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# The Ets2 Transcription Factor Inhibits Apoptosis Induced by Colony-Stimulating Factor 1 Deprivation of Macrophages through a Bcl-$x_L$-Dependent Mechanism

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**Bcl-$x_L$, a member of the Bcl-2 family, inhibits apoptosis, and its expression is regulated at the transcriptional level, yet nothing is known about the transcription factors specifically activating this promoter. The *bcl-x* promoter contains potential Ets binding sites, and we show that the transcription factor, Ets2, first identified by its sequence identity to v-*ets* of the E26 retrovirus, can transactivate the *bcl-x* promoter. Transient expression of Ets2 results in the upregulation of Bcl-$x_L$ but not of Bcl-$x_S$, an alternatively spliced gene product which induces apoptosis. Ets2 is ubiquitously expressed at low levels in a variety of cell types and tissues but is specifically induced to abundant levels during macrophage differentiation. Since Bcl-$x_L$ is also upregulated during macrophage differentiation, we asked whether the *bcl-x* could be a direct downstream target gene of Ets2 in macrophages. BAC1.2F5 macrophages, which are dependent on macrophage colony-stimulating factor 1 (CSF-1) for their growth and survival, were used in these studies. We show that CSF-1 stimulation of BAC1.2F5 macrophages results in the upregulation of expression of *ets2* and *bcl-$x_L$* with similar kinetics of induction. In the absence of CSF-1, these macrophages undergo cell death by apoptosis, whereas constitutive expression of Ets2 rescues these cells from cell death, and *bcl-$x_L$* is upregulated. These results strongly suggest a novel role of Ets2 in affecting apoptosis through its regulation of Bcl-$x_L$ transcription.**

Cell death by apoptosis is a process essential for normal development and maintenance of cell homeostasis in organisms. Although the mechanisms of inducing or inhibiting cell death are not well understood, several proteins have been identified as initiators or inhibitors of apoptosis. Antiapoptotic proteins include Bcl-2, Bcl-$x_L$ (5), Bcl-w (21), A1 (30), and Mcl-1 (27). Bcl-2, the first antiapoptotic protein identified, and the closely related Bcl-$x_L$ are probably the best characterized. Their genomic structures are similar and are believed to have arisen from a common ancestral gene or by gene duplication (23). Expression of Bcl-$x_L$ is regulated at the transcriptional level, yet the specific transcription factors activating this promoter have not yet been characterized.

The *bcl-x* gene encodes several alternatively spliced mRNAs, including *bcl-$x_L$*, *bcl-$x_S$*, bclΔTM, and *bcl*-β (23, 43). Bcl-$x_L$ suppresses, whereas Bcl-$x_S$ induces, apoptosis (5, 17, 22). The functions of the other two transcripts have not yet been well documented. Bcl-$x_L$ is upregulated in different myeloid cells as they differentiate toward macrophages, including murine leukemic M1 myeloblasts (31), human promyelocytic leukemia HL60 cells, human myeloid U937 cells, and human peripheral blood monocytes exposed to phorbol esters (12). In contrast, Bcl-2 is downregulated in these systems. Both the 5′ regulatory sequences found upstream of the first noncoding exon of Bcl-$x_L$ and the first facultative intron contain potential Ets binding sites (EBS) for Ets transcription factors, and both of these regions have been shown to have promoter activity (23).

The nuclear proto-oncogene Ets2 was first identified by its sequence identity (7, 19, 49, 50) to the v-Ets portion of the gag-Myb-Ets fusion protein of the E26 avian retrovirus (29, 37). Ets2 is a member of a large family of transcription factors known as the *ets* family. The most highly conserved domain of Ets first identified by sequence comparisons (7, 49) was shown to contain nuclear localization signals and to be the DNA binding domain (8). This domain of approximately 85 amino acids is known as the Ets domain, and it recognizes a GGA consensus core sequence (reviewed in reference 48). The specificity of Ets family members is provided by sequences flanking the GGA core. Although Ets2 binds to DNA in its monomeric form, it can bind in conjunction with transcription factors binding to adjacent sites to activate transcription (18).

Ets2 expression correlates with cell proliferation (4) and differentiation (9, 19) and with different stages during *Xenopus* (13, 33) and mouse development (32). Recent studies substantiate these correlations by showing that Ets2 is necessary for early embryonic development (51) and plays a role in cartilage and bone development (45) and in macrophage differentiation (2, 25). Macrophages represent the final step in myelomonocytic differentiation, and they play an essential role in inflammatory responses and in defense mechanisms of the organism against infectious diseases and neoplasia. Ets2 expression correlates with the later stages of myelomonocytic differentiation toward macrophages (9, 19), and constitutive expression of Ets2 in an immature myeloblastic leukemic cell is sufficient to induce the onset of macrophage differentiation (2). Ets2 expression also correlates with the induction of macrophage functions (9), yet the significance of this still remains unknown. Recent in vivo studies show normal macrophage development

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in adult *ets2*-deficient mice (51) when they are rescued from early embryonic death. However, transgenic studies show that a dominant-negative Ets2 mutant under the control of a monocyte/macrophage-specific promoter results in aberrant monocyte/macrophage development only during the first 40 days after birth (25). Taken together, these results indicate that Ets2 plays a role in macrophage differentiation, yet it is likely that other *ets* family members can compensate for a loss of functional Ets2.

BAC1.2F5 is a macrophage cell line dependent on macrophage colony-stimulating factor 1 (CSF-1) for its growth and survival (35). Interestingly, both *ets2* and *bcl-x*$_L$ are coinduced upon CSF-1 stimulation with similar kinetics. We thus investigated whether the *bcl-x* promoter could be a physiological target of Ets2.

## MATERIALS AND METHODS

**Transactivation studies.** The 5′ regulatory sequences of the *bcl-x* gene, including the first facultative intron, were cloned by nested PCR. By using human DNA from the HT29 cell line, the first PCR was performed with the following primers: GTCCAAAACACCTGCTCACTCACT and CTCCCTGCGTCCCTCACTGA AACC. After denaturation at 94°C for 5 min, *Taq* (Goldstar Red; Eurogente) was added and amplification was performed through 30 cycles (1 min at 94°C, 1 min at 55°C, and 1 min at 72°C). A second, nested PCR was performed with primers CCAAAGCCAAGATAAGATTCTGAA and CAAAAACCAACTAA ATCCATACCA under the same conditions as those for the first PCR. The 700-bp PCR product was sequenced, and its sequence is identical to that reported in the DDBJ/EMBL/GenBank databases (accession no. D30746). This PCR fragment, corresponding to the *bcl-x* promoter containing the nine EBS, was first introduced into pUAg, digested with *Eco*RI, and excised from an agarose gel. This excised *Eco*RI/*Eco*RI fragment was then subcloned into pBSKII(−), digested with *Bam*HI/*Hin*dIII, and excised. Finally, the excised *bcl-x* *Bam*HI/*Hin*dIII fragment was subcloned in the *Bgl*II/*Hin*dIII sites of the promoterless pXP vector (pXP1) upstream of the luciferase gene to generate pXP-Bcl-x$^{Pr}$.

A truncated promoter fragment deleting a cluster of seven upstream EBS was generated by digestions of pBSKII-*bcl-x* with *Bgl*II and *Sac*I. This 422-bp fragment, containing only two EBS, was then subcloned into *Bgl*II and *Sac*I sites of pXP1 to generate pXP-ΔBcl-x$^{Pr}$. Another promoter construct, containing six upstream EBS, was generated by digesting pXPBcl-x$^{Pr}$ with *Sma*I and *Bgl*II. This fragment was subcloned into the thymidine kinase promoter reporter construct, pTK-Luc, which was digested with *Hin*dIII (blunted with Klenow enzyme) and then digested with *Bam*HI to generate Bcl-x$^{Pr}$TK-Luc. These reporter constructs were used in the transactivation experiments. Ets2 or a dominant-negative mutant of Ets2, Δ1-238Ets2, was cloned into pRK5 (42) to generate pRK5-*ets2* or pRK5Δ1-238*ets2*, respectively, as previously described (2). 293 cells were transfected by the calcium phosphate coprecipitation method in 24-well dishes with 150 ng of the reporter construct in the presence of pRK5 (150 ng), pRK5-*ets2* (150 ng), or pRK5Δ1-238*ets2* (150 ng) and 20 ng of pCMV-βGal as an internal control for transfection efficiency.

AP1 activity was also measured by using the full-length (pXP-Bcl-x$^{Pr}$) and truncated promoter (pXP-ΔBcl-x$^{Pr}$) reporter constructs. pXP-Bcl-x$^{Pr}$ or pXP-ΔBcl-x$^{Pr}$ was cotransfected with 200 ng of pRK5, pRK-*fos*, or pRK-*jun* (39) and 20 ng of pCMV-βGal, which served as an internal control as described above. pRK-*ets1* was generated by digestion of pBSK-*ets1* and pRK7 with *Eco*RI and *Bam*HI, and pRK-*PU1* was made by digestion of pBSK-*PU1* and pRK7 with *Eco*RI and *Bam*HI. pXP-Bcl-x$^{Pr}$ (150 ng/ml) was cotransfected in 293 cells with either pRK-*ets1* (150 ng/ml) or pRK-*PU1* (150 ng/ml) and 20 ng of pCMV-βGal as described above.

