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Myc-Mediated Apoptosis Is Blocked by Ectopic Expression of Bcl-2

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The product of the c-*myc* proto-oncogene is an important positive regulator of cell growth and proliferation. Recently, c-Myc has also been demonstrated to be a potent inducer of apoptosis when expressed in the absence of serum or growth factors. To further examine Myc-induced apoptosis, we coexpressed the proto-oncogene *bcl2*, which has been shown to block apoptosis in other systems, with c-*myc* in serum-deprived Rat 1a fibroblasts. Here we report that ectopic expression of *bcl2* specifically blocks apoptosis induced by constitutive c-*myc* expression. Constitutive c-*myc* expression in serum-deprived Rat 1a cells caused a >15-fold increase in the number of dead cells, accompanied by DNA fragmentation. However, coexpression of *bcl2* with c-*myc* in these cells led to a 10-fold increase in the number of live cells and a significant decrease in DNA fragmentation. Thus, Bcl-2 effectively inhibits Myc-induced apoptosis in serum-deprived Rat 1a fibroblasts without blocking entry into the cell cycle. These results imply that apoptosis serves as a protective mechanism to prevent tumorigenicity elicited by deregulated Myc expression. This protective mechanism is abrogated, however, by Bcl-2 and therefore may explain the synergism between Myc and Bcl-2 observed in certain tumor cells.

Expression of the c-*myc* proto-oncogene is strongly implicated in the control of cell growth and proliferation. Expression of c-*myc* is necessary and in some cases sufficient for entry into the S phase of the cell cycle (4, 13, 14, 37), whereas shutoff of c-*myc* expression is associated with quiescence or cellular differentiation. Correspondingly, addition of growth factors to quiescent cells leads to a transient increase in the level of c-*myc* expression, and deprivation of growth factors leads to virtually undetectable levels of the RNA and protein. In addition, c-*myc* expression is frequently deregulated in neoplasias and is often implicated in their genesis (for reviews, see references 4a, 10, 11, 26, and 43). The mechanism(s) by which Myc can influence cell growth is as yet unknown but may include its activity as a transcriptional regulator (3, 13, 22, 23a, 24, 31a), its ability to bind growth suppressors such as the retinoblastoma gene product (38), and its ability to induce DNA replication (8, 21).

Paradoxically, despite the apparent importance of Myc in the induction of cell proliferation, expression of Myc has recently been linked to the induction of cell death (4, 14, 40). In two of these reports (4, 14), cell death was accelerated or induced by inappropriate expression of Myc. These studies demonstrated that ectopic expression of Myc under conditions where Myc is not normally expressed (i.e., in growth factor withdrawal or serum deprivation) resulted in cell death by apoptosis, a process typified by nuclear condensation and DNA fragmentation into oligonucleosomal fragments (for reviews, see references 51 to 53 and 56).

To further study Myc-induced apoptosis, we examined the effect of coexpression of the proto-oncogene *bcl2* in Rat 1a fibroblasts constitutively expressing high levels of Myc. These cells have been shown to undergo apoptosis when deprived of serum (14). We chose to examine the effect of Bcl-2 on the basis of its ability to inhibit apoptosis in many hematopoietic systems. *bcl2* was discovered as a result of its translocation to the immunoglobulin heavy-chain enhancer in the t(14;18) translocation present in more than 80% of human follicular lymphomas (7, 9). These neoplasias are characterized by an accumulation of mature resting B cells presumed to result from a block of apoptosis which would normally cause turnover of these cells; transgenic mice expressing Bcl-2 under the control of the Eμ enhancer similarly develop follicular lymphomas which have a high risk of developing into malignant lymphomas (20, 27, 44). Bcl-2 expression has been shown to exhibit protective effects on cells under a variety of conditions, including glucocorticoid- and cyclic AMP-induced apoptosis of T and B cells (2) and growth factor withdrawal-induced apoptosis in hematopoietic cells (5, 20, 48). Additionally, Bcl-2 expression has been demonstrated to reduce growth factor requirements in fibroblasts (34) and to increase cell survival during ethanol and heat shocks (46). We report here that Bcl-2 expression is sufficient to inhibit apoptosis induced by Myc expression in serum-starved fibroblasts.

