same filter was hybridized to c-*fos* and c-*jun* probes. The *bottom panel* shows the RNAs blotted onto the filter.

6 or, as control, in the same buffer medium containing IL-6 in which they had been previously cultivated, and RNA was examined at various time intervals. As early as 60 min after growth factor deprivation, both CTLL and 7TD1 cells, IL-2- and IL-6-dependent, respectively, expressed c-*fos* and c-*jun* transcripts (Figs. 3 and 4). The c-*myc* protooncogene was not

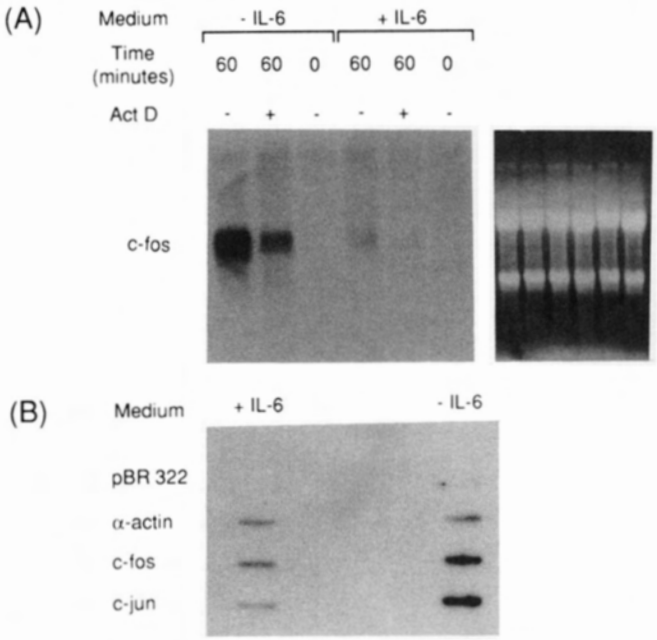

FIG. 5. **Transcriptional induction of protooncogenes in growth factor-deprived cells.** *A*, Northern blot analysis of c-*fos* expression in 7TD1 cells after deprivation of IL-6 in the presence or absence of a transcriptional inhibitor (actinomycin D (*Act D*), 1 μg/ml). Cells were cultivated as detailed under "Experimental Procedures" and in the legend of Fig. 3 and then resuspended either in the same medium in which they had been cultivated or in medium deprived of IL-6 with or without actinomycin D. RNAs were then extracted at time 0 or after 1 h and examined for c-*fos* expression. The ethidium bromide-stained RNAs are shown on the right. *B*, run-off analysis of protooncogene induction by growth factor deprivation. Cells were cultivated as detailed above, and nuclei were isolated 45 min after resuspension in either the same medium containing IL-6 in which they were cultivated or in medium lacking IL-6. Nuclear transcripts were then hybridized to specific probes as shown, pBR322 was used as a negative control.

found to be expressed in CTLL cells (Fig. 3) under these experimental conditions. Expression of c-*fos* and c-*jun* was not a consequence of cell handling since cells processed in parallel but resuspended in the same medium containing IL-6 failed to express both c-*fos* and c-*jun* (Figs. 3 and 4). Expression of c-*fos* and c-*jun* was transient, being undetectable 120 min after growth factor removal (Figs. 3 and 4).

To ascertain whether the induction of c-*fos* and c-*jun* in growth factor-deprived cells was dependent upon gene transcription, we treated cells with actinomycin D. As shown in Fig. 5*A*, actinomycin D reduced c-*fos* expression induced by growth factor deprivation. This finding tentatively suggests that growth factor deprivation activates protooncogene expression at the transcriptional level. This conclusion was then confirmed by nuclear run-off analysis in which, as shown in Fig. 5*B*, the transcription of c-*fos* and c-*jun* (but not of α-actin) genes was augmented in cells subjected to growth factor deprivation.