Several independent experiments using 293 cells were performed in duplicate, triplicate, or quadruplicate. Cell lysates were prepared as described previously (1). Briefly, 48 to 72 h after transfections, cell lysates were prepared in 25 mM Tris (pH 7.5)–10% glycerol–1% Triton X-100–2 mM dithiothreitol and analyzed for luciferase and β-galactosidase activities as described by the respective manufacturers, Promega and Tropix (Galactolight). All luciferase activities were corrected according to pCMV-βGal, used as an internal control for transfection efficiency.

**Cell culture and establishment of BAC-Ets2 clones.** BAC1.2F5 cells (35) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Gibco-BRL) and 20% L-cell-conditioned medium as a source of CSF-1 (44).

The complete human c-*ets2* cDNA was inserted into the murine retroviral vector pLXSN containing the *neo* gene for selection (34) as previously described (2) with the following modifications. After transfection of the murine ecotropic retrovirus packaging cell line PE501 (34) with the retroviral construct, the viral supernatant obtained 24 h posttransfection was used to infect BAC1.2F5 cells. Neomycin-resistant clones of BAC1.2F5 cells constitutively expressing Ets2 were obtained upon selection with 200 μg of active G418 (Geneticin; Sigma)/ml.

**Northern (RNA) hybridization analysis.** Cells were CSF-1 starved for 24 h or 3 days as indicated in the text. Cells were treated either with conditioned media from L cells as a source of CSF-1 or with 60 ng of purified recombinant CSF-1 (rCSF-1)/ml for the times indicated. Treatments with actinomycin D Boehringer Mannheim and cycloheximide (Sigma) were carried out at 5 and 10 μg/ml, respectively. Cells were pretreated with dimethyl sulfoxide, actinomycin D, or cycloheximide for 30 min and were then either maintained in the absence of CSF-1 or restimulated with 60 ng of purified rCSF-1/ml for an additional 2 h.

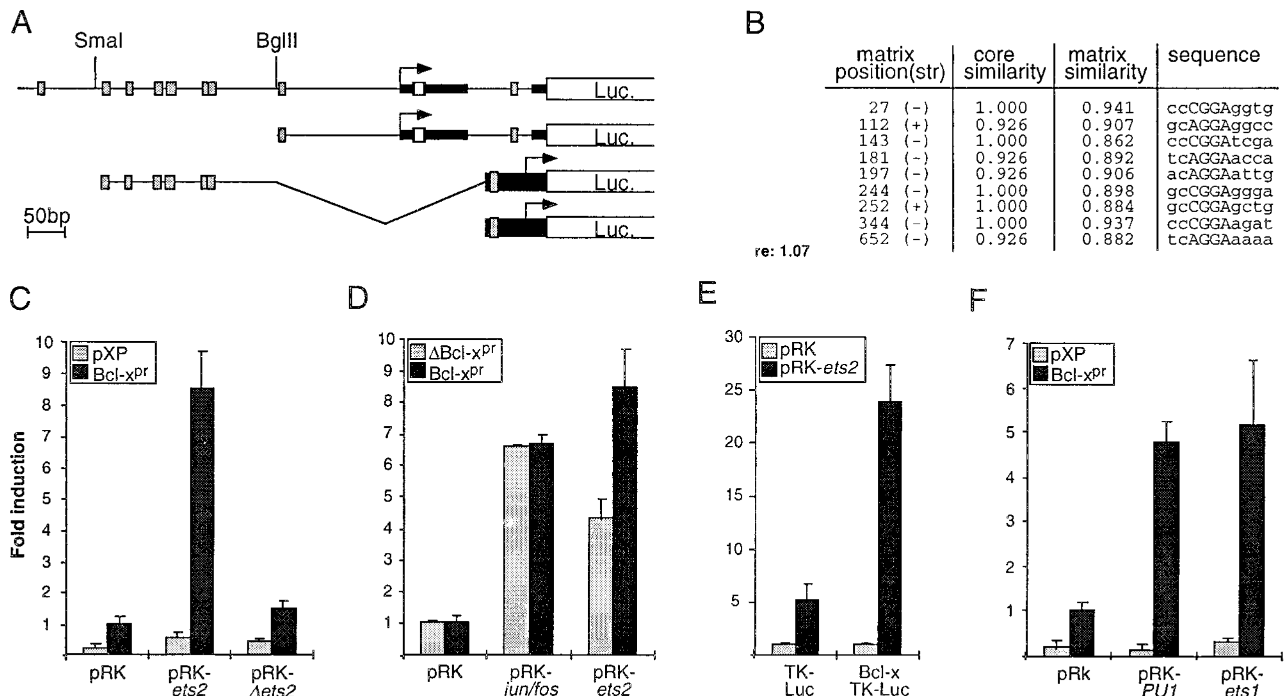
Cells were washed twice with 1× phosphate-buffered saline (PBS) and then lysed in RNA Insta-Pure (Eurogentec) as described by the manufacturer. Five micrograms of total RNA was loaded and electrophoresed on a 2.2 M formaldehyde–1% agarose gel and then transferred to a nylon membrane (Amersham) as described by the manufacturer. Purified *ets2*, *ets1*, *PU-1/spi.1*, *bcl-x*$_L$, *bcl-2*, CSF-1, interleukin 1β (IL-1β), JE (also known as monocyte chemoattractant protein 1 [MCP-1]), tumor necrosis factor alpha (TNF-α), and S26 cDNA fragments were used as probes. High-specific-activity probes were generated by using the Stratagene Prime-It kit as described by the manufacturer. Prehybridization and hybridization were carried out at 42°C in a solution of 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's solution–0.5% sodium dodecyl sulfate (SDS)–50% formamide containing 20 μg of denatured salmon sperm DNA per ml. Washing was performed under normal-stringency conditions at 50°C by using 0.1× SSC–0.1% SDS. All mRNA transcripts except CSF-1 were visualized after a 4- to 12-h exposure to BIOMAX film (Kodak) at −80°C with Dupont Quanta Fast intensifying screens and with a phosphorimager system (Fuji). CSF-1 was visualized after a 4-day exposure. All signals were quantified by using MacBAS version 2.2 software, and fold inductions were calculated by taking into account the S26 signal as a control for RNA loading (47).

**Detection of Ets2-dependent induction of Bcl-x$_L$ in 293 cells.** The internal ribosomal entry site sequence of poliovirus 1 (nucleotides 1 to 633) was cloned into the *Sma*I site of pEGFP-N1 (Clontech) to generate pCIG. The cDNA encoding the full-length human Ets2 in pBSKII(−) was digested with *Xho*I and *Sma*I and subcloned into *Xho*I and *Sac*II (blunted) sites of pCIG to generate pCIG-Ets2. The cDNA encoding ΔEts2 was digested with *Bam*HI and *Apa*I and was subcloned into the *Bgl*II and *Apa*I sites of pCIG to generate pCIG-ΔEts2. 293 cells were transiently transfected with Ets2, ΔEts2, or the empty pCIG vector containing green fluorescent protein (GFP). After 48 h, cells were washed in PBS, and GFP was visualized by fluorescence to determine the efficiency of transfections. Cells were then lysed, and 10 μg of total protein from each lysate in Laemmli buffer was electrophoresed on a 10% polyacrylamide–bisacrylamide gel. Migrated proteins were transferred to a polyscreen polyvinylidene difluoride (PVDF) transfer membrane as described by the manufacturer (Dupont-NEN), immunoblotted by using Bcl-x and Bcl-2 antibodies, and revealed by ECL as described by the manufacturer (Amersham).

The detection of Ets2 was performed in the following manner. Proliferating neomycin-resistant BAC1.2F5 cells infected with the empty retroviral vector and neomycin-resistant cells infected with an Ets2 retrovirus were washed twice in cold 1× PBS. Cells were scraped and centrifuged, and the remaining PBS was removed. Cell pellets were immediately frozen in liquid nitrogen and stored in a −80°C freezer until use. Cells were thawed and sonicated, and total protein was quantified by using the Bio-Rad Protein Assay in 1× radioimmunoprecipitation assay buffer. Equal amounts of total protein of each cell lysate were then immunoprecipitated with an Ets-specific antibody corresponding to the C-terminal region of v-Ets recognizing both Ets1 and Ets2 proteins as previously described (20). Following electrophoresis on an SDS–10% polyacrylamide gel and transfer, the membrane was then subjected to ECL relevation (Amersham) using the same Ets antiserum as a primary antibody.