MATERIALS AND METHODS

Retroviruses and cell lines. To generate human c-*myc*- and human *bcl2*-expressing retroviruses, we cloned the corresponding cDNAs into retroviral expression vectors by standard methods. The c-*myc* cDNA was obtained by digestion of plasmid pMV6myc/neo (a gift of W. Lee, University of Pennsylvania) with *Xho*I and *Eco*RI. The resulting 4.3-kb fragment was cloned into the *Eco*RI-*Xho*I sites of pGEM7Z (Promega) and digested with *Hin*dIII to generate the 1.4-kb c-*myc* cDNA. This fragment was ligated into the *Hin*dIII site of plasmid pMV12 (a gift of R. Krauss and B. Weinstein, Columbia University) to create pMV12-Myc (Fig. 1A). The 950-bp *Eco*RI fragment of pGEMBcl2 (a gift of G. Getz, University of Chicago) contains the entire Bcl-2 cDNA

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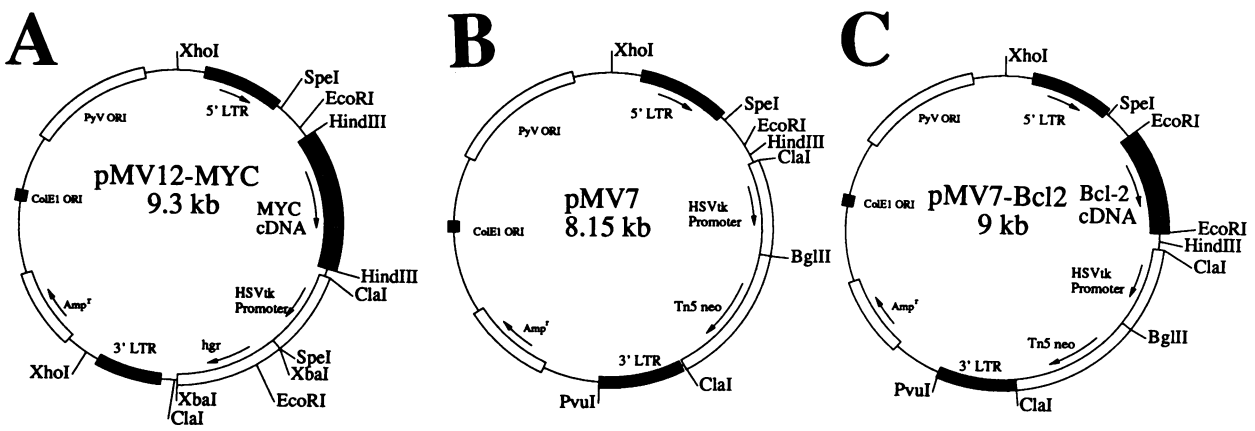

FIG. 1. pMV retrovirus expression constructs. The 5′ and 3′ Moloney murine sarcoma virus long terminal repeats are indicated by black boxes, and cDNA coding regions of human c-*myc* (panel A) and human *bcl2* (panel C) are indicated by stippled boxes. The pMV12 vector (A) contains the gene for hygromycin resistance (hgr), and the pMV7 (B) and pMV7-Bcl2 (C) vectors contain the gene encoding G418 resistance (Tn5 neo).

coding sequence and was subcloned into the *Eco*RI site of the retroviral vector pMV7 (Fig. 1B and C) (23). These plasmids, in addition to the parental pMV7, were individually stably transfected into ψ2 cells by the calcium phosphate coprecipitation method (17). Selection for cells expressing the virus-encoded genes was begun after 48 h by 1:10 dilution of the cells into medium containing 1,000 μg of G418 per ml (for pMV7 transfections) or 400 μg of hygromycin B per ml (for pMV12 transfections). Two days following initiation of selection, antibiotic concentrations were reduced to 500 μg of G418 per ml and 200 μg of hygromycin B per ml. After 1 week of selection, surviving cells were pooled and used to produce retrovirus.

The Rat 1a cell line was infected for 4 h in 10 μg of Polybrene per ml with the pMV12-Myc retrovirus by standard methods (6). Infected cells were diluted 1:10 after 48 h and selected for 2 days in medium containing 400 μg of hygromycin B per ml; they were then maintained in medium containing 200 μg of hygromycin B per ml. Resistant clones were selected and expanded as clonal cell lines; a single line, designated RAiMH3-1a, was used in this study. RAiMH3-1a cells were infected with pMV7-Bcl2 retrovirus or with pMV7 retrovirus as a control to generate the polyclonal cell lines Rat 1a/*myc*/*bcl2* and Rat 1a/*myc*/*neo*, respectively. Cells from each line were pooled to eliminate clonal variation. All cells were maintained in 5% CO_2 in Dulbecco's modified E4 medium supplemented with 10% fetal calf serum, 100 U of penicillin G per ml, and 100 μg of streptomycin sulfate per ml. Rat 1a/*myc*/*bcl2* and Rat 1a/*myc*/*neo* cells were maintained in medium supplemented with 200 μg of hygromycin B per ml and 500 μg of G418 per ml.

