Induction of Apoptosis by a Secreted Lipocalin That is Transcriptionally Regulated by IL-3 Deprivation

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Many hematopoietic cells undergo apoptosis when deprived of specific cytokines, and this process requires de novo RNA/protein synthesis. Using DNA microarrays to analyze interleukin-3 (IL-3)–dependent murine FL5.12 pro–B cells, we found that the gene undergoing maximal transcriptional induction after cytokine withdrawal is *24p3*, which encodes a secreted lipocalin. Conditioned medium from IL-3–deprived FL5.12 cells contained 24p3 and induced apoptosis in naïve FL5.12 cells even when IL-3 was present. 24p3 also induced apoptosis in a wide variety of leukocytes but not other cell types. Apoptotic sensitivity correlated with the presence of a putative 24p3 cell surface receptor. We conclude that IL-3 deprivation activates *24p3* transcription, leading to synthesis and secretion of 24p3, which induces apoptosis through an autocrine pathway.

Apoptosis is a physiological form of cell death that has been implicated in diverse biological processes, including normal development, tissue homeostasis, and defense against pathogens (*1*–*3*). The mechanisms governing apoptosis are deregulated in several human diseases, including cancer, autoimmunity, and degenerative disorders (*4*).

Two general forms of apoptosis can be distinguished by their requirement for de novo transcription and translation. Fas ligand and tumor necrosis factor promote cell death by recruiting and activating caspases at the plasma membrane and do not require macromolecular synthesis (*5*). In contrast, apoptosis induced by genotoxic agents requires transcriptional activation of p53-dependent genes (*6*). Other transcription-dependent apoptotic programs include glucocorticoid-induced killing of thymocytes (*7*, *8*) and cell death induced by the T cell receptor (*9*).

Apoptosis of hematopoietic cells can be induced by withdrawal of cytokines such as interleukin 3 (IL-3). Apoptosis is blocked by actinomycin D or cycloheximide, implying a requirement for gene expression (*10*). We used high-density DNA microarrays to search for death-promoting genes that are transcriptionally activated in IL-3–deprived hematopoietic cells.

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Genes transcriptionally activated after IL-3 deprivation. The mouse pro–B lymphocytic cell line FL5.12 is dependent on IL-3 for growth. About 6 hours after IL-3 withdrawal, the cells begin to undergo apoptosis in the absence of cytokine (*11*, *12*). We isolated polyadenylated [poly(A)$^+$] mRNA from these cells 8 hours after IL-3 withdrawal and used it to interrogate Affymetrix DNA microarrays representing ~30,000 genes. Transcription profiles of cells grown in the presence or absence of IL-3 were compared. The gene that showed the largest increase in expression in IL-3–deprived cells (12.6-fold) was *24p3* (*13*), which encodes a lipocalin (*14*). Genes *mNip3*, which encodes a Bcl-2 family member (*15*), and *p40Phox*, which encodes a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunit (*16*), also showed a substantial increase in expression, whereas other genes showed reduced expression upon IL-3 withdrawal (*13*). Notably, the gene for the transcription factor ATFx (*17*) was repressed by 50-fold.

Northern blots (Fig. 1) revealed that transcriptional induction of *24p3* was first detectable in FL5.12 cells within 2 hours of IL-3 withdrawal and that 24p3 was similarly activated in IL-3–dependent murine 32D cells (*18*) and IL-3–dependent murine primary bone marrow cells (*19*).

Secreted 24p3 induces and is required for apoptosis. Lipocalins are small secreted proteins that play a role in diverse biological processes through binding of small hydrophobic molecules, interaction with cell surface receptors, and formation of macromolecular complexes (*14*). We detected 24p3 in the culture medium of FL5.12 cells after IL-3 withdrawal (Fig. 2A, inset) (*20*). To investigate whether the secreted 24p3 participates in apoptosis, we tested conditioned

Table 1. Cell type specificity of 24p3-mediated apoptosis. Cells were considered sensitive to 24p3-mediated apoptosis if >40% were annexin V–FITC positive when cultured in the presence of 24p3.

Cell type	Susceptibility to 24p3
Leukocytic cell lines	
Cytokine-dependent	
IL-3	
Mouse FL5.12	+
Mouse FL5.12/Bcl-x_L	−
Mouse 32D	+
Mouse Ba/F3	+
Mouse Lyd9	+
IL-2	
Mouse HT-2	+
IL-7	
Mouse D1-F4	−
Cytokine-independent	
Human MT-4	+
Mouse WEHI 7.1.C.4	+
Human Jurkat	−
Nonleukocytic cell lines	
Human HeLa	−
Monkey COS-7	−
Mouse NIH 3T3	−
Human U20S	−
Primary cells	
Murine primary thymocytes	+
Murine primary spienocytes	+
Murine primary bone marrow cells	+
Human primary neutrophils	+
Human peripheral blood lymphocytes	+
Human primary macrophages	−

medium from IL-3–deprived cells for apoptosis-inducing activity. We collected medium from FL5.12 cells cultured with or without IL-3, supplemented it with recombinant IL-3, added the medium to naïve FL5.12 cells, and analyzed cell viability by a trypan blue exclusion assay (*21*). Conditioned medium from FL5.12 cells cultured in the absence of IL-3 induced cell death even though IL-3 was present, whereas the medium from FL5.12 cells cultured in the presence of IL-3 had no effect on cell viability (Fig. 2A). An annexin V–fluorescein isothiocyanate/propidium iodide (FITC/PI) staining (Fig. 2B) and a DNA fragmentation assay (Fig. 2C) confirmed that cell death was apoptotic (*22*). Conditioned medium from IL-3–deprived primary bone marrow cells also induced apoptosis in FL5.12 cells and primary bone marrow cells (*13*).