**Cell cycle immunoblot analyses and kinase assays.** Detection of cell cycle proteins cyclin A, cyclin B1, cyclin D1, cyclin E, p27, p21, Cdk4 and Cdk2, and pRb was performed as follows. Cell pellets were obtained as described above from populations starved of CSF-1 for 3 days or from populations starved of CSF-1 for 4 days and restimulated with CSF-1 for 10 or 22 h. Preparation of whole-cell extracts, the conditions for immunoprecipitation, histone H1 kinase assays, and immunoblotting have been described previously (15). Western blot analysis of cyclin immunoprecipitates was performed as described (16). When both immunoprecipitating and immunoblotting antibodies were generated in the same species, the immunocomplexes were not boiled but only incubated in the Laemmli SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer at 37°C (15 min), and horseradish peroxidase-conjugated ImmunoPure protein A/G (Pierce) was used for detection. The proteins were visualized by using the ECL detection system (Amersham). Most of the primary antibodies used in this study have been described previously (16). pRb-P-Ser780-specific antibodies, described by Kitagawa et al. (26), were purchased from MBL.

**Detection of apoptotic cells.** Apoptotic cells were detected both with Annexin V and by terminal deoxynucleotide transferase (TdT)-mediated dUTP nick end labeling (TUNEL). Annexin V interactions with phosphatidylserines on the outer surfaces of cells were determined as described by the manufacturer (Boehringer Ingelheim) with the following modifications. After cells were incubated with Annexin V conjugated with fluorescein isothiocyanate (FITC) and washed in binding buffer, they were fixed in PBS containing 3% paraformaldehyde for 15 min at 20°C. Cells were then washed in 1× PBS, then incubated with




| matrix position(str) | core similarity | matrix similarity | sequence |
|---|---|---|---|
| 27 (-) | 1.000 | 0.941 | ccCGGAggtg |
| 112 (+) | 0.926 | 0.907 | gcAGGAggcc |
| 143 (-) | 1.000 | 0.862 | ccCGGAtcga |
| 181 (-) | 0.926 | 0.892 | tcAGGAacca |
| 197 (-) | 0.926 | 0.906 | acAGGAattg |
| 244 (-) | 1.000 | 0.898 | gcCGGAggga |
| 252 (+) | 1.000 | 0.884 | gcCGGAgctg |
| 344 (-) | 1.000 | 0.937 | ccCGGAagat |
| 652 (-) | 0.926 | 0.882 | tcAGGAaaaa |

FIG. 1. Transactivation studies. (A) The full-length human *bcl-x* promoter containing nine EBS and a truncated promoter containing only two EBS cloned upstream of the luciferase gene (Luc) are schematically represented. Solid boxes represent the first exon and part of the second exon, and the arrow represents the transcriptional start site based on the sequence in the databases (accession no. D30746). EBS were identified by using the MatInspector version 2.2 program (40) and are represented by shaded boxes. The open box in the first exon corresponds to an AP1 site. Also schematically represented is a thymidine kinase promoter reporter construct (TK-Luc) alone or with a cluster of upstream EBS. The shaded box in the thymidine kinase promoter corresponds to an EBS. (B) EBS sequences (capital letters, core), positions, and orientations on the positive (+) or minus (−) strand (str) are indicated. The prediction of EBS found randomly over 700 bp is indicated (random event [re], 1.07). Sequences with matrix similarities higher than 0.85 were selected. (C) 293 cells were transfected with the empty reporter, pXP, or the full-length *bcl-x* promoter construct (Bcl-$x^{Pr}$) in the presence of pRK5 (pRK), pRK5-*ets2*, or pRK5Δ1-238*ets2* (pRK-Δ*ets2*). Transcriptional activities are reported as fold inductions compared to the activity of Bcl-$x^{Pr}$ without an exogenous transactivator, arbitrarily set at 1. pCMV-βGal was used in all experiments as an internal control for transfection efficiency. All experiments were repeated three to eight times, and standard deviations are indicated. (D) Cotransfections of 293 cells with the full-length (Bcl-$x^{Pr}$) and truncated promoter (ΔBcl-$x^{Pr}$; see panel A) constructs in the presence of pRK or pRK-*ets2* or pRK-*jun*/*fos* were performed as described for panel C. Transcriptional activities are reported as fold induction compared with the activities of both promoter constructs in the absence of exogenous factors, set arbitrarily at 1. (E) The TK-Luc reporter construct alone or containing a cluster of upstream EBS (Bcl-$x^{Pr}$ TK-Luc) was cotransfected in 293 cells with pRK or pRK-*ets2*. Transcriptional activities are reported as fold induction compared to the activity of TK-Luc in the absence of exogenous factors, set at 1 arbitrary unit. pCMV-βGal was used as described above as an internal control for transfection efficiency. (F) pXP or Bcl-$x^{Pr}$ was cotransfected with the pRK, pRK-*PU1*, or pRK-*ets1* expression construct. Transcriptional activities are reported as fold inductions compared to the activity of Bcl-$x^{Pr}$ without an exogenous transactivator, arbitrarily set at 1. pCMV-βGal was used in all experiments as an internal control for transfection efficiency.

a 1/5,000 dilution of 4′,6-diamidino-2-phenylindole (DAPI) for 5 min at 37°C. Cells were then washed three times in 1× PBS and twice in water; then Mowiol was added to the slide and cells were mounted.

For detection of DNA breaks, TUNEL was performed. Cells were fixed in 3.7% formalin for 10 min at 20°C. Following PBS washes, cells were permeabilized in 0.1% sodium citrate and 0.1% Triton X-100 for 2 min at 4°C. Cells were then washed in 1× PBS and water and incubated with 75 U of TdT from calf thymus and biotin-16-dUTP (both from Boehringer Mannheim) for 60 min at 37°C in a humid chamber. Following washes in PBS and PBS containing 5% fetal calf serum, cells were incubated with a 1/5,000 dilution of streptavidin-Texas Red and DAPI for 30 min at 37°C in the dark. Cells were washed and mounted as described above.

## RESULTS

**Ets2 transactivates the *bcl-x* promoter and upregulates Bcl-$x_L$ expression.** We previously showed that Ets2 expression correlates with macrophage differentiation and function (9, 19). Since Bcl-$x_L$ expression is upregulated in cells as they differentiate toward macrophages (12, 31), we asked whether Ets2 could upregulate *bcl-$x_L$* expression by transactivating the *bcl-x* promoter. To address this question, we cloned and sequenced the human *bcl-x* promoter. The sequence obtained is identical (data not shown) to the sequence found in the databases (accession no. D30746). To determine whether this promoter does indeed respond to Ets2, we cloned it into a promoterless reporter vector, pXPLuc. Cotransfection into human 293 cells of pRK-*ets2*, a human Ets2 expression vector, with this full-length promoter construct, pXP-Bcl-$x^{Pr}$, resulted in an eightfold increase in transcriptional activity compared to the activity observed in the absence of exogenously expressed Ets2 (Fig. 1C). Cotransfections with an expression vector coding for a dominant-negative mutant of Ets2 (pRK-Δ*ets2*, corresponding to the DNA binding domain devoid of the majority of the transactivation domain [2]) resulted in a transcriptional activity similar to that observed with the empty expression vector (Fig. 1C). It is worth noting that, in agreement with previous results reported by Grillot et al. for the murine *bcl-x* promoter in the absence of exogenously added transcription factors (23), the fragment encompassing the *bcl-x* promoter cloned in the opposite orientation also showed some activity (data not shown).

To further characterize the response of the *bcl-x* promoter to Ets2, we performed a computer search for EBS using MatInspector (40). This analysis revealed that an unusually high number of EBS are found within this 700-bp promoter fragment. Eight EBS are found upstream of the first noncoding exon, and one is found in the first intron (Fig. 1A), while the predicted number of EBS in a random 700-bp sequence is 1.07. The nine EBS sequences, as well as their locations and orien-

tations, are listed in Fig. 1B. This promoter region is highly conserved between humans and mice (over 70%) (23). Like the human *bcl-x* promoter, the murine *bcl-x* promoter also contains a high concentration of sites (11 EBS in 700 bp [data not shown]). To determine the role of these EBS, we first deleted the cluster of seven upstream EBS sites (Fig. 1A), resulting in a truncated promoter still containing two EBS ($\Delta$Bcl-$x^{Pr}$). In 293 cells, this truncated promoter displayed a fivefold-decreased basal activity compared to that of the full-length *bcl-x* promoter (data not shown). Furthermore, its relative response to Ets2 is decreased by 50% compared to that of the full-length promoter (Fig. 1D). To validate this decrease in Ets2 stimulation and rule out the possibility that our deletion resulted in a total disruption of *bcl-x* promoter activity, we took advantage of an AP1 site which is present in the first exon and thus is found in both the truncated and the full-length promoter constructs. The responses of the two promoters to AP1 (Fos plus Jun) in 293 cells were identical (Fig. 1D), demonstrating that the removal of the EBS cluster specifically affected the response of the promoter to Ets2 and not to AP1. Finally, we cloned the cluster of six upstream EBS upstream of a thymidine kinase promoter construct to generate Bcl-xTK-Luc. The presence of this cluster rendered the thymidine kinase promoter 5 times more responsive to Ets2. The induction observed with pRK-*ets2* and TK-Luc in the absence of the EBS cluster results from an internal EBS found in the thymidine kinase promoter (Fig. 1A). Taken together, these results indicate that (i) the *bcl-x* promoter responds to Ets2 and (ii) Ets2 transactivates this promoter through both the distal upstream cluster and the two proximal EBS.