RNA isolation and Northern (RNA) analysis. Total cellular RNA was isolated from proliferating cells by the guanidinium isothiocyanate method followed by centrifugation through a CsCl cushion (47). Electrophoresis of 15 μg of total RNA through a 1% agarose–2.2 M formaldehyde gel, transfer to nitrocellulose, and hybridization to [α-^{32}P]dCTP-labeled probes were performed as described previously (50). The 1.4-kb *Eco*RI-*Hin*dIII fragment of pGEMMYC (50) was used for random-primed labeling (Multiprime; Amersham) of a human c-Myc cDNA probe, a 950-bp *Eco*RI fragment of pGEMBcl2 was used to synthesize a human Bcl-2 cDNA probe, and a 1.2-kb *Xba*I fragment containing rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (a gift of H. Singh, University of Chicago) was used for control probe synthesis.

Induction and analysis of apoptosis. A total of 10^6 cells from each cell line were plated on 100-mm-diameter plates 15 h prior to starvation for serum, at which time the medium was replaced with Dulbecco's modified E4 medium containing only penicillin and streptomycin. Cells were examined microscopically, and representative fields were photographed at each time point to observe morphological changes and the extent of cell death as represented by the relative numbers of floating and adherent cells. Additionally, at each time point, floating cells were collected by aspiration of the medium and a 10-ml phosphate-buffered saline (PBS) wash, followed by centrifugation for 5 min in a clinical centrifuge. Cells were washed in Ca^{2+}-Mg^{2+}-free PBS containing 1 mM EDTA, pelleted, and lysed by resuspension in 1 mM EDTA–0.6% sodium dodecyl sulfate. Sodium chloride was added to 1 M, and the solution was mixed by gentle inversion and incubated at 4°C overnight. Following centrifugation for 20 min in a microcentrifuge at 4°C, low-molecular-weight DNA was ethanol precipitated from the supernatant. DNA was analyzed for fragmentation by electrophoresis in 1.5% agarose in Tris-acetate-EDTA buffer, stained with ethidium bromide, and photographed under UV illumination.

Determination of cell viability. Triplicate cultures of 10^4 cells from each cell line were plated on 60-mm-diameter plates 15 h prior to serum deprivation and were treated as described above. At each time point, floating cells were collected by centrifugation of the aspirated medium and a 5-ml PBS wash and were resuspended in 1 ml of PBS. Adherent cells were trypsinized, collected in 10 ml of PBS, centrifuged, and resuspended in 1 ml of PBS. Numbers of viable and nonviable cells from each time point were determined by trypan blue exclusion.

Cell cycle analysis. Subconfluent proliferating cells and cells starved of serum for 48 h (as described above) were washed twice in PBS and then scraped in 1 ml of PBS. The cells were fixed in 50% ethanol–50% PBS, digested with RNase A, and stained with 100 μg of propidium iodide per ml. Cell cycle profiles and distributions were determined by flow-cytometric analysis of 10^4 cells by using the LysisII program on a FACScan flow cytometer (Becton-Dickinson).

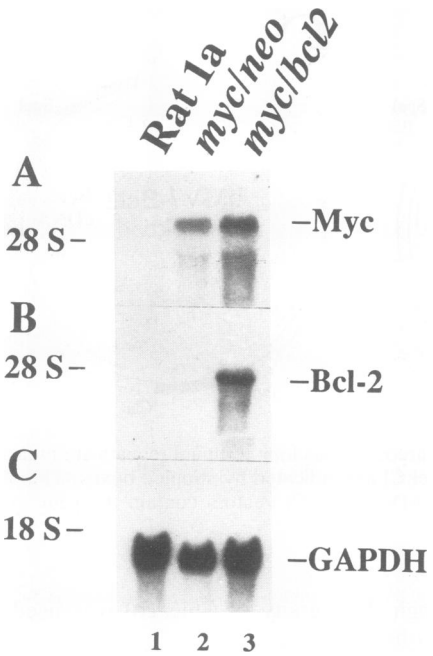

FIG. 2. Analysis of RNA from Rat 1a (lane 1), Rat 1a *myc/neo* (lane 2), and Rat 1a *myc/bcl2* (lane 3) cell lines. Total RNA (15 μg) from each cell line was subjected to RNA blot analysis and sequentially probed with ^{32}P-labeled DNA probes of human c-Myc (A), human Bcl-2 (B), and rat GAPDH (C) cDNAs. The positions of 28S and 18S rRNAs, retrovirally expressed Myc and Bcl-2, and endogenous GAPDH are indicated.

Cell debris and clumped cells were excluded from cell cycle distribution analysis by gating.