Three experimental strategies were used to verify that the apoptosis-inducing activity in the conditioned medium was 24p3. First, 24p3 was ectopically expressed in COS-7 monkey kidney cells, which are resistant to 24p3-mediated apoptosis (Table 1) (*23*). Conditioned medium from these 24p3-transfected cells induced apoptosis in FL5.12 cells (Fig. 3, A through C), whereas conditioned medium from COS-7 cells transfected with expression vector alone had no apoptotic activity. Second, we tested whether ectopic expression of 24p3 in FL5.12 cells could induce apoptosis. The *24p3* gene was placed under the control of an ecdysone-inducible promoter and stably introduced into FL5.12 cells. The addition of ecdysone resulted in expression and secretion of 24p3 (*24*) and apoptosis (Fig. 3D) (*25*). Finally, we showed that recombinant 24p3 purified from *Escherichia coli* (*26*) induced apoptosis in FL5.12 cells and IL-3–dependent primary bone marrow cells (Fig. 3E).

To investigate whether 24p3 expression is required for apoptosis induced by IL-3 deprivation of FL5.12 cells, we performed antisense experiments with two phosphorothioate antisense (AS) oligonucleotides and, as a control, a phosphorothioate sense oligonucleotide (*27*). Both 24p3 AS oligonucleotides substantially reduced 24p3 levels, whereas the sense oligonucleotide had no effect (Fig. 4A). The AS-1 oligonucleotide was particularly effective, preventing apoptosis in ~75% of cells (Fig. 4B). Moreover, after treatment with 24p3 AS oligonucleotides, the conditioned medium from IL-3–deprived FL5.12 cells was no longer pro-apoptotic (Fig. 4C).

Finally, we demonstrated that an antibody to 24p3 blocked apoptosis in cytokine-deprived, IL-3–dependent, primary bone marrow cells (Fig. 4D), confirming that 24p3 is required to promote apoptosis.

Specificity of 24p3-mediated apoptosis. We next explored the cell-type specificity of the 24p3 pro-apoptotic activity. Based on

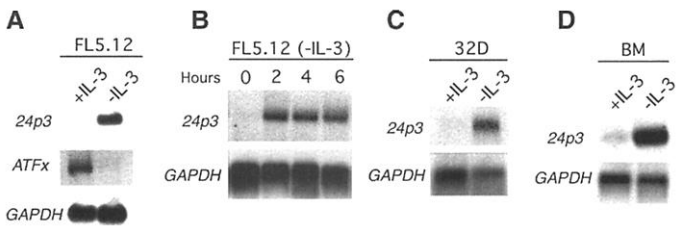
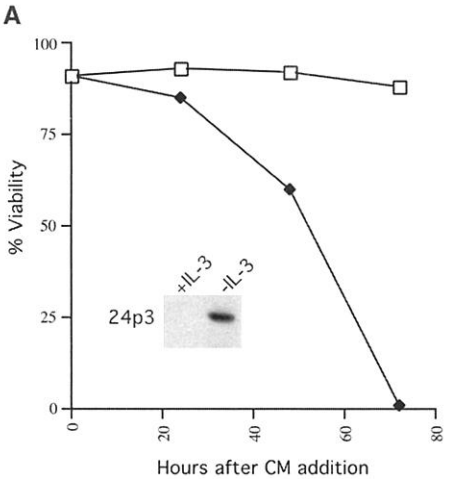
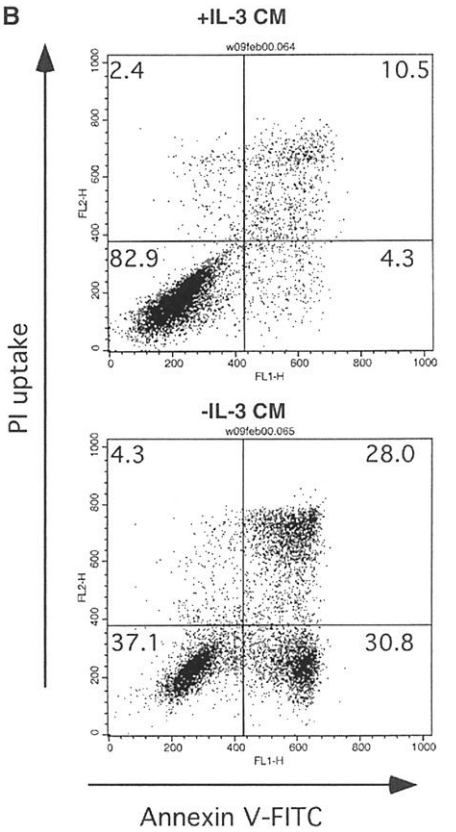


Fig. 1. Transcriptional activation of *24p3* after IL-3 deprivation as assessed by Northern blots. Poly(A)$^+$ RNA (2 μg) or total RNA (10 μg) was analyzed on denaturing formaldehyde agarose gels. Blots were probed with the indicated probes and washed under high-stringency conditions. (**A**) Expression of *24p3*, *ATFx*, and *GAPDH* (glyceraldehyde phosphate dehydrogenase) in FL5.12 cells was analyzed 8 hours after IL-3 withdrawal. (**B**) Time course of *24p3* induction. RNA was isolated from FL5.12 cells at the indicated times after IL-3 withdrawal. (**C**) Expression of *24p3* in 32D cells 8 hours after IL-3 withdrawal. (**D**) Expression of *24p3* in IL-3–dependent primary bone marrow (BM) cells 6.5 hours after IL-3 withdrawal.