Since Ets2 can transactivate the *bcl-x* promoter through the nine EBS found within its sequence, it was probable that other *ets* family members might as well. We performed transactivation studies using the most closely related Ets2 family member, Ets1, and a more distantly related member which is expressed in macrophages, PU1/Spi.1. As predicted, both Ets1 and PU1/Spi.1 could transactivate the *bcl-x* promoter with similar efficiencies in 293 cells (Fig. 1F). However, it is unlikely that these proteins affect *bcl-x* transcription in BAC1.2F5 macrophages (see below).

Next we wanted to determine whether transient expression of Ets2 could result in the upregulation of Bcl-$x_L$ protein. To this end, 293 cells were transiently transfected with one of the bicistronic vectors pCIG-Ets2 and pCIG-$\Delta$Ets2 or with an empty pCIG vector. Transfection efficiencies were similar in these experiments, as determined by the percentages of GFP-positive cells (Fig. 2). Cells were then lysed, and 10 $\mu$g of total protein from each lysate was electrophoresed on SDS–10% polyacrylamide gels and immunoblotted by using Bcl-x and Bcl-2 antibodies. A protein with an apparent molecular size of approximately 32.5 kDa, corresponding to the antiapoptotic Bcl-$x_L$ gene product, is upregulated 4.5-fold in 293 cells transiently expressing Ets2 (Fig. 2) compared to 293 cells transfected with the control vector (arbitrarily set at 1) or one containing $\Delta$Ets2 (0.6-fold). Although the Bcl-x antibody used in these studies can recognize the proapoptotic Bcl-$x_S$ gene product of approximately 25 kDa, Bcl-$x_S$ is not detected in these experiments. In addition, low levels of Bcl-2 are detected, and these levels of Bcl-2 expression remain unchanged in 293 cells transfected with any of the three plasmid constructs (data not shown). Together, these results show that Ets2 can transactivate the *bcl-x* gene and can specifically upregulate Bcl-$x_L$ protein expression.

Since Ets2 expression correlates with macrophage differentiation and function (9, 19), and Bcl-$x_L$ expression is upregulated in cells as they differentiate toward macrophages (12, 31),

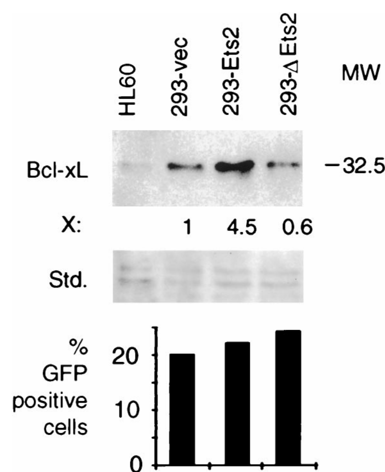



FIG. 2. Bcl-$x_L$ is upregulated by transient Ets2 expression. The level of Bcl-$x_L$ is upregulated in 293 cells transiently transfected with Ets2 (293-Ets2) but not with $\Delta$Ets2 (293-$\Delta$Ets2) or with an empty vector (293-vec). Before cell lysis, GFP was visualized and the efficiencies of the transfections were determined as the percentages of GFP-positive cells, ranging from 20 to 24%. Ten micrograms of total protein from each lysate was analyzed on an SDS–10% polyacrylamide gel, transferred to a membrane, incubated with Bcl-x and Bcl-2 antibodies, and processed by ECL as described in Materials and Methods. Fold inductions of Bcl-$x_L$, determined by using the program MacBAS, version 2.2, are given ($\times$), with 1 corresponding to the level of Bcl-$x_L$ in 293 cells transfected with an empty vector. Detection of nonspecific background bands has been included as a control for loading and membrane transfer (Std.). Lysates from untreated HL60 cells expressing detectable levels of Bcl-$x_L$ and high levels of Bcl-2 were also included (12). Transient expression of Ets2 and $\Delta$Ets2 proteins migrating at approximately 60 and 18 kDa, respectively, were detected in 293 cells by [$^{35}$S]methionine labelling and immunoprecipitation analyses using an Ets antibody (7) which specifically detects Ets2 proteins (data not shown). MW, molecular weight (in thousands).

we asked whether *ets2* induction correlates with *bcl-$x_L$* expression in macrophages. We chose murine BAC1.2F5 macrophages, since these macrophages are dependent on CSF-1 for their growth and survival (35) and since restimulation with CSF-1 of BAC1.2F5 cells starved of CSF-1 for 24 h results in a rapid induction of *ets2* expression (10). BAC1.2F5 cells were CSF-1 starved for 24 h and then were restimulated with CSF-1. Northern analysis of RNA isolated from these cells revealed that the induction of *bcl-$x_L$* expression correlates with the induction of *ets2* expression upon CSF-1 restimulation (Fig. 3). We did not detect *bcl-2* under the conditions tested (data not shown).

Since we showed that both Ets1 and PU1/Spi.1 can transactivate the *bcl-x* promoter, we asked whether these *ets* family members could be implicated in upregulating *bcl-$x_L$* in BAC1.2F5 macrophages restimulated with CSF-1. Northern analyses using *ets1*- and *PU1/spi.1*-specific probes were performed. A major *ets1* transcript migrating at 5.3 kb and minor transcripts at 4.0, 2.2, and 2.0 kb have been observed in mouse cells (4). However, these messages were not detected in either unstimulated or CSF-1-stimulated BAC1.2F5 cells (Fig. 3). Therefore, although Ets1 could potentially affect the activation of *bcl-x*, its absence in macrophages rules out its implication in the regulation of *bcl-$x_L$* in these cells. In contrast, *PU1* is readily expressed in BAC1.2F5 macrophages in the absence of CSF-1, and this expression does not vary greatly upon restimulation with CSF-1. We conclude that PU1 is not a key component of *bcl-$x_L$* response to CSF-1 since (i) the level of *PU1* mRNA is unaffected by CSF-1 starvation and (ii) macrophages die in the absence of CSF-1 despite the presence of *PU1*.

Since *ets2* expression correlates with that of *bcl-$x_L$* and since

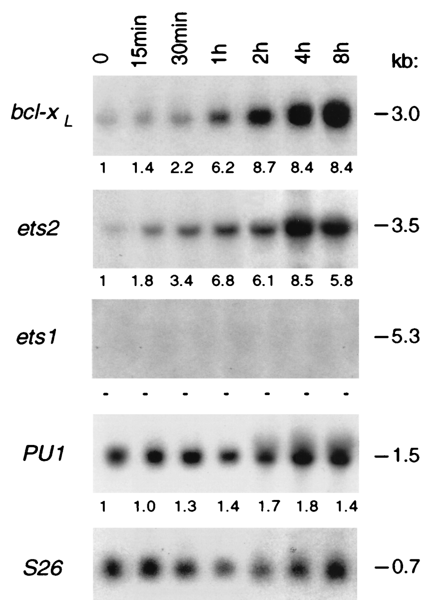



FIG. 3. CSF-1 upregulates the expression of *ets2* and *bcl-x*$_L$. *ets2* and *bcl-x*$_L$ are upregulated with similar kinetics upon restimulation with CSF-1 of BAC1.2F5 macrophages that had been starved of CSF-1 for 24 h. RNA was isolated from BAC1.2F5 cells that had been CSF-1 starved for 24 h and left untreated (0) or restimulated with rCSF-1 for 15 min, 30 min, 1 h, 2 h, 4 h, or 8 h. Five micrograms of each sample was electrophoresed on a 1% formaldehyde gel, transferred to a nylon membrane, and hybridized to purified full-length *bcl-x*$_L$, *ets2*, *ets1*, *PU1*/*spi.1* (*PU1*), S26 cDNA fragments as probes. The size (in kilobases) of each transcript is indicated. The expression of *bcl-x*$_L$, *ets2*, and *PU1* in CSF-1-starved cells, was arbitrarily set as 1. Fold inductions of *bcl-x*$_L$, *ets2*, and *PU1* upon restimulation are corrected according to S26 ribosomal protein mRNA hybridization, included as a control for RNA loading (47). Quantification was performed by using the program MacBAS, version 2.2.

*ets2* can transactivate the *bcl-x* promoter, we asked whether *bcl-x*$_L$ could be a direct target gene of Ets2 in BAC1.2F5 macrophages and whether Ets2 may play a role in inhibiting a poptosis in macrophages deprived of CSF-1 by activating *bcl-x*$_L$ at the transcriptional level. To address these questions, we established BAC1.2F5 cell clones constitutively expressing Ets2.