RESULTS

Overexpression of c-*myc* and *bcl2* in Rat 1a cells. To assay the effects of expression of Myc and coexpression of Myc and Bcl-2 in serum-starved fibroblasts, we used retroviruses to enforce high levels of constitutive expression of the recombinant genes. We first used the retrovirus pMV12-Myc (Fig. 1A) to infect Rat 1a cells so as to generate a cell line overexpressing human c-*myc*. Hygromycin-resistant clones exhibited morphological characteristics of transformed cells (as was previously shown [42]) and were tested for growth in soft agarose. One clone which grew in soft agarose, designated RAiMH3-1a, was shown by Southern analysis to have a single integrated provirus (41) and was used for further cell line construction. The retroviruses pMV7 and pMV7-Bcl2 (Fig. 1B and C; see Materials and Methods for details of their construction) were then used to infect RAiMH3-1a cells to generate the cell lines Rat 1a/*myc/neo* and Rat 1a/*myc/bcl2*, respectively, established from pooled G418-resistant cells. Both cell lines constitutively express high levels of human Myc under control of the Moloney murine sarcoma virus long terminal repeat, and the cell line Rat 1a/*myc/bcl2* additionally expresses high levels of human Bcl-2. Expression of these genes was verified by Northern blot analysis of RNA extracted from these cells and the parental Rat 1a cell line (Fig. 2). Retrovirally expressed c-Myc mRNA is present at high levels in the derived cell lines Rat 1a/*myc/neo* and Rat 1a/*myc/bcl2* but not in Rat 1a cells (Fig. 2A), and Bcl-2 mRNA is similarly expressed at high levels in only the Rat 1a/*myc/bcl2* cells (Fig. 2B). When normalized for GAPDH hybridization, approximately equal amounts of Myc were expressed in the Rat 1a/*myc/neo* and Rat 1a/*myc/bcl2* cell lines (Fig. 2C). The expression of the endogenous c-*myc* and *bcl2* genes is not detectable at this exposure and thus constitutes a minor proportion of the total expression of these genes in the cells.

Increased cell death is caused by Myc expression and blocked by Bcl-2 coexpression in serum-starved fibroblasts. Expression of c-*myc* during growth factor withdrawal has been shown to induce cells to activate a cell death pathway known as apoptosis (4, 14). To evaluate our cell lines for induction of apoptosis, we placed equivalent numbers of cells in medium lacking serum and analyzed the cells at various times following starvation for changes resembling apoptosis. Specifically, we examined the cells for morphological changes by light microscopy and for DNA fragmentation by agarose gel electrophoresis. In addition, in parallel experiments we counted the number of live and dead cells in both the floating and adherent populations.

Microscopic analysis of proliferating cells (Fig. 3, 0-h time point) demonstrated the transformed appearance of the cell lines overexpressing c-*myc*. That is, the Rat 1a/*myc/neo* and Rat 1a/*myc/bcl2* cells appear more refractile and rounder than the flatter parental Rat 1a cells. This observation is in agreement with previous studies of this cell line, which demonstrated transformation by expression of c-*myc* alone (42). Coexpression of *bcl2* did not change the transformed phenotype when compared with the phenotype of cells expressing *myc* alone, as determined by cell morphology (Fig. 3, 0 h, middle and bottom panels) and by the ability of the cells to grow in soft agarose (data not shown).

Starvation of the parental Rat 1a cells resulted in a decrease in refractility and a more pronounced flattening. This flattening, coupled with completion of mitosis of cells already committed to the cell cycle at the initiation of starvation, resulted in the appearance of continued cell proliferation (Fig. 3, 48 h, top panel). Some cell death did occur during starvation, although this fraction was minor compared with the proportion of cells that remained adherent (Fig. 4A). Constitutive expression of c-*myc*, however, resulted in a rapid induction of detachment and cell death which increased with prolonged starvation, reaching a 15-fold increase after 48 h (Fig. 3 [middle panels] and 4A). The dying cells underwent a transition from being tightly attached to loosely attached to detached from the tissue culture plate. This effect was much more apparent and dramatic in cells expressing c-*myc* alone than in the parental Rat 1a cells or in cells coexpressing c-*myc* and *bcl2* (Fig. 3). Reproducibly, most of the Rat 1a/*myc/neo* cells had died by 48 h of serum starvation, and essentially no cells remained viable following 1 week of starvation (49).

Coexpression of Bcl-2, however, greatly inhibited cell death induced by c-*myc* expression during serum starvation (Fig. 3 [bottom panels] and 4A). Although some cell death did occur, a pronounced difference was observed between Rat 1a/*myc/neo* cells and Rat 1a/*myc/bcl2* cells at 48 h of starvation (Fig. 3 [middle and bottom panels] and 4). Furthermore, we observed a 10-fold increase in the number of live Rat 1a/*myc/bcl2* cells following 48 h of serum deprivation but no significant increase in the number of live Rat 1a or Rat 1a/*myc/neo* cells (Fig. 4B). This difference was increased by prolonged starvation; Bcl-2-expressing cells remained viable for at least 1 week of starvation (49).