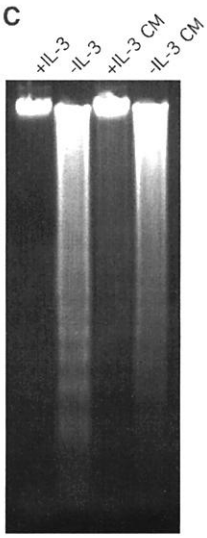


Fig. 2. Induction of apoptosis by conditioned medium from IL-3–deprived FL5.12 cells. (**A**) Conditioned medium from IL-3–deprived FL5.12 cells promotes death of naïve FL5.12 cells. Inset shows immunoblot of medium from FL5.12 cells cultured with IL-3 or deprived of IL-3 for 24 hours. Cell viability was quantitated by trypan blue exclusion. CM, conditioned medium supplemented with IL-3 (3 ng/ml). Squares, +IL-3 CM; diamonds, –IL-3 CM. (**B**) FL5.12 cells were analyzed by annexin V–FITC/PI staining 48 hours after addition of CM. (**C**) FL5.12 cells were subjected to DNA fragmentation analysis 24 hours after IL-3 withdrawal (lane 2) or 48 hours after addition of CM (lanes 3 and 4).

trypan blue exclusion and annexin V–FITC/PI staining, 24p3 promoted apoptosis in many but not all leukocytic cell lines, primary thymocytes, primary lymphocytes, and neutrophils (Table 1) (*13*). In contrast, nonhematopoietic cells and monocyte-derived macrophages were resistant. Thus, the ability of 24p3 to induce apoptosis is cell type–specific.

To test the possibility that 24p3 functions

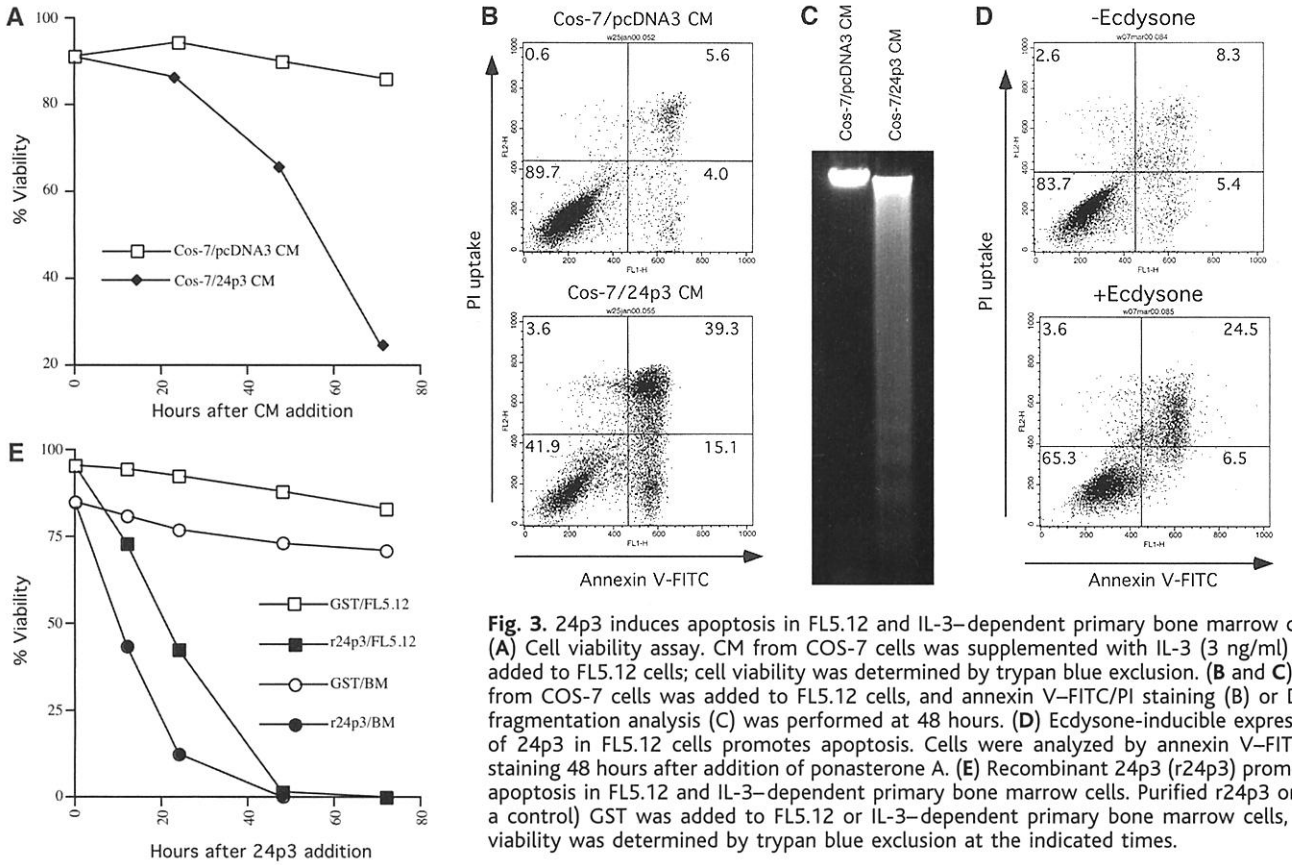


Fig. 3. 24p3 induces apoptosis in FL5.12 and IL-3–dependent primary bone marrow cells. (**A**) Cell viability assay. CM from COS-7 cells was supplemented with IL-3 (3 ng/ml) and added to FL5.12 cells; cell viability was determined by trypan blue exclusion. (**B** and **C**) CM from COS-7 cells was added to FL5.12 cells, and annexin V–FITC/PI staining (B) or DNA fragmentation analysis (C) was performed at 48 hours. (**D**) Ecdysone-inducible expression of 24p3 in FL5.12 cells promotes apoptosis. Cells were analyzed by annexin V–FITC/PI staining 48 hours after addition of ponasterone A. (**E**) Recombinant 24p3 (r24p3) promotes apoptosis in FL5.12 and IL-3–dependent primary bone marrow cells. Purified r24p3 or (as a control) GST was added to FL5.12 or IL-3–dependent primary bone marrow cells, and viability was determined by trypan blue exclusion at the indicated times.