**Constitutive Ets2 expression does not alter growth factor-induced macrophage proliferation.** Following retroviral infection of BAC1.2F5 cells and G418 selection, neomycin-resistant clones were obtained. Two of these Ets2-expressing clones, verified as independent by Southern analysis (data not shown), were further characterized and are referred to as BAC-Ets2.1D and BAC-Ets2.6C. Northern analysis showed that the retroviral *ets2* transcript distinguishable by size (approximately 5.0 kb) from the endogenous *ets2* transcript (3.5 kb) is present in BAC-Ets2.1D and BAC-Ets2.6C (Fig. 4). Analysis by immunoblotting with an anti-Ets antibody of cell lysates resolved by SDS-PAGE revealed a 60-kDa signal corresponding to the endogenous Ets2 protein visible in control BAC1.2F5 cells infected with an empty retroviral vector (BAC-vec) in the presence of CSF-1 (Fig. 4). In both neomycin-resistant Ets2-expressing clones, the same 60-kDa signal was detected. However, the level of Ets2 expression is slightly higher in BAC-Ets2.1D (5.1-fold) and BAC-Ets2.6C (2.3-fold) than in control BAC-vec cells. These results indicate that both clones do express exogenous Ets2 and do so at levels found within physiological range (9). In fact, very low levels of Ets2 overexpression are sufficient to induce major morphological changes, since it was shown that an *ets2* transgene expressed at less than twice the level of endogenous *ets2* was sufficient to produce severe bone and cartilage deformations in mice (45). The closely related Ets1 protein was not detected in any of these cell lysates.

Based on phase-contrast microscopy, the constitutive expression of Ets2 in BAC1.2F5 cells does not alter cell morphology in the presence of CSF-1 (see Fig. 6A). In addition, BAC-Ets2.1D and BAC-Ets2.6C cells grow at rates similar to that of parental BAC1.2F5 cells when CSF-1 is present in the medium (data not shown). Many macrophage-specific genes are rapidly upregulated upon CSF-1 restimulation of BAC1.2F5 cells that have been starved of CSF-1 for 24 h (10). Northern analysis reveals that genes coding for JE/MCP-1 or cytokines IL-1β and TNF-α are barely detectable in control (BAC-vec) and Ets2-expressing cells (BAC-Ets2.1D) in the absence of CSF-1. However, all are induced with relatively similar kinetics in control and Ets2-expressing cells upon restimulation with CSF-1 (Fig. 5A), although relative levels might vary. Together, these results indicate that constitutive expression of Ets2 in BAC1.2F5 cells does not qualitatively alter the responses of these cells to CSF-1.

**Constitutive Ets2 expression permits cell survival in the absence of growth factor.** Since long-term CSF-1 starvation of parental BAC1.2F5 cells results in increased cell death (35), we asked what would be the effects of CSF-1 deprivation on BAC-Ets2.1D and BAC-Ets2.6C cells. When BAC1.2F5 cells are stably infected with the empty retroviral vector and maintained in the absence of CSF-1 for 7 days, these cells die in a manner similar to that of parental BAC1.2F5 cells (35) (Fig. 6A). Constitutive expression of Ets2 in BAC-Ets2.1D and BAC-Ets2.6C cells, on the other hand, results in cell survival (Fig. 6A), which was maintained for at least 2 to 3 weeks. Cell

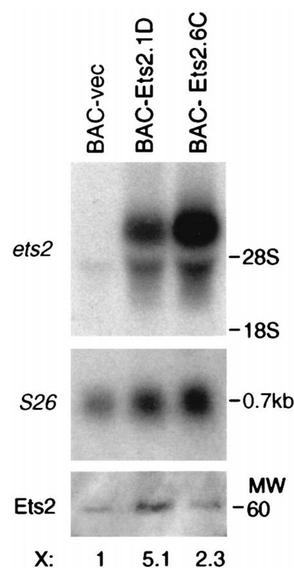



FIG. 4. *ets2* mRNA and Ets2 protein expression in control and Ets2-expressing clones. Total RNA was isolated from BAC-vec, BAC-Ets2.1D, or BAC-Ets2.6C cells, and Northern analysis was performed as described for Fig. 3. The endogenous *ets2* transcript migrates at approximately 3.5 kb (just below 28S), whereas the retroviral *ets2* transcript migrates at approximately 5 kb (just above 28S). 28S and 18S RNAs are indicated, and hybridization with S26 has been included as a control for RNA loading. The signal detected under 28S in both Ets2-expressing lines is due to the trailing of the viral signal. Immunoprecipitations of total cell lysates from BAC-vec, BAC-Ets2.1D, or BAC-Ets2.6C were performed by using an antibody against both Ets1 and Ets2 proteins. Following transfer onto PVDF membranes, Western blot analysis was performed with the same antibody. The Ets2 protein migrating at approximately 60 kDa, but not Ets1, is detected, and its level of expression is higher in both Ets2-expressing clones, with fold inductions (×) indicated. MW, molecular weight (in thousands).

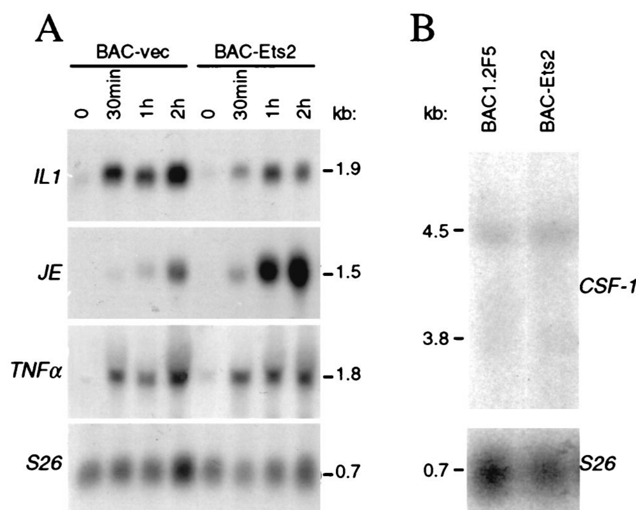



FIG. 5. (A) CSF-1 induction of macrophage-specific markers in control and Ets2-expressing macrophages. RNA was isolated from BAC-vec or BAC-Ets2.1D cells in the 24-h absence of CSF-1 (0) or upon restimulation with CSF-1 for 30 min, 1 h, or 2 h, as indicated. Five micrograms of each sample was electrophoresed as described for Fig. 3 and hybridized to purified full-length IL-1β (IL1), JE/MCP-1 (JE), and TNF-α cDNA fragments as $^{32}$P-labelled probes. S26 was used as a control for RNA loading. (B) CSF-1 expression in control and Ets2-expressing macrophages. RNA was isolated from BAC1.2F5 or BAC-Ets2.1D cells in the 3-day absence of CSF-1. Five micrograms of each sample was electrophoresed as described for Fig. 3 and hybridized by using a purified CSF-1 cDNA fragment as a $^{32}$P-labelled probe. Fold inductions of the major 4.5-kb CSF-1 transcript, corrected according to S26, are 1 and 1.3 in BAC1.2F5 and BAC-Ets2, respectively. Quantification was performed as described for Fig. 3.

growth studies, graphically represented in Fig. 6B, indicate that while control BAC-vec cells die in the absence of CSF-1, cell numbers of BAC-Ets2.1D and BAC-Ets2.6C clones remain relatively constant. This effect is independent of initial cell densities (data not shown).

We previously showed that exogenous Ets2 expression in immature myeloblast leukemic cells results in the secretion of CSF-1 (2). Northern analysis was thus performed to determine the level of CSF-1 expression in CSF-1-starved control and Ets2-expressing cells. As can be seen in Fig. 5B, the level of CSF-1 mRNA expression is low but detectable in control cells and remains low when Ets2 is constitutively expressed. This low level of CSF-1 mRNA expression is not sufficient to support BAC1.2F5 cell survival, since (i) BAC1.2F5 cells die in the absence of exogenously added CSF-1, (ii) conditioned media prepared from both BAC-Ets2 clones were not able to support BAC1.2F5 cell survival in contrast to that of Ets2-expressing myeloblasts under the same conditions, and (iii) antibodies blocking the effects of CSF-1 signaling did not affect the cell survival of either Ets2-expressing BAC1.2F5 clone, whereas parental BAC1.2F5 cells died (data not shown). Together, these results demonstrate that the cell survival of BAC-Ets2 macrophages in the absence of exogenously added CSF-1 is not due to an autocrine loop induced by CSF-1 secretion in Ets2-expressing macrophages.

**Constitutive Ets2 expression alters some cell cycle responses, including pRb phosphorylation, upon growth factor deprivation and restimulation.** The survival behavior that we observe with Ets2-expressing cells upon CSF-1 deprivation may reflect an alteration in the cell cycle control events. To address this issue, we analyzed several principal cell cycle regulators, such as $G_1$ (D1 and E), S-phase (A), and mitotic (B1) cyclins and the cyclin-dependent kinase (Cdk) inhibitors (CKI) $p27^{Kip1}$ and $p21^{Waf1/Cip1}$, whose expression or activities may be affected by constitutive Ets2 expression. In addition, we analyzed the phosphorylation status of pRb, a growth suppressor that is underphosphorylated in $G_1$ (and quiescent) cells and undergoes cyclin-Cdk-dependent phosphorylation at or after the $G_1$/S phase transition (14). To this end we performed Western blot analysis of the cell extracts prepared from exponentially growing cells as well as from macrophages that were starved for several days and subsequently stimulated with rCSF-1 for 10 (mid-$G_1$) or 22 (S/$G_2$) h. Cyclin D-specific pRb phosphorylation was examined by using specific antibodies that recognize phospho-S780, which is phosphorylated exclusively by cyclin D1-Cdk4 (26). In addition, we analyzed immunoprecipitates of $G_1$ (cyclin D1 and cyclin E)- and S (cyclin A)-phase cyclins for their kinase activities and/or Cdk presence.