Taken together, these data indicate that c-*fos* and c-*jun* are induced in the early phases after a message of programed cell death has been delivered to cells. To ascertain whether these genes may play a regulatory role in the control of the genetic program of cell death, we decided to use the approach of antisense oligonucleotides to block c-*fos* and c-*jun* expression and then analyze the effect of this inhibition on growth factor deprivation-induced cell death.

In a first series of experiments, we wanted to verify whether treatment of cells with antisense oligonucleotides can indeed

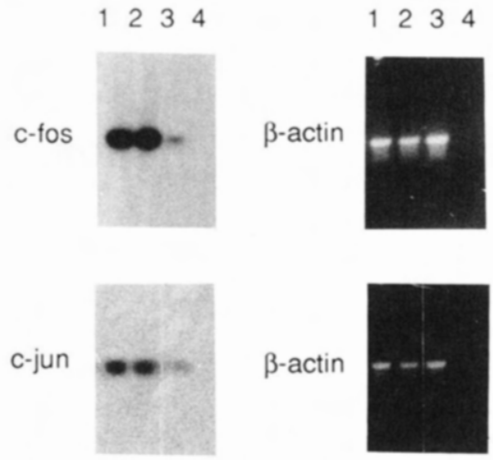

FIG. 6. **Expression of c-*fos* and c-*jun* in growth factor-deprived 7TD1 cells treated with sense or antisense oligonucleotides against c-*fos* and c-*jun* protooncogenes.** Cells were grown and resuspended in IL-6-deprived medium as described under "Experimental Procedures" and added with sense (control) or antisense oligonucleotides against c-*fos* and c-*jun* at a final concentration of 40 μg/ml. After 1 h, RNA was extracted and reverse-transcribed to cDNA. The same cDNA synthesis reaction was then amplified with c-*fos* (for cells incubated with sense or antisense oligonucleotides against c-*fos*) or c-*jun* (for cells incubated with oligonucleotides against c-*jun*) and, as a control, β-actin-specific primers. After 15 cycles, the amplification reaction was electrophoresed through 1% agarose gel, blotted onto nitrocellulose filters, and then hybridized to c-*fos* or c-*jun* probes labeled by nick translation and [α-^{32}P]dCTP. *Lane 1*, cells cultivated in growth factor-deprived medium for 1 h; *lane 2*, same as *lane 1* with the addition of sense oligonucleotides against c-*fos* or c-*jun*; *lane 3*, same as *lane 1* with the addition of antisense oligonucleotides against c-*fos* or c-*jun*; *lane 4*, negative control (no RNA). The *right panels* show the ethidium bromide-stained amplified DNA obtained with β-actin-specific primers.

TABLE II

Survival of 7TD1 and CTLL cells deprived of growth factors and treated with sense or antisense oligonucleotides against c-fos and c-jun protooncogenes

Cells were cultivated in growth factor-deprived medium in the presence or absence of specific oligonucleotides, as detailed under "Experimental Procedures," for 48 h, and then viable cells were evaluated by trypan blue dye exclusion. Values are mean ± S.D. of three different experiments. In each experiment, each experimental group was counted in triplicate after coding samples. Data are expressed as percent increase of viable cells over cells resuspended in growth factor-deprived medium in the absence of oligonucleotides. Oligonucleotides did not affect the viability of cells resuspended in IL-6 or IL-2-supplemented medium (data not shown).