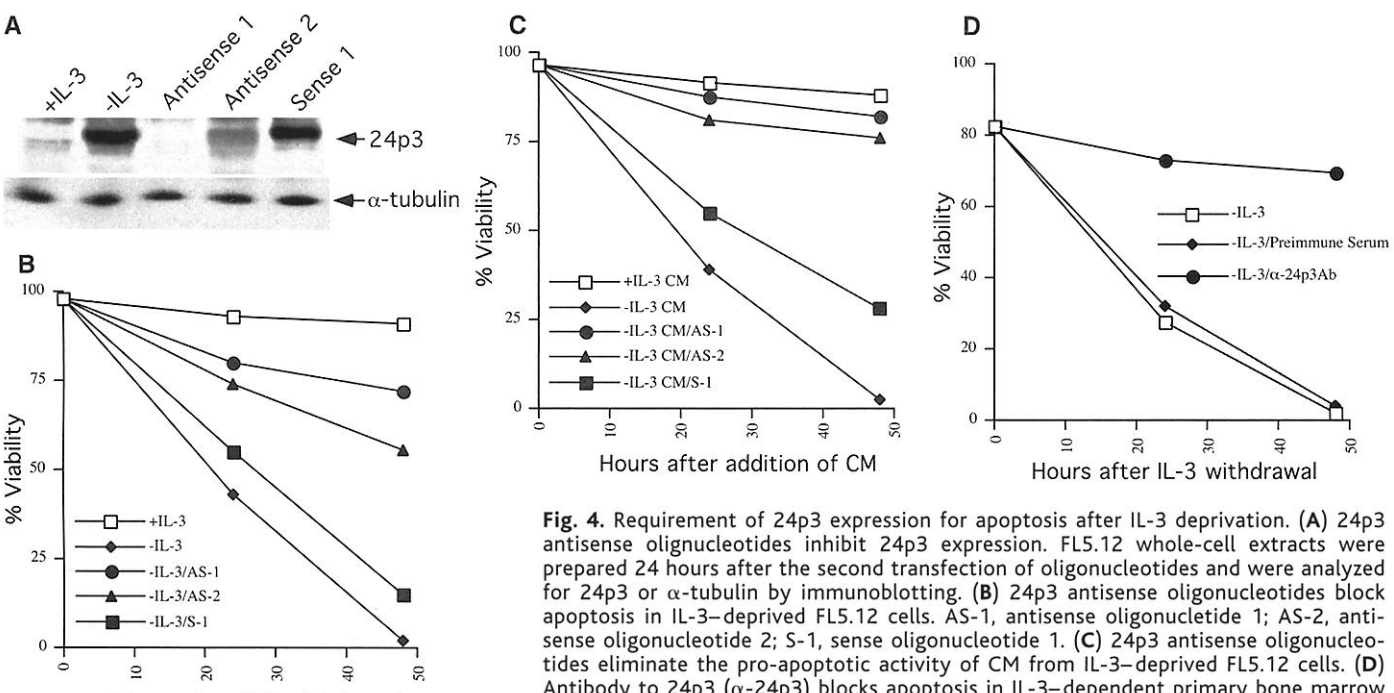


Fig. 4. Requirement of 24p3 expression for apoptosis after IL-3 deprivation. (**A**) 24p3 antisense oligonucleotides inhibit 24p3 expression. FL5.12 whole-cell extracts were prepared 24 hours after the second transfection of oligonucleotides and were analyzed for 24p3 or α-tubulin by immunoblotting. (**B**) 24p3 antisense oligonucleotides block apoptosis in IL-3–deprived FL5.12 cells. AS-1, antisense oligonucleotide 1; AS-2, antisense oligonucleotide 2; S-1, sense oligonucleotide 1. (**C**) 24p3 antisense oligonucleotides eliminate the pro-apoptotic activity of CM from IL-3–deprived FL5.12 cells. (**D**) Antibody to 24p3 (α-24p3) blocks apoptosis in IL-3–dependent primary bone marrow cells; 0.5 μg of affinity-purified antibody to 24p3 (*43*) or 2 μg of preimmune serum was added after IL-3 withdrawal, and cell viability was determined by annexin V–FITC/PI staining at the indicated times.

through a cell surface receptor that might be cell type–specific, we performed ligand-cell binding experiments. We found that ^{32}P-labeled 24p3 bound to FL5.12 cells and that >98% of binding could be blocked by addition of a 1000-fold molar excess of unlabeled 24p3 (Fig. 5A). 24p3 also bound specifically to IL-3–dependent primary bone marrow cells and to other leukocytic cell lines, such as 32D and murine HT-2, which are susceptible to 24p3–mediated apoptosis but not to nonhematopoietic cell lines, such as murine NIH 3T3 and COS-7, which are resistant. Human Jurkat T cells and murine D1-F4 cells, which are resistant to 24p3-induced apoptosis (Table 1) did not bind 24p3. Thus, for all cells tested, binding of 24p3 correlated with susceptibility to 24p3-mediated apoptosis.