Initial observations showing that CSF-1-starved BAC1.2F5 cells contain a significant proportion of cells in S phase (35) were confirmed by the abundant presence of cyclin A, a typical S-phase cyclin (Fig. 7A). Although the persistent presence of cyclin A after starvation might suggest that the cell cycle machinery in these cells cannot "sense" CSF-1 removal, both the levels of the CKI p21 and p27 and those of cyclin D1 (Fig. 7A and C), as well as pRb phosphorylation, appeared to be CSF-1 dependent. Thus, CSF-1 starvation results in increased p27 levels and decreased cyclin D1 levels (Fig. 7C) and an accumulation of hypophosphorylated pRb (Fig. 7A). Conversely, CSF-1 stimulation resulted in strong cyclin D-dependent pRb phosphorylation (Fig. 7A). Thus, it is possible that, compared to growth-arrested fibroblasts (15), BAC1.2F5 macrophages exhibit uncoupled cell cycle-regulatory pathways: one, represented by cyclin D1 (and, to a certain extent, cyclin B1) and CKI, that is invariably CSF-1 dependent and another, represented by cyclin A, that is not. Even though CSF-1 removal did not appreciably affect cellular levels of cyclin A-Cdk2 and cyclin E-Cdk2 complexes (Fig. 7C), their activities, as measured by in vitro phosphorylation of histone H1, decreased considerably and increased again upon CSF-1 stimulation (Fig. 7B), suggesting that Cdk2 may be, in part, responsible for pRb phosphorylation. Western blot analysis of cyclin immunocomplexes showed elevated amounts of p27 associated with both cyclin A-Cdk2 and cyclin E-Cdk2 complexes, accounting for their inactivation (Fig. 7C).

In Ets2-expressing cells, cell cycle regulation seemed to be altered in several respects. Like control cells, they also failed to express cyclin D1 in the absence of CSF-1, and the levels of cyclin A remained unchanged (Fig. 7C). However, levels of cyclins E and B1 did not appear to be affected by CSF-1 removal. CSF-1 stimulation resulted in increases in cyclin D1 levels, but to a much lower extent than in control cells. Unlike the effects in control cells, CSF-1 withdrawal and/or addition did not significantly affect cyclin A- and cyclin E-associated kinase activities (Fig. 7B) in spite of marked accumulation of p27 and its increased presence in cyclin-Cdk2 complexes (Fig. 7C). Another important difference in Ets2-expressing cells is the apparent absence of regulation of pRb phosphorylation. In contrast to control cells, where underphosphorylated pRb accumulated after CSF-1 withdrawal, no significant change in pRb phosphorylation occurred in Ets2-expressing cells after starvation. In addition, both pRb expression and phosphorylation levels in stimulated (22) and exponentially growing cells were inferior to those observed in control cells (Fig. 7A). This may be due to lower levels of cyclin D-Cdk4 complexes in these cells (Fig. 7C). Since these cells contain high Cdk2-associated kinase activity, it seems that the observed pRb phosphorylation may be primarily due to the activity of cyclin D1-Cdk4 complexes (we could not detect Cdk6 in these cells). This notion is further supported by our results showing that, in contrast to the

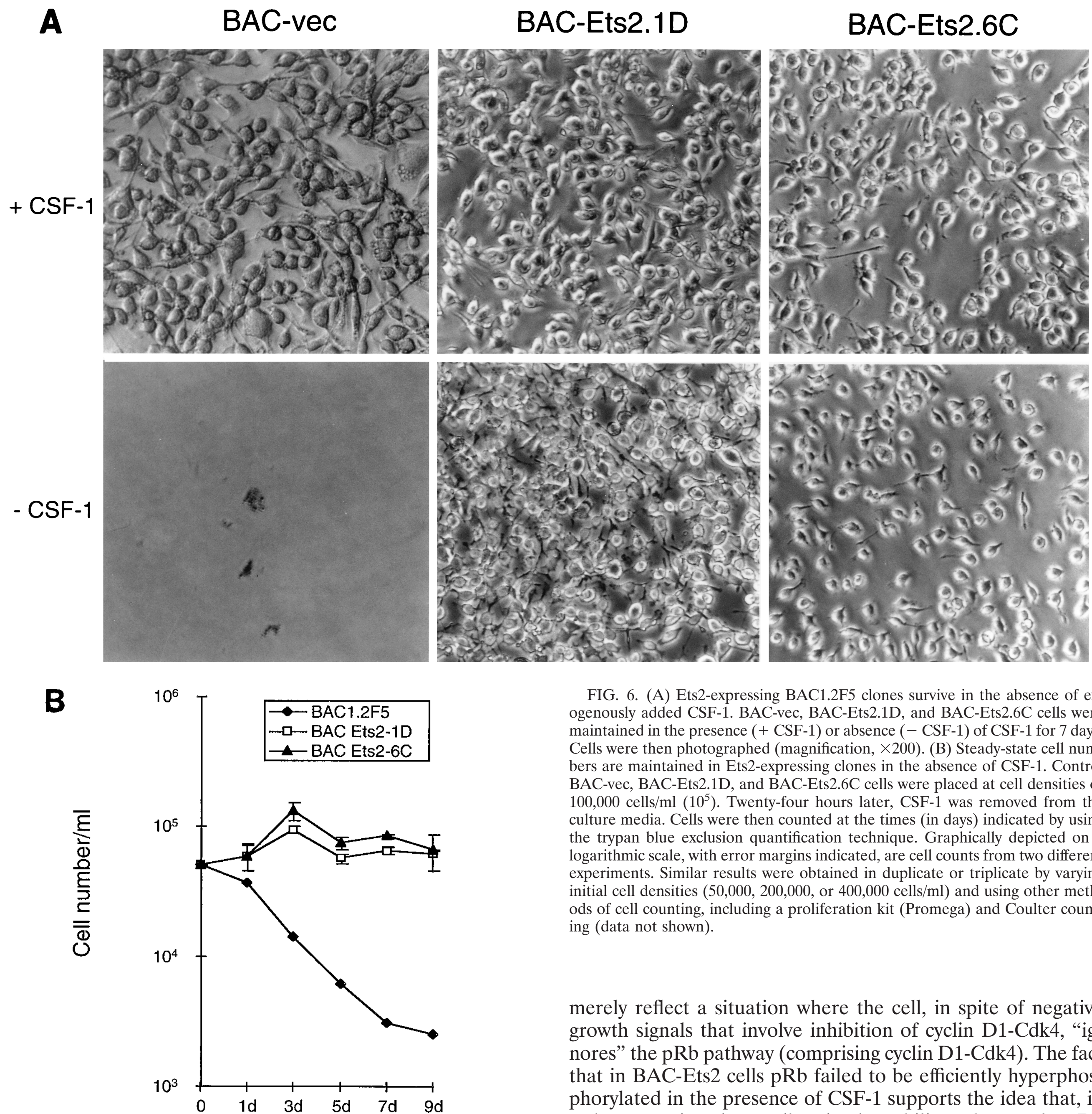



FIG. 6. (A) Ets2-expressing BAC1.2F5 clones survive in the absence of exogenously added CSF-1. BAC-vec, BAC-Ets2.1D, and BAC-Ets2.6C cells were maintained in the presence (+ CSF-1) or absence (− CSF-1) of CSF-1 for 7 days. Cells were then photographed (magnification, ×200). (B) Steady-state cell numbers are maintained in Ets2-expressing clones in the absence of CSF-1. Control BAC-vec, BAC-Ets2.1D, and BAC-Ets2.6C cells were placed at cell densities of 100,000 cells/ml ($10^5$). Twenty-four hours later, CSF-1 was removed from the culture media. Cells were then counted at the times (in days) indicated by using the trypan blue exclusion quantification technique. Graphically depicted on a logarithmic scale, with error margins indicated, are cell counts from two different experiments. Similar results were obtained in duplicate or triplicate by varying initial cell densities (50,000, 200,000, or 400,000 cells/ml) and using other methods of cell counting, including a proliferation kit (Promega) and Coulter counting (data not shown).

effects in control cells, where CSF-1 stimulation led to a strong increase in cyclin D1-Cdk4 levels (Fig. 7C), leading to subsequent accumulation of cyclin D-specific pRb phosphorylation (Fig. 7A), in Ets2-expressing cells this accumulation and pRb phosphorylation were only transient, as though these cells exhibited a defective cyclin D1-pRb pathway. Hence, the observed dynamics of pRb phosphorylation in control and BAC-Ets2 cells suggest that it stems from cyclin D1-Cdk4 activity.

Our results suggest that constitutive Ets2 expression in BAC1.2F5 macrophages results in changes in the cell cycle regulation that, in turn, may somehow contribute to cell survival following CSF-1 removal. At this point we cannot say whether these changes directly play a role in cell survival or merely reflect a situation where the cell, in spite of negative growth signals that involve inhibition of cyclin D1-Cdk4, "ignores" the pRb pathway (comprising cyclin D1-Cdk4). The fact that in BAC-Ets2 cells pRb failed to be efficiently hyperphosphorylated in the presence of CSF-1 supports the idea that, in order to survive, these cells gained an ability to bypass the pRb pathway. However, for the time being, the link between the Ets2 pathway and this alteration of cell cycle machinery is not well understood.