Cells undergoing death by apoptosis exhibit a characteristic DNA fragmentation pattern consisting of degradation of DNA into discrete oligonucleosomal fragments. To deter-

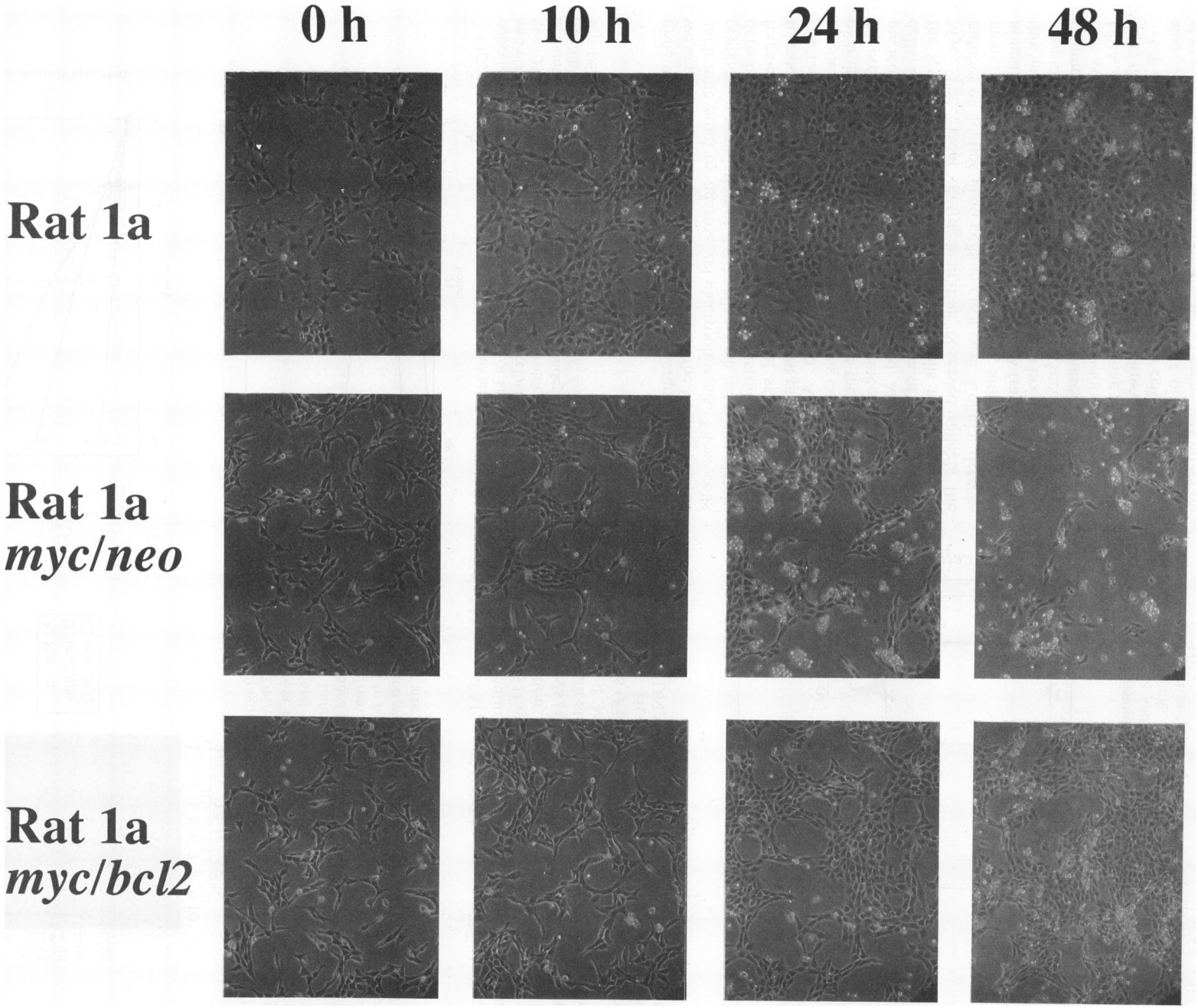

FIG. 3. Cell morphology following serum starvation. Rat 1a, Rat 1a *myc/neo*, and Rat 1a *myc/bcl2* cells were placed in serum-free medium, and representative fields were photographed at various time points, as indicated.