Binding of 24p3 to FL5.12 cells was saturable (Fig. 5B, left), consistent with the protein competition results (Fig. 5A). On the basis of Scatchard plot analysis (Fig. 5B, right), we estimate that FL5.12 cells contain approximately 230,000 24p3 receptors, to which 24p3 binds with a dissociation constant of ~92 pM. These results reveal the existence of a high-affinity 24p3 receptor whose selective cellular distribution might explain the cell type specificity of 24p3-mediated apoptosis.

***24p3* transcriptional regulation.** To investigate whether the *24p3* gene is transcriptionally activated in cells dependent on cytokines other than IL-3, we analyzed the IL-2–dependent murine cell line HT-2 (*28*) and the IL-7–dependent murine cell line D1-F4 (*29*). Like FL5.12 cells, these cell lines are dependent on their respective cytokines for growth and undergo apoptosis upon cytokine deprivation. Transcription of *24p3* was not activated in HT-2 cells deprived of IL-2 or in D1-F4 cells deprived of IL-7, and when cultured in the absence of cytokine, the media from these cells was not pro-apoptotic (*13*). We also examined several mammalian cell lines that undergo apoptosis when deprived of serum. Transcription of *24p3* was not activated upon withdrawal of serum from human HL-60, Jurkat, or NIH 3T3 cells, even though apoptosis occurred (*13*). Thus, transcriptional activation of *24p3* after growth factor withdrawal appears to be highly specific (*30*).

Apoptosis of primary thymocytes can be induced by 24p3 (Table 1) and glucocorticoids (*31*). The *24p3* promoter has a glucocorticoid response element (*32*), which led us to test whether *24p3* was transcriptionally activated in primary thymocytes treated with the synthetic glucocorticoid dexamethasone. Untreated primary thymocytes had a low level of *24p3* transcription (Fig. 6A, lanes 1 through 5), perhaps explaining their low level of spontaneous apoptosis, whereas treatment with dexamethasone substantially increased *24p3* transcription (Fig. 6A, lanes 6 through 9). These observations suggest a possible mechanism by which glucocorticoids induce apoptosis in primary thymocytes.

Insulin-like growth factor 1 (IGF-1) stimulates proliferation and differentiation of a variety of cell types and can inhibit apoptosis resulting from deprivation of serum or cytokines (*33*). We therefore investigated the effect of IGF-1 on 24p3-mediated apoptosis. Unexpectedly, IGF-1 had no effect on apoptosis resulting from direct addition of 24p3 to FL5.12 cells (Fig. 6B). To help resolve this apparent paradox, we analyzed transcription of *24p3* under these different conditions. We found that IGF-1 blocked the transcriptional activation of *24p3* that normally occurs after IL-3 withdrawal (Fig. 6C, lane 2 versus 3). These results strongly suggest that IGF-1 promotes survival after IL-3 withdrawal by inhibiting *24p3* transcriptional activation. Direct addition of 24p3 bypasses this transcriptional block, and therefore IGF-1 has no effect.

Role of Bcl-2 family members. Previous studies have shown that several apoptotic pathways function by regulating phosphorylation of Bad, a pro-apoptotic member of the Bcl-2 family (*34*). Phosphorylation of Bad blocks its pro-apoptotic activity, which is promoted by IL-3 through a pathway involving phosphatatidylinositide-3′-OH kinase (PI3K) and Akt. IL-3 deprivation results in dephosphorylation of Bad and apoptosis (*2*, *34*).

The finding that 24p3 induced apoptosis even when IL-3 was present prompted us to analyze the effect of 24p3 on Bad phosphorylation (*35*). As expected, Bad was phosphorylated when cells were cultured with IL-3 (Fig. 6D, lane 1) and was unphosphorylated after IL-3 withdrawal (Fig. 6D, lane 2). Addition of 24p3 also led to dephosphorylation of Bad, even though IL-3 was present (Fig. 6D, lane 4). Thus, 24p3 can override the normal IL-3 signaling pathway, leading to dephosphorylation of Bad and apoptosis.

Another Bcl-2 family member, Bcl-X_L, inhibits apoptosis induced by a variety of stimuli, including IL-3 withdrawal (*11*, *36*). We analyzed the activity of 24p3 in FL5.12 cells expressing Bcl-X_L. Culture media from IL-3–deprived FL5.12 cells and COS-7 cells expressing 24p3 failed to induce apoptosis of FL5.12/Bcl-X_L cells (Fig. 6E), indicating that