Treatment	Increase of viable cells over control	
	7TD1	CTLL
	%	
Sense c-*fos*	0 ± 2.1	0 ± 1.9
Antisense c-*fos*	27.6 ± 3.4	47.0 ± 5.9
Sense c-*jun*	8.1 ± 2.1	9.0 ± 1.8
Antisense c-*jun*	36.6 ± 5.1	52.8 ± 4.9
Sense c-*jun* + sense c-*fos*	2.1 ± 1.5	8.4 ± 1.4
Antisense c-*jun* + antisense c-*fos*	71.0 ± 6.9	54.6 ± 7.3

reduce the levels of mRNA coding for these two protooncogenes. To this aim we used, along the line of other reports (31, 32), a polymerase chain reaction-based semiquantitative approach (30). As shown in Fig. 6, the amount of c-*fos* and c-*jun* transcripts was considerably reduced in cells treated with antisense oligonucleotides. Sense oligonucleotide-treated cells expressed the same levels of transcripts found in untreated cells. As expected, when the same cDNA was amplified with β-actin-specific sequences, no appreciable difference was evident in the levels of transcripts among experimental groups.

Having established that antisense oligonucleotides were effective in reducing c-*fos* and c-*jun* transcripts, we examined whether anti-*fos* and anti-*jun* oligonucleotides prevent cell death after growth factor deprivation. All samples were counted in triplicate and after coding. As shown in Table II, in which the mean ± S.D. from three separate experiments has been reported, control (sense) oligonucleotides did not appreciable modify the percentage of viable cells. By contrast, the percentage of viable cells was considerably increased in antisense oligonucleotide-treated cells. This effect was present when cells were treated with anti-*fos* and anti-*jun* oligonucleotides separately, but it was more evident when both antisense sequences were added simultaneously, following an approach already described by others (33). The percentage of viable cells was augmented, with respect to untreated deprived cells, in anti-*fos* oligonucleotide-treated cells by 27.7 ± 3.4% for 7TD1 and by 47.0 ± 5.9% for CTLL cells and in anti-c-*jun* oligonucleotide-treated cells by 36.6 ± 5.1 and 52.8 ± 4.9%, respectively. When both antisense oligonucleotides were added in the same well, the percentages were 71.0 ± 6.9% for 7TD1 cells and 54.6 ± 7.3% for CTLL cells.

DISCUSSION

The results presented here indicate that an early event that occurs in lymphoid cells undergoing programed cell death after growth factor deprivation is represented by the rapid and transient induction of c-*fos* and c-*jun*. Incubation of growth factor-deprived cells with antisense oligonucleotides directed against c-*fos* and c-*jun* genes protects hematopoietic cells from death, thus indicating an important role of these protooncogenes in the early phases of programed cell death induced by growth factor deprivation of lymphoid cells.

The process of apoptosis is physiologically controlled, being activated in certain cell types in response to a definite set of external signals (1). These may be represented by the appearance of a "positive" signal, such as glucocorticoids in thymocytes, or the removal of environmental signals, such as hormones or growth factors (1–9). Indeed, cell death by apoptosis has been described in many cell lines dependent upon the external supply of IL-2, IL-3, and colony-stimulating factors (2–9) and, as demonstrated herein, also of IL-6.

Whatever the nature of the external signal that triggers programed cell death, available information indicates that this process consists of a self-destructive process, in which the cell plays an active role, that requires gene transcription and translation, as suggested by the fact that the process is prevented by inhibitors of RNA or protein synthesis (2, 3, 10). This has prompted investigators to find genes specifically induced in programed cell death. Although specific sequences have been described in different models of apoptosis (11–18), the actual role played by these sequences in the process of cell death is unknown; and thus, the molecular basis of the genetic program of cell death is still largely obscure. Most recently, two genes have been shown to be somehow implicated in the regulation of the apoptotic process. On the one hand, bcl-2 expression can prolong the survival of B cells and delay the onset of death in IL-3- and IL-4-dependent hemopoietic cell lines (19–21), and antisense oligonucleotides against this protooncogene inhibit the survival of a human leukemia cell line (34). On the other hand, forced expression of the p53 gene in a murine myeloid leukemia cell line induces

cell death by apoptosis in the absence of growth or differentiation signals (9). The mechanism of action of these genes in apoptosis remains a matter of investigation.