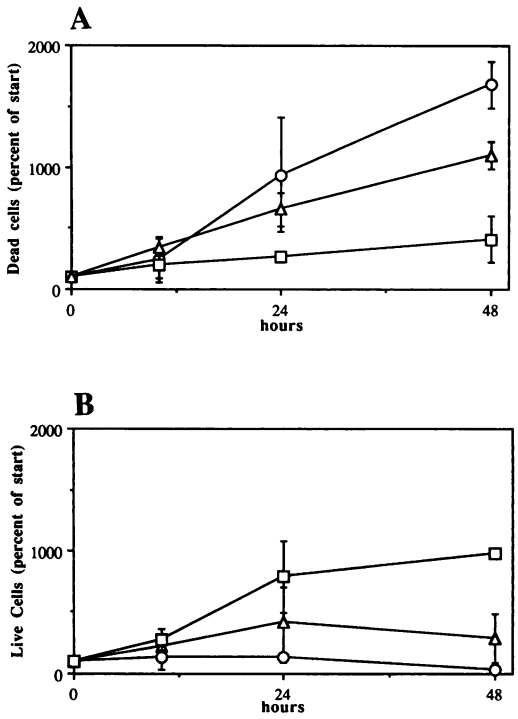

FIG. 4. Cell viability following serum starvation. Cells were collected at various times following initiation of serum starvation, and viability was determined by trypan blue exclusion. Dead (A) or live (B) cells as the percentage found in proliferating cells are demonstrated. Values are means of triplicate independent determinations ± standard errors of the mean. Symbols: △, Rat 1a cells; ○, Rat 1a *myc/neo*; □, Rat 1a *myc/bcl2*.

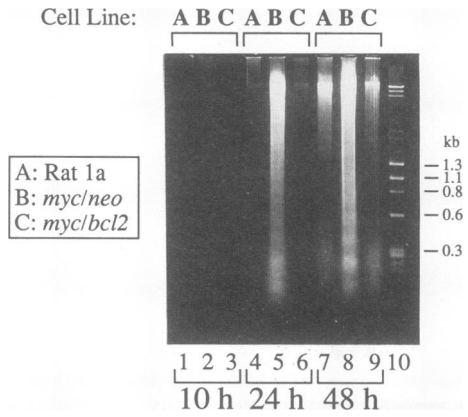

FIG. 5. DNA fragmentation following serum starvation. Low-molecular-weight DNA was collected from detached cells (from the experiment shown in Fig. 3), fractionated by electrophoresis in a 1.5% agarose gel, and visualized by ethidium bromide staining and UV fluorescence. Lanes: 1, 4, and 7 (lanes A), DNA from Rat 1a cells; 2, 5, and 8 (lanes B), DNA from Rat 1a *myc/neo* cells; 3, 6, and 9 (lanes C), DNA from Rat 1a *myc/bcl2* cells following 10 h (lanes 1 to 3), 24 h (lanes 4 to 6), and 48 h (lanes 7 to 9) of serum starvation; 10, λ DNA (digested with *Hin*dIII) and φX174 DNA (digested with *Hae*III) molecular size markers.

mine whether these cells were in fact undergoing apoptosis, we analyzed genomic DNA isolated from the floating cells at each time point by agarose gel electrophoresis. These cells represent a population of dead cells which have completely detached from the plate. Figure 5 demonstrates observable fragmentation in all cell lines at 24 h of starvation, with fragmentation in Rat 1a/*myc/neo* cells most prominent. This contrast is more pronounced after 48 h. Although some fragmentation is present in Bcl-2-expressing cells, the degree of fragmentation is comparable to that of the control cells. As previously reported (14), DNA is fragmented into oligonucleosomal fragments in serum-starved Myc-expressing cells, but here we observe that expression of Bcl-2 reduces the level of fragmentation to that of cells not expressing Myc. The extent of fragmentation observed reflects the number of floating dead cells in each case. Cellular detachment is a late event in apoptosis (14), but, although only the nonadherent cells were analyzed for DNA fragmentation by this assay, fragmentation in apoptosis has been shown to be a rapidly induced event in many systems. Accordingly, DNA fragmentation is also occurring in the adherent-cell population as observed by electrophoresis of low-molecular-weight DNA isolated from adherent Myc-expressing serum-starved cells (21a) and by flow cytometry (see below).

Effect of expression of Myc and Bcl-2 on cell cycle progression. To further evaluate the consequence of Bcl-2 expression on serum-starved fibroblasts, we examined its effect on the distribution of cells throughout the cell cycle. To this end, we analyzed the cell lines for the effects of expression of Myc and Bcl-2 as determined by flow cytometry. Expression of Myc or of Myc and Bcl-2 had no significant effect on cell cycle distribution in proliferating cells when compared with Rat 1a cells (Fig. 6a to c; Table 1). However, after 48 h of serum starvation, a distinct difference was observable in the three cell lines (Fig. 6d to f; Table 1). The growth arrest observed in serum-starved Rat 1a cells was partially blocked by expression of Myc in both Rat 1a/*myc/neo* and Rat 1a/*myc/bcl2* cells (Fig. 6e; Table 1) but not by expression of Bcl-2 alone (49). However, an additional peak (labeled A_0) appeared on the DNA histogram following 48 h of starvation.