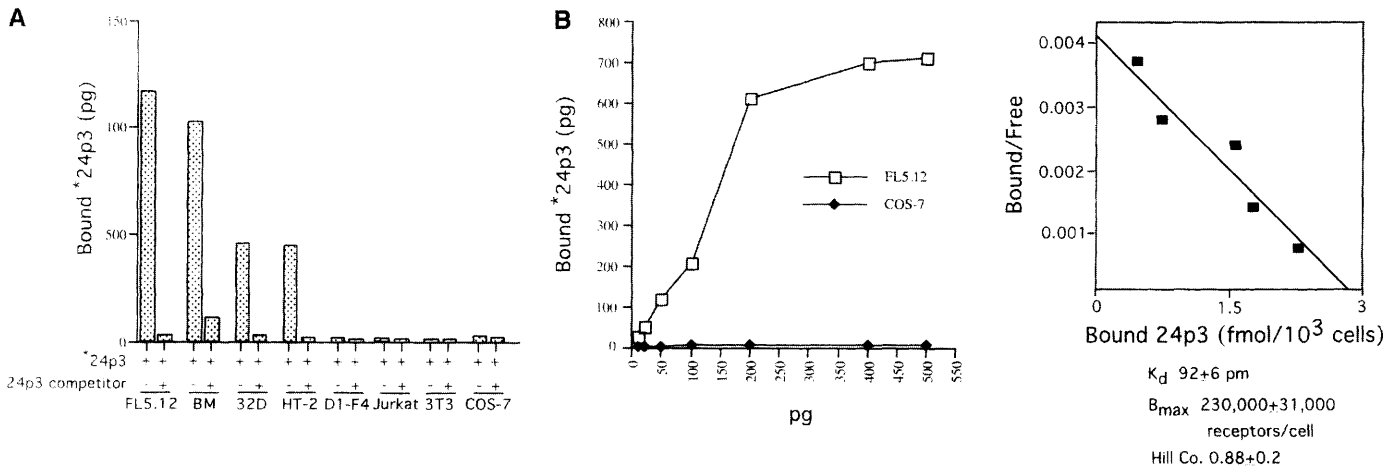


Fig. 5. A putative 24p3 receptor on cells susceptible to 24p3-mediated apoptosis. GST-24p3 was labeled by phosphorylation with heart myosin kinase (Sigma) and $^{32}\gamma$-ATP (specific activity ~7.7 dpm/μmole), and the GST moiety was removed by thrombin cleavage. 20,000 cells were incubated with ^{32}P-labeled 24p3 in 500 μl of RPMI supplemented with 10% FCS. After a 30-min incubation at 37°C, cells were pelleted and washed three times with RPMI containing 10% FCS, and the pellet and supernatant were assayed for radioactivity. Specific binding was defined as the difference between total binding and the nonspecific binding that remained after addition of a 1000-fold excess of unlabeled 24p3. (**A**) Specific binding of 24p3 to cells. *24p3, ^{32}P-labeled 24p3; 24p3 competitor, a 1000-fold molar excess of 24p3. (**B**) Scatchard analysis. A binding curve (left) and a Scatchard plot (right) are shown.

$Bcl\text{-}X_L$ blocks apoptosis induced both by IL-3 withdrawal and by 24p3 addition (*37*).

Discussion. Previous studies of apoptosis induced by IL-3 withdrawal have focused on posttranslational modifications of Bcl-2 family members. These studies have revealed important steps in this apoptotic pathway but have not explained the requirement for de novo transcription and translation. Our results reveal a transcriptionally regulated apoptotic program that can be broadly divided into two stages: In the first stage, IL-3 withdrawal transcriptionally activates the *24p3* gene, followed by synthesis and secretion of 24p3; in the second stage, secreted 24p3 interacts with a 24p3 cell surface receptor and induces apoptosis by an autocrine mechanism. 24p3 was not identified or predicted in numerous prior hematopoiesis and cytokine studies, which illustrates the power of expression profiling.

Our results imply that a major function of IL-3 in promoting cell viability is to maintain the *24p3* gene in a transcriptionally repressed state. Factors in addition to 24p3 may also contribute to the efficiency of apoptosis after IL-3 withdrawal. In this regard, *mNip3* and *p40Phox*, which have been previously implicated in apoptosis (*15*, *16*), were also transcriptionally activated (*13*).

24p3 is a member of the lipocalin family of small secreted polypeptides. The lipocalin family was originally identified and classified on the basis of amino acid similarity. It is now clear, however, that despite this sequence similarity, lipocalins have remarkably diverse functions, including, for example, retinol transport, cryptic coloration, olfaction, pheromone transport, and prostaglandin synthesis (*14*).

A detailed understanding of the physiological role(s) of 24p3 will require further studies. However, we have found that leukocytes are a major target of 24p3, which fits in well with several previous studies. For example, plasma levels of 24p3 are elevated during the acute phase response (*38*), which involves a massive expansion of neutrophils. Neutrophils are susceptible to 24p3-mediated apoptosis (Table 1), and 24p3 plasma levels may set an upper limit on neutrophil number. The likely human homolog of 24p3 is neutrophil-associated lipocalin (NGAL) (*14*), which is present in neutrophilic granules and could play a role in the neutrophilic apoptosis that follows an inflammatory response. *NGAL/24p3* expression has also been detected in inflamed epithelia (*39*) and in the mouse uterus during parturition (*40*). In both instances, 24p3 could help control inflammation. Our finding that 24p3 is expressed in primary thymocytes after dexamethasone treatment (Fig. 6A) raises the possibility that 24p3 may participate in thymocyte selection.

Secreted 24p3 may also be involved in immune system homeostasis, which requires that expanded cell populations be rapidly eliminated after their functions are completed. IL-3 is produced and secreted primarily by activated T cells; thus, as the immune response begins to terminate, IL-3 levels decrease (*41*). It has been previously recognized that declining IL-3 levels would prevent maturation of certain hematopoietic precursors and lead to apoptotic death of IL-3–dependent cells (*42*). Our results reveal that declining IL-3 levels also induce 24p3 expression and secretion, providing an independent mechanism to facilitate termination of the immune response.