Since programed cell death seems to require intact transcriptional activity, we reasoned that induction of genes coding for transcriptional factors may represent an important early event in programed cell death. We (22) and others (23–26) have described that cells exposed to environmental stress signals express c-*fos* and c-*jun* protooncogenes, genes that encode for proteins forming transcriptional complexes. Thus, we decided to investigate the possibility that these genes can be induced in the early phases of programed cell death. Our data indicate that genes coding for the transcriptional factors *fos* and *jun* are induced in the very early phases of death after growth factor deprivation of hematopoietic cells, and that blocking of these sequences by antisense oligonucleotides can prevent cell death, thus indicating that this induction has an important regulatory role in the process of cell death.

Gene induction during apoptosis has been investigated in various cell types under different experimental conditions, including glucocorticoid treatment of thymocytes or lymphoma cells (17, 18). Since we were concerned that positive treatment of cells with compounds like glucocorticoids may result in the induction of a set of genes not necessarily related to the activation of cell death, we decided to investigate gene induction in growth factor-deprived cells. Although expression of protooncogenes has been described also in hormone-deprived prostatic cells (16), whether c-*fos* and c-*jun* are expressed also in different models of apoptosis remains to be established.

c-*fos* and c-*jun* protooncogenes are rapidly activated by a large series of signals involved in proliferation, differentiation and functional activation (for review, see Ref. 35). Since it is known that these genes code for proteins that form complexes with properties of transcriptional factors, these genes are viewed as a "master switch" for turning on other genes in response to a wide range of stimuli. Thus, these genes may act as a step in the chain of events that convert signals at the cell membrane to long-lasting responses that require gene activity. The data presented here, by showing that reduction of c-*fos* and c-*jun* transcripts by anti-sense oligonucleotides prevents apoptosis, suggest also that "death signals" may cause c-*fos* and c-*jun* to be transduced into the cells.

That c-*fos* and c-*jun* protooncogenes are induced by two seemingly opposite processes, cell death and proliferation, is puzzling. One possible explanation is that the induction of a single gene is not significant *per se*, but must be viewed in the context of the more complex modifications induced by a given signal in the cell. In this regard, it is interesting that in lymphoid cells the same external signal can be sensed in cells of the same lineage as the growth or death signal depending upon the differentiation state of the cell (36).

Cell death by apoptosis is a highly complex phenomenon subjected to a strict environmental control that requires the activation of a specific genetic program. Our data indicate that certain transcriptional factors may be important in the regulation of this phenomenon. The identification of genes involved in the process of programed cell death may contribute to a more in-depth understanding of the molecular basis of the regulation of cell death and provide a basis for the modulation of the process of cell death.