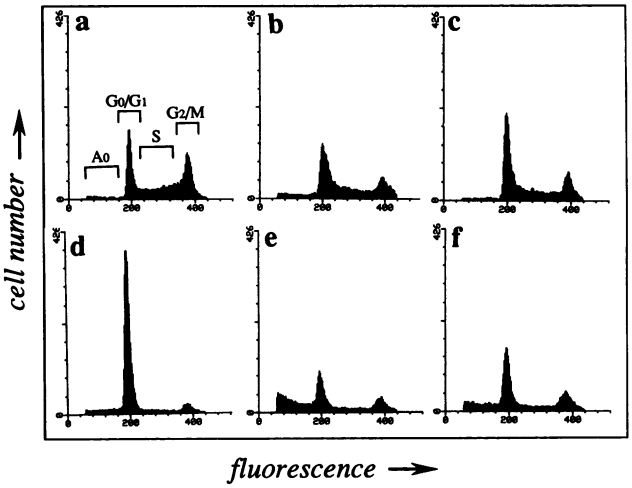

FIG. 6. DNA histograms of cells during serum starvation. Ethanol-fixed Rat 1a (a and d), Rat 1a *myc/neo* (b and e), and Rat 1a *myc/bcl2* (c and f) cells, proliferating (panels a to c) or serum starved for 48 h (panels d to f), were stained with propidium iodide and analyzed for DNA content (fluorescence). A total of 1.5×10^4 cells were analyzed to create each histogram. Peaks representing fragmented DNA (A_0) and the G_0/G_1, S, and G_2/M phases of the cell cycle are indicated.

TABLE 1. Cell cycle analysis of proliferating and serum-starved cells

Cell line	Serum[a]	% of cells (mean ± SE) in[b]:			
		A_0	G_0/G_1	S	G_2/M
Rat 1a	+	5.1 ± 0.4	33.4 ± 0.1	29.8 ± 0.4	32.3 ± 0.2
Rat 1a *myc/neo*	+	8.7 ± 3.1	41.3 ± 0.2	25.8 ± 0.4	22.9 ± 0.2
Rat 1a *myc/bcl2*	+	4.6 ± 0.1	49.4 ± 0.1	24.1 ± 0.4	23.6 ± 0.2
Rat 1a	−	12.8 ± 0.5	73.1 ± 0.6	6.6 ± 0.1	7.8 ± 0.1
Rat 1a *myc/neo*	−	47.0 ± 0.2	29.4 ± 0.5	9.3 ± 0.3	14.8 ± 0.1
Rat 1a *myc/bcl2*	−	27.1 ± 0.2	42.3 ± 0.1	12.3 ± 0.0	19.1 ± 0.3

[a] Cells were maintained in 10% fetal calf serum (+) or serum starved (−) for 48 h.
[b] Results of duplicate experiments involving 10^4 cells.

This peak represents cells with <2c DNA content; specifically, these are cells with fragmented DNA (1, 30). These cells constituted 47.0% ± 0.2% of total Rat 1a/*myc/neo* cells (Table 1) and demonstrate apoptosis in the adherent-cell population. This peak also developed in the serum-starved Rat 1a and Rat 1a/*myc/bcl2* cell lines (Fig. 6d and f) but to a lesser degree (12.8% ± 0.5% for Rat 1a cells and 27.1% ± 0.2% for Rat 1a/*myc/bcl2* cells [Table 1]), in agreement with our data on fragmented DNA in the floating-cell population observed by agarose gel electrophoresis. Thus, coexpression of *bcl2* with c-*myc* reduced the Myc-mediated induction of DNA fragmentation observed during serum deprivation without blocking entry into the cell cycle, providing further evidence that Bcl-2 expression can block apoptosis induced by enforced high-level expression of Myc in serum-starved fibroblasts.

DISCUSSION

In this report we observe that constitutive overexpression of Myc in serum-deprived Rat 1a fibroblasts induces apoptosis and demonstrate that expression of Bcl-2 is sufficient to inhibit this cell death. This effect was observed both at the level of light microscopy of cells in culture and by the degree of DNA fragmentation as monitored by agarose gel electrophoresis and DNA content analysis. Induction of apoptosis by Myc and inhibition by Bcl-2 were distinctly observable by 24 h of serum starvation, and protection by Bcl-2 persisted for at least as long as the time it took for all Rat 1a/*myc/neo* cells to die (approximately 1 week). Furthermore, Bcl-2 coexpression enabled serum-deprived cells to enter and traverse the cell cycle with a distribution similar to that for proliferating cells.