**Growth factor deprivation induces apoptosis, whereas constitutive expression of Ets2 inhibits this process.** One physiologically relevant signaling pathway inducing programmed cell death is growth factor deprivation. The striking dependence of BAC1.2F5 cells on CSF-1 is evidently reflected by cell death in its absence (35) (Fig. 6A). To determine whether decreases in BAC1.2F5 cell numbers in the absence of CSF-1 are indeed due to programmed cell death, interactions of Annexin V with phosphatidylserine were determined. Upon the onset of apoptosis, phosphatidylserines are rapidly expressed on the outer surfaces of cells, which allows recognition and subsequent phagocytosis by macrophages of these apoptotic cells, thereby

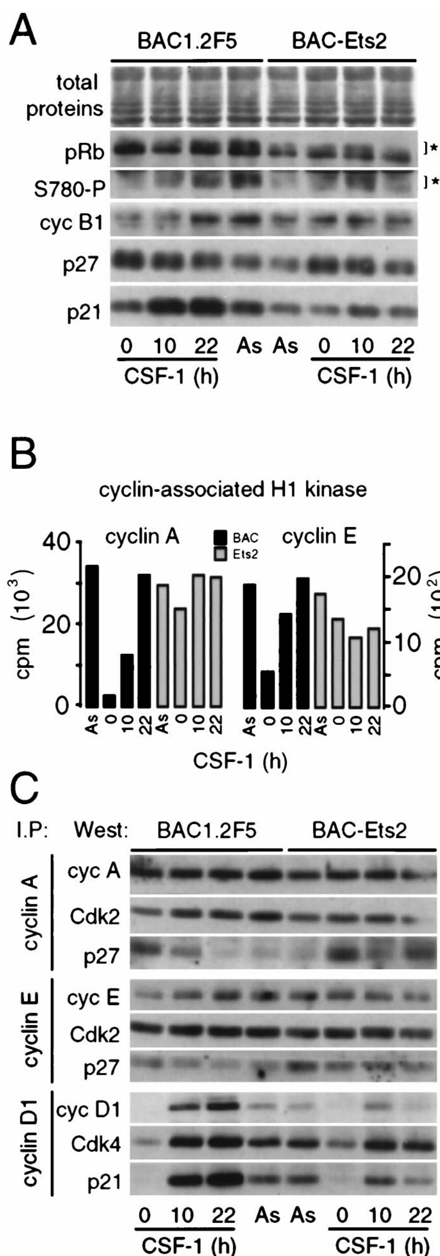



FIG. 7. Differential cell cycle regulation in control and Ets2-expressing macrophages. (A) Relative levels of pRb, cyclin (cyc) B1, and CKI $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ in total cell extracts prepared from exponentially growing (As), starved (0 h), and CSF-1-stimulated (10 and 22 h) control (BAC1.2F5) and Ets2-expressing (BAC-Ets2) macrophages. The proteins were separated by SDS–7.5% or –12% PAGE and detected by Western blot analysis using the antibodies indicated. The amido black-stained membrane after the transfer (total proteins) shows equal loading. Cyclin D1-specific pRb phosphorylation was revealed by an antibody directed against phospho-S780 (S780-P) (26). Asterisks indicate the bands resulting from pRb hyperphosphorylation. (B) Cyclin A- and cyclin E-associated kinase activities in exponentially growing, starved, and CSF-1-stimulated macrophages. Cyclin complexes were serially immunoprecipitated from equal amounts of the indicated extracts (150 μg) and tested for histone H1 kinase activity. Immunoprecipitates were separated by SDS–10% PAGE, and either they were transferred to a PVDF membrane for immunoblot analysis of their contents or the Coomassie blue-stained histone H1 bands were excised (after drying) and directly counted (Cerenkov). (C) Western blot analysis (West) of cyclin A, E, and D1 immunoprecipitates (I.P.) isolated from the indicated cell extracts.

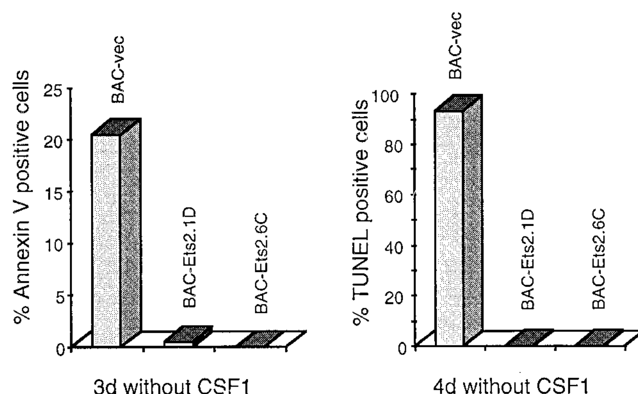



FIG. 8. In the absence of CSF-1, BAC1.2F5 cells die by apoptosis, whereas constitutive expression of Ets2 inhibits apoptosis. Cells were CSF-1 starved for 3 days, then incubated with Annexin V conjugated with FITC, fixed, and incubated with DAPI. The level of Annexin V-positive cells was calculated as a percentage of the total number of DAPI-positive cells. Cells were CSF-1 starved for 4 days; then cells were fixed and permeabilized, and TUNEL was performed. Following washes, cells were incubated with streptavidin-Texas Red and DAPI. As above, the level of TUNEL-positive cells was calculated as a percentage of the total number of DAPI-positive cells. A total of 200 cells were counted, and these experiments were repeated three times.

preventing an inflammatory response. Cells were maintained in the presence or in a 3-day absence of CSF-1 and then were incubated with FITC-conjugated Annexin V and fixed, and cell death was visualized by immunofluorescence. Costaining with DAPI was performed as a control for nuclear staining of the cells. Although no difference in Annexin V staining was observed for the three cell populations maintained in medium containing CSF-1 (data not shown), many control BAC-vec cells in the absence of CSF-1 were positively labelled, indicating that these cells were undergoing the early stages of programmed cell death. Background, nonspecific levels of staining with FITC-conjugated Annexin V were observed in Ets2-expressing BAC1.2F5 clones. The levels of Annexin V-positive cells, expressed as percentages of the total number of DAPI-positive cells, were calculated from different experiments and are graphically represented in Fig. 8. Approximately 20% of BAC1.2F5 cells were undergoing early stages of cell death in a 3-day absence of CSF-1, whereas fewer than 1% of Ets2-expressing BAC1.2F5 cells were dying under these conditions.

TUNEL was used as a second method of detection by measuring fragmentation occurring within the nucleus. No staining by TUNEL was observed in the three asynchronous cell populations maintained in CSF-1 (data not shown). However, in the 4-day absence of CSF-1, only 25 to 50% of the control BAC-vec cells were viable, and the majority of these nuclei were positively stained with TUNEL. In contrast, BAC-Ets2.1D and BAC-Ets2.6C cell numbers were maintained over the 4-day starvation period, and few of these nuclei were positively stained under the same conditions. These results demonstrate that later stages of apoptosis are occurring only with CSF-1-starved BAC-vec cells. The percentage of apoptotic versus viable cells were calculated from different experiments (Fig. 8). More than 90% of the remaining viable BAC1.2F5 cells were dying in the 4-day absence of CSF-1, whereas fewer than 2% of Ets2-expressing BAC1.2F5 clones were dying under the same conditions. These results indicate that constitutive Ets2 expression inhibits the onset of the apoptotic process in the absence of CSF-1 survival signals.

**Constitutive Ets2 expression results in an upregulation of *bcl-x*$_L$ expression.** Since Ets2 can transactivate the *bcl-x* promoter, we asked whether the regulation of *bcl-x*$_L$ expression

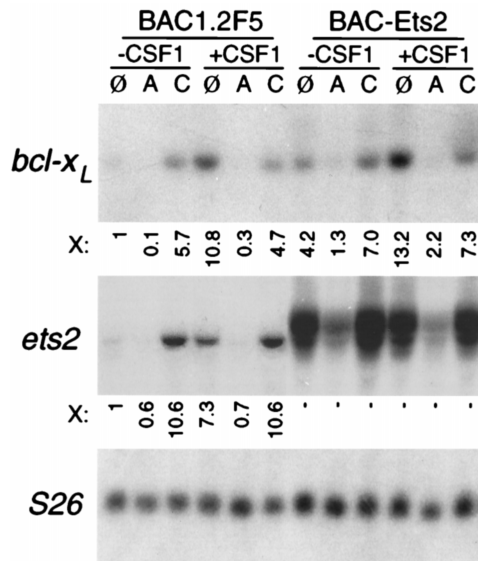



FIG. 9. $bcl\text{-}x_L$ upregulation occurs at the transcriptional level and depends on de novo protein synthesis. RNA was isolated from BAC1.2F5 cells starved of CSF-1 for 3 days (Ø), then pretreated with actinomycin D (A) or cycloheximide (C), and then maintained alone or treated with rCSF-1 for 2 h as described in Materials and Methods. Five micrograms of each sample was electrophoresed and hybridized to *ets2*, $bcl\text{-}x_L$, and S26 cDNA fragments as probes. Fold inductions of *ets2* and $bcl\text{-}x_L$ were determined as described above for Fig. 3.