REFERENCES

1. Kerr, J. F. R., Wyllie, A. H., and Currie, A. R. (1972) *Br. J. Cancer* **26,** 239–257
2. Wyllie, A. H. (1980) *Nature* **284,** 555–556
3. Sellins, K. S., and Cohen, J. J. (1987) *J. Immunol.* **139,** 3199–3206
4. Duke, R. C., and Cohen, J. J. (1986) *Lymphokine Res.* **5,** 289–299
5. Williams, G. T., Smith, C. A., Spooncer, E., Dexter, T. M., and Taylor, D. R. (1990) *Nature* **343,** 76–79
6. Koury, M. J., and Bondurant, M. C. (1990) *Science* **248,** 378–381
7. Lopez, A. F., Williamson, D. J., Gamble, J. R., Begley, C. G., Harlan, J. N., Klebanoff, S. J., Watlersdorph, A., Wong, G., Clark, S. C., and Vadas, M. A. (1986) *J. Clin. Invest.* **78,** 1220–1228
8. Yamaguchi, Y., Suda, T., Ohta, S., Tominaga, K., Miura, Y., and Kasahara, T. (1991) *Blood* **10,** 2542–2547
9. Yonish-Rouach, E., Resnitzky, D., Lotem, J., Sachs, L., Kimichi, A., and Oren, M. (1991) *Nature* **352,** 345–347
10. Wyllie, A. H., Morris, R. G., Smith, A. L., and Dunlop, D. (1984) *J. Pathol.* **142,** 67–77
11. Pierce, G. B., Lewellyn, A. L., and Parchment, R. E. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 3654–3658
12. Ellis, H. M., and Horvitz, H. E. (1986) *Cell* **44,** 817–829
13. Fesus, L., Thomazy, V., and Falus, A. (1987) *FEBS Lett.* **224,** 104–108
14. Fesus, L., Thomazy, V., Autuori, F., Ceru, M. P., Taresa, E., and Piacentini, M. (1989) *FEBS Lett.* **245,** 150–156
15. Wadewitz, A. G., and Lockshin, R. A. (1988) *FEBS Lett.* **241,** 19–23
16. Buttyan, R., Zakeri, Z., Lockshin, R., and Wolgemuth, D. (1988) *Mol. Endocrinol.* **2,** 650–657
17. Harrigan, M. T., Baughman, G., Campbell, N. F., and Bourgeois, S. (1990) *Mol. Cell. Biol.* **9,** 3438–3446
18. Owens, G. P., Hahn, W. E., and Cohen, J. J. (1991) *Mol. Cell. Biol.* **11,** 4177–4188
19. Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R. D., and Korsmeyer, S. J. (1990) *Nature* **348,** 334–336
20. Henderson, S., Rowe, M., Gregory, C., Croom-Cater, D., Wang, F., Longnecker, R., Kieff, E., and Rickinson, A. (1991) *Cell* **65,** 1107–1115
21. Nunez, G., London, K., Hockenbery, D., Alexander, M., McKearn, J. P., and Korsmeyer, S. J. (1990) *J. Immunol.* **144,** 3602–3610
22. Colotta, F., Polentarutti, N., Staffico, M., Fincato, G., and Mantovani, A. (1990) *Biochem. Biophys. Res. Commun.* **168,** 1013–1019
23. Andrews, G. K., Harding, M. A., Calvet, J. P., and Adamson, E. D. (1987) *Mol. Cell. Biol.* **7,** 3452–3458
24. Hollander, M. C., and Fornace, A. J., Jr. (1989) *Cancer Res.* **49,** 1687–1692
25. Datta, R., Sherman, M. L., and Kufe, D. W. (1990) *Blood* **76,** 298–301
26. Kharbanda, S. M., Sherman, M. L., and Kufe, D. W. (1990) *J. Clin. Invest.* **86,** 1517–1523
27. Burton, K. (1956) *Biochem. J.* **62,** 315–321
28. Chirgwin, J. M., Przybyla, A. E., Mac Donald, R. J., Rutter, W. J. (1979) *Biochemistry* **18,** 5294–5299
29. Colotta, F., Wang, J. M., Polentarutti, N., and Mantovani, A. (1987) *J. Exp. Med.* **165,** 1224–1229
30. Golay, J., Passerini, F., and Introna, M. (1991) *PCR Methods Applicat.* **1,** 144–145
31. Venturelli, D., Travali, S., and Calabretta, B. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 5963–5967
32. Maier, J. A. M., Vaulalas, S. A., Roeder, P. D., and Maciag, T. (1990) *Science* **249,** 1570–1572, and references therein
33. Fagarasan, M. O., Aiello, F., Muegge, K., Durum, S., and Axelrod, J. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 7871–7874
34. Reed, J. C., Stein, C., Subasinghe, C., Haldar, S., Croce, C. M., Yum, S. and Cohen, J. (1990) *Cancer Res.* **50,** 6565–6570
35. Curran, T., and Franza, R. F., Jr. (1988) *Cell* **55,** 395–397
36. Smith, C. A., Williams, G. T., Kingston, R., Jenkinson, R., and Owen, E. J. (1989) *Nature* **337,** 181–184