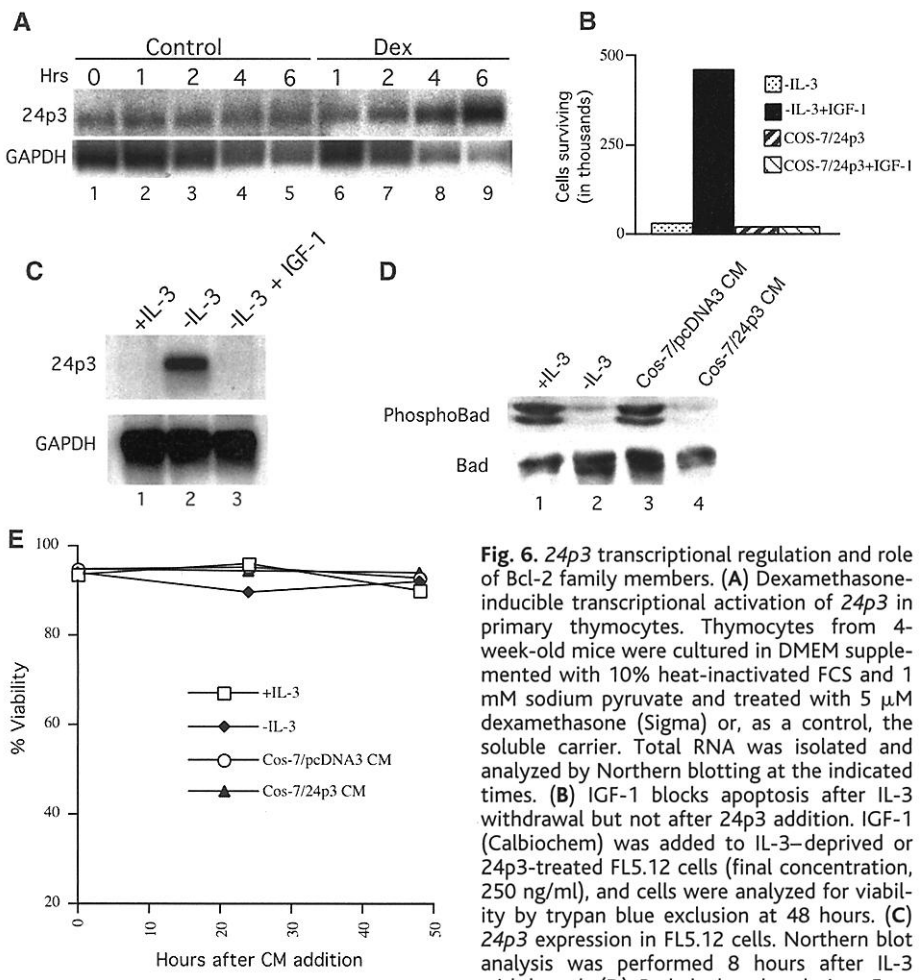


Fig. 6. *24p3* transcriptional regulation and role of Bcl-2 family members. (**A**) Dexamethasone-inducible transcriptional activation of *24p3* in primary thymocytes. Thymocytes from 4-week-old mice were cultured in DMEM supplemented with 10% heat-inactivated FCS and 1 mM sodium pyruvate and treated with 5 μM dexamethasone (Sigma) or, as a control, the soluble carrier. Total RNA was isolated and analyzed by Northern blotting at the indicated times. (**B**) IGF-1 blocks apoptosis after IL-3 withdrawal but not after 24p3 addition. IGF-1 (Calbiochem) was added to IL-3–deprived or 24p3-treated FL5.12 cells (final concentration, 250 ng/ml), and cells were analyzed for viability by trypan blue exclusion at 48 hours. (**C**) *24p3* expression in FL5.12 cells. Northern blot analysis was performed 8 hours after IL-3 withdrawal. (**D**) Bad dephosphorylation. Four hours after IL-3 withdrawal or 48 hours after 24p3 addition, immunoblot analysis was performed with an antibody that recognizes either total Bad or is specific for phosphorylated Bad as indicated. (**E**) Both IL-3 withdrawal and 24p3 addition fail to induce cell death in FL5.12 cells expressing $Bcl\text{-}X_L$. Cell viability was quantitated by trypan blue exclusion.

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20. Conditioned medium (2 ml) from cells grown in the presence or absence of IL-3 was concentrated in Centricon YM-10 filters (Millipore), and the retentates were collected and fractionated on a 12% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) gel. The membrane was incubated with an antibody to 24p3 (*43*) and developed with an ECL kit from Amersham.
21. IL-3 withdrawal and cell viability determinations were performed as in (*11*). For cell death assays, 2×10^5 hematopoietic cells or 8×10^5 fibroblasts in 60-mm dishes were incubated with conditioned medium from IL-3–deprived FL5.12 cells or COS-7 cells transfected with pcDNA3 or pcDNA3/24p3, supplemented with IL-3 (3 ng/ml). For fibroblasts, both floating and adherent cells (after trypsinization) were collected at the indicated time points and analyzed by a trypan blue (Sigma) dye exclusion assay. A