occurs at the transcriptional level and depends on de novo protein synthesis in macrophages. To address this question, BAC1.2F5 and BAC-Ets2-expressing cells were first starved of CSF-1 for 3 days and then pretreated in the absence of CSF-1 with an inhibitor of transcription, actinomycin D, or an inhibitor of protein synthesis, cycloheximide, for 30 min. After this pretreatment, cells were either maintained in the absence of CSF-1 or restimulated with 60 ng of CSF-1/ml for 2 h. RNA was isolated from these cells, and Northern analysis was performed. Results are shown in Fig. 9. From these experiments, several conclusions can be drawn. First, in contrast to BAC1.2F5 cells, the level of *ets2* expression was not downregulated in Ets2-expressing cells following CSF-1 deprivation, as would be expected from a constitutively active retroviral promoter. Second, in the absence of CSF-1 treatment, although $bcl\text{-}x_L$ mRNA was detected in BAC1.2F5 cells, it was upregulated in macrophages constitutively expressing Ets2. Third, like *ets2* expression, $bcl\text{-}x_L$ expression in both BAC1.2F5 and Ets2-expressing cells was decreased upon actinomycin D treatment, showing that $bcl\text{-}x_L$ mRNA has a relatively short half-life. In addition, the increased $bcl\text{-}x_L$ mRNA signal detected following CSF-1 treatment is completely blocked by actinomycin D, indicating that the $bcl\text{-}x_L$ mRNA level is due to an increase in *bcl-x* promoter activity and not to stabilization of the transcript. Fourth, cycloheximide treatment stabilizes both $bcl\text{-}x_L$ and *ets2* transcripts in the absence of CSF-1. However, CSF-1 treatment in the presence of cycloheximide reduced $bcl\text{-}x_L$ expression, indicating that de novo protein synthesis is required for $bcl\text{-}x_L$ transcription. From these results, we can conclude that (i) $bcl\text{-}x_L$ induction following CSF-1 treatment stems from an increase in *bcl-x* promoter activity and (ii) de novo protein synthesis is required for this transcriptional activation.

To summarize, the level of $bcl\text{-}x_L$ expression in CSF-1-starved control cells is not high enough to protect against cell death. However, $bcl\text{-}x_L$ upregulation by constitutive *ets2* expression now permits protection against growth factor deprivation-induced apoptosis. Taken together, these results show that Ets2 can inhibit apoptosis in the absence of growth factor and that at least one mechanism of inhibition involves the capacity of Ets2 to transactivate the *bcl-x* gene, resulting in the upregulation of the $bcl\text{-}x_L$ transcript.

## DISCUSSION

Many transcription factors are involved in inducing proliferative and/or differentiation responses, and some regulate processes of programmed cell death, demonstrating that they play an essential role in determining the fate of a cell. Under certain conditions, some of these factors are capable of deregulating either the cell cycle or programmed cell death, resulting in uncontrolled growth of the cell. In this paper we show that (i) the Ets2 transcription factor can transactivate the *bcl-x* promoter; (ii) depriving BAC1.2F5 macrophages of CSF-1, a factor necessary for the growth and survival of these cells, results in programmed cell death; (iii) constitutive expression of Ets2 in these macrophages inhibits this apoptotic process in the absence of survival factor stimuli; and (iv) constitutive Ets2 expression is accompanied by an upregulation of the expression of $bcl\text{-}x_L$ but not of *bcl-2*. Although other mechanisms may be involved, this suggests that Ets2-dependent protection against apoptosis passes through the $Bcl\text{-}x_L$-dependent survival pathway in macrophages.

Transient expression of Ets2 in 293 cells results in transactivation of the *bcl-x* promoter and upregulation of $Bcl\text{-}x_L$ protein. We therefore were interested in determining whether Ets2 is an upstream effector of $Bcl\text{-}x_L$. There is a correlation of expression of Ets2 and $Bcl\text{-}x_L$ in different myeloid cells. $Bcl\text{-}x_L$ and Ets2 are upregulated both in human U937 and HL60 cells as these cells differentiate toward macrophages and in human peripheral blood monocytes exposed to phorbol ester (9, 12); we show in this report that $bcl\text{-}x_L$ and *ets2* are upregulated in a similar manner in murine CSF-1-dependent macrophages.

The constitutive expression of Ets2 in BAC1.2F5 cells has no effect on cell proliferation when CSF-1 is present, indicating that there is no synergy in signaling between Ets2 and CSF-1 in these macrophages. However, constitutive expression of Ets2 in the absence of CSF-1 permits cell survival of these macrophages. These results differ from those obtained in fibroblasts exogenously expressing CSF-1R, where CSF-1 induces proliferation through an Ets2- and Myc-dependent pathway (28, 41). In the absence of CSF-1 signaling, we observe cell survival of our CSF-1-dependent macrophages when Ets2 is constitutively expressed, but no immediate proliferative response.

Macrophages constitutively expressing Ets2 are a model system for distinguishing between cell survival and proliferation. CSF-1 allows these cells to proliferate, whereas Ets2 compensates for its absence only by preventing these cells from undergoing apoptosis and not by allowing proliferation, as is the case with fibroblasts exogenously expressing CSF-1R. A possible explanation for this difference is that CSF-1 signaling is intrinsic to macrophages and that part of the CSF-1 signaling cascade in CSF-1R-expressing fibroblasts may not reflect a bona fide macrophage CSF-1 signaling pathway, since fibroblasts do not express CSF-1R under physiological conditions. Thus, even though Ets2 appears to play a role in CSF-1 signaling in both cell types, Ets2 is not sufficient to completely mimic the action of CSF-1 in macrophages.

Our attempts to establish BAC1.2F5 macrophages constitutively expressing a dominant-negative mutant form of Ets2 were unsuccessful. Yet the same retroviral supernatants were successfully used to infect CSF-1-independent BAC1.2F5 cells constitutively expressing v-*raf* (1a, 10). Although negative, these

results suggested to us that the expression of a dominant-negative form of Ets2 is incompatible with cell survival in this system. This is in agreement with the findings of Langer et al. (28), showing that CSF-1-treated fibroblasts exogenously expressing CSF-1R lose their ability to form colonies in soft agar when a dominant-negative form of Ets2 is coexpressed. Therefore, expression of a dominant-negative form of Ets2 inhibits cell growth in fibroblasts and may be incompatible with cell growth or survival in BAC1.2F5 macrophages, thus explaining the impossibility of obtaining BAC1.2F5 cells constitutively expressing a dominant-negative form of Ets2.

When CSF-1 binds to its receptor, a series of signaling events occurs, including the activation of a cytoplasmic kinase, Raf (3). BAC1.2F5 macrophages expressing v-*raf* have been generated, and these cells differ from control cells in terms of morphology and gene expression and by their proliferation independent of CSF-1 (10). In addition, c-*myc*, *ets2*, TNF-α, and IL-1β are constitutively active in *raf*-expressing BAC1.2F5 cells (10). By using this system it was shown that *raf* activates at least two independent signaling pathways (11). It is possible that one of these includes Ets2 and the other includes Myc and that the activation of Ets2 in one pathway would be insufficient to induce proliferation without the parallel activation of Myc. We are currently investigating whether the activation of Myc with Ets2 will be sufficient to induce these changes exhibited by Raf expression.

In this report, we show that macrophages dependent on CSF-1 for their growth and survival die by programmed cell death upon removal of CSF-1 and that constitutive Ets2 expression in these macrophages inhibits this apoptotic process. We propose a novel role for Ets2 in inhibiting apoptosis. It appears that other *ets* family members are involved in controlling programmed cell death as well. Ets1 is the progenitor to the v-Ets portion of the E26 retroviral fusion product and is the family member most closely related to Ets2. The *ets1* gene has been disrupted in embryonic stem cells, and by using the recombination activating gene complementation assay with RAG2−/− blastocysts, it was shown that *ets1*-deficient T cells die by apoptosis (6, 36). In addition an Ets1 variant can induce apoptosis in human colon cancer cells (24). More distantly related *ets* family members, *erg* and Fli-1, inhibit apoptosis in serum-deprived fibroblasts (52), and Spi-1/PU.1 cooperates with an activated erythropoietin receptor to inhibit apoptosis in primary erythroblasts (46), suggesting that the role in inhibiting programmed cell death may be a common function of members of the *ets* family. Yet the mechanism of inhibition of apoptosis remained undetermined. The promoter regions of *bcl-2* and *bcl-x* have been identified, yet little is known about the role of specific transcription factors in activating these genes. We show that Ets2 can transactivate the *bcl-x* promoter and that constitutive Ets2 expression results in the upregulation of $bcl\text{-}x_L$, showing that *bcl-x* is indeed a downstream target gene of Ets2 in macrophages. While this paper was under review, an article further supporting our results, describing $Bcl\text{-}x_L$ as the key antiapoptotic protein during cytokine-regulated myelopoiesis (38), was published.

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