minimum of 400 cells were counted, and all experiments were performed in duplicate.
22. Apoptosis was assessed by staining with annexin V–FITC/PI (Calbiochem). Stained cells were analyzed in a Beckton-Dickinson flow cytometer. For DNA fragmentation analysis, DNA from 2×10^6 cells was isolated by phenol extraction and analyzed on a 1% agarose gel (*19*).
23. For construction of a stable cell line expressing 24p3, the 24p3 cDNA was amplified by polymerase chain reaction and cloned into the Eco RI and Bam HI sites of pcDNA3 vector (Invitrogen). COS-7 cells were transfected with pcDNA3/24p3 and selected with G418 at 600 μg/ml. G418-resistant colonies were isolated and screened for 24p3 expression by Northern blotting and immunoblotting.
24. For establishment of a 24p3 ecdysone-inducible cell line, the 24p3 cDNA containing a hemagglutinin (HA) tag at the COOH-terminus was cloned into the ecdysone-inducible vector pIND (Invitrogen). FL5.12 cells were transfected with Superfect (Qiagen). FL5.12 cells were first transfected with pVgRXR expressing the subunits of the receptor and selected with Zeocin (600 μg/ml) (Invitrogen). The resulting clones were then transfected with pIND/24p3-HA and selected with G418 (800 μg/ml) (Gibco-BRL). 24p3 expression was induced by addition of 10 μM ponasterone A (Invitrogen).
25. Comparable expression of a 24p3 derivative lacking the NH2-terminal signal sequence failed to induce cell death (*44*), suggesting that secretion of 24p3 is required for apoptosis.
26. Recombinant 24p3 was synthesized as a glutathione *S*-transferase (GST) fusion protein (pGST-2TK-24p3) and isolated to >90% purity. The GST moiety was removed by thrombin digestion. Purified 24p3 was added to cells to a final concentration of 0.8 to 10 μg/ml.
27. Phosphorothioate oligonucleotides were purchased from Genosys. Sense and antisense 1 oligonucleotides span −12 to −5 and antisense 2 oligonucleotide spans +585 to +593 of 24p3 mRNA (+1, translation start site). FL5.12 cells were transfected with 2 μM of each oligonucleotide using lipofectamine (Gibco-BRL). After 24 hours, cells were washed with RPMI medium plus 10% fetal calf serum (FCS) and again transfected with 2 μM of oligonucleotide. Transfected cells were deprived of IL-3 24 hours after the second transfection.
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35. For analysis of Bad phosphorylation, FL5.12 cells were treated with conditioned medium from COS-7 cells transfected with pcDNA3 or the 24p3 expression vector. Cells were lysed in 1% NP-40 lysis buffer containing 20 mM tris-HCl (pH 7.4), 150 mM NaCl, 1.5 mM MgCl2, 1 mM EDTA, 10 mM NaF, 0.2 mM Na_3VO_4, 1 mM Na_3MoO_4, and protease inhibitor tablets from Boehringer-Mannheim. Cell lysates were incubated with 2 μg of Bad antibody (Transduction Labs, Lexington, KY). Immune complexes were resolved by 12% SDS-PAGE and transferred onto a polyvinylidene diflouride membrane (Millipore). Blots were incubated with either a phospho-specific antibody to Bad (New England Biolabs) or an antibody to Bad and were developed with an ECL kit (Amersham).
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37. FL5.12/Bcl-X_L cells synthesize and secrete 24p3 after IL-3 deprivation (*44*).
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A Cellular Function for the RNA-Interference Enzyme Dicer in the Maturation of the *let-7* Small Temporal RNA

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The 21-nucleotide small temporal RNA (stRNA) *let-7* regulates developmental timing in *Caenorhabditis elegans* and probably in other bilateral animals. We present in vivo and in vitro evidence that in *Drosophila melanogaster* a developmentally regulated precursor RNA is cleaved by an RNA interference-like mechanism to produce mature *let-7* stRNA. Targeted destruction in cultured human cells of the messenger RNA encoding the enzyme Dicer, which acts in the RNA interference pathway, leads to accumulation of the *let-7* precursor. Thus, the RNA interference and stRNA pathways intersect. Both pathways require the RNA-processing enzyme Dicer to produce the active small-RNA component that represses gene expression.

Two small temporal RNAs (stRNAs), *lin-4* and *let-7*, regulate the timing of development in *Caenorhabditis elegans* (*1–3*). stRNAs encode no protein, but instead appear to block the productive translation of mRNA by binding sequences in the 3′-untranslated region of their target mRNAs (*1*, *2*, *4–11*). *let-7* is present in most if not all bilaterally symmetric animals, including *Drosophila melanogaster* and humans (*12*). In *Drosophila*, *let-7* first appears at the end of the third larval instar, accumulates to high levels in pupae, and persists in adult flies (*12*).

The mechanism by which stRNAs are synthesized is unknown. The ~21-nucleotide (nt) *let-7* RNA has been proposed to be cleaved from a larger precursor transcript (*12*). The generation of small RNAs from a longer, structured precursor—double-stranded RNA (dsRNA)—is an essential feature of the RNA interference (RNAi) pathway, raising the possibility that stRNAs are generated by mechanisms similar to the initial steps in RNAi and suggesting that enzymes such as the *Drosophila* protein Dicer might play a role in generating stRNAs (*13–18*).

A candidate RNA for the *Drosophila let-7* precursor. Examination of the developmental expression of *let-7* in *Drosophila* revealed a candidate for a *let-7* precursor RNA, *let-7L* (*19*). *let-7L* was detected at the end of the third larval instar and at the beginning of pupation, the same developmental stages where *let-7* itself is first expressed (Fig. 1A) (*12*). Consistent with the transcript being a *let-7* precursor, the amount of *let-7L* RNA declined as *let-7* accumulated. *let-7L* RNA was slightly shorter than a 76-nt RNA standard. Previous analysis of the genomic sequence flanking *Drosophila let-7* led to the proposal that a 72-nt RNA hairpin might be a *let-7* precursor (*12*) (Fig. 1B).

let-7 is also expressed in human tissues (*12*) and in cultured human HeLa cells, but not in *Drosophila* embryos or cultured *Drosophila* S2 cells (Fig. 1C). Primer extension analyses confirmed that the mature *let-7* RNA detected by Northern hybridization was bona fide *let-7* (*20*). Primer extension products corresponding to the 5′ ends of mature *let-7* RNAs were detected in total RNA from early and unstaged *Drosophila* pupae and from human HeLa cells (Fig. 1D). Primer extension analysis of total RNA from un-

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