These studies confirm and extend the work of Evan et al. (14) describing the induction of apoptosis in quiescent fibroblasts by constitutive overexpression of c-Myc. Similarly, c-Myc has been demonstrated to accelerate induction of apoptosis during interleukin-3 (IL-3) withdrawal from an IL-3-dependent myeloid cell line (4) and blockage of c-Myc expression by antisense oligonucleotides was sufficient to inhibit apoptosis induced by anti-CD3 treatment of a hybridoma cell line (40). These observations, coupled with evidence that Myc is essential for cell replication, lead to a paradox: how is Myc capable of regulating two processes with distinctly opposite outcomes, cell growth and cell death? One possibility is that a factor present in serum generates an intracellular signal which activates a pathway complementing Myc and activating cell growth. This signal may represent the initiation of specific gene induction or protein modification which produces or activates other proteins to act in concert with Myc to induce cell growth. However, in the absence of this second pathway (i.e., under conditions of growth factor deprivation), Myc induces cell death. In several systems, induction of apoptosis has been demonstrated to be dependent on new RNA and protein synthesis. Evidence that Myc can serve as a sequence-specific DNA-binding protein and transcriptional activator implicates activation of specific genes as a mechanism by which Myc can induce either proliferation or apoptosis. Indeed, activation of apoptosis by Myc has been shown to be dependent on regions of Myc that were also shown to be essential for transformation (14), and these domains have recently been demonstrated to be essential for *trans* activation (3, 22). However, although Myc has been shown to activate the transcription of specific genes (13, 31), no connection has been established between these genes and genes induced during apoptosis.

We chose to overexpress Bcl-2 to examine its effect on Myc-induced apoptosis on the basis of its ability to inhibit apoptosis in a variety of hematopoietic cell lines and tissues. This effect has been extensively demonstrated in promyeloid, pre-B-cell, and pro-B-cell lines induced to undergo apoptosis by growth factor withdrawal (5, 20, 48) or glucocorticoid treatment (2) and in T cells of transgenic animals (44). Bcl-2 expression has also been found to reduce the growth factor requirements of NIH 3T3 fibroblasts for DNA synthesis (33), although the level of DNA synthesis obtained was lower than that in cells treated with growth factors. Similarly, expression of Bcl-2 in B-lymphoblastoid cell lines resulted in an increased tolerance to stresses of serum deprivation, ethanol and methotrexate treatment, and heat shock (46). The protective effect of Bcl-2 does not appear to be a result of altering cell cycle progression since we did not observe a change in cell cycle profile attributable to expression of Bcl-2, and other studies have reported that Bcl-2 expression has no effect on cell cycle progression in pro-B cells (20) and does not block establishment of quiescence in serum-starved NIH 3T3 cells (34). Although we observed that expression of Bcl-2 enhanced cell survival during Myc-induced cell cycle progression in serum-deprived fibroblasts, coexpression of Myc and Bcl-2 did not lead to IL-3-independent proliferation in FDC-P1 myeloid cells (48) and did not block phorbol ester-induced growth arrest in the human B-cell leukemia cell line 380 (25). One explanation for this difference is that enforced Myc expression may not be sufficient for cell cycle progression in all cell lines and that Bcl-2 expression maintains cell viability without promoting growth. Although many hematopoietic cell lines and at least one fibroblast cell line can be protected from apoptosis by Bcl-2 expression, this phenomenon may not be universal, since Bcl-2 overexpression was not sufficient to make an

IL-2-dependent T-cell line or an IL-6-dependent myeloma line interleukin independent (20).

The ability of Bcl-2 expression to repress Myc-induced apoptosis in serum-starved fibroblasts suggests that this form of apoptosis shares a common mechanism with apoptosis induced in hematopoietic lines as described above. How Bcl-2 actually inhibits apoptosis is unclear; the protein has been localized to the inner mitochondrial membrane and has been reported to have no kinase or GTP-binding activity (20). It is normally constitutively expressed throughout the cell cycle (35), although its expression is induced following mitogenic activation of peripheral blood lymphocytes (36). One suggested role of Bcl-2 is as a regulator of intracellular Ca^{2+} concentration, since the mitochondrion is a major store of cytosolic Ca^{2+} and Ca^{2+} acts both as a second messenger in the signal pathway from growth factor receptors to the nucleus and as an inducer of apoptosis (2).

Activation of a cell death pathway by inappropriate expression of Myc may have evolved as a mechanism to prevent development of neoplasias from cells with deregulated Myc expression (see also reference 14). That is, cells which aberrantly express high levels of Myc without the activation of complementary pathways by signaling from growth factors would be selectively destroyed instead of proliferating. This would represent a rapid and efficient mechanism for protecting an organism from unchecked cell growth, since Myc expression alone enables cells to enter the cell cycle (13, 14). According to this model, for tumors to develop, additional mutations or deregulations which would either activate a complementary pathway or block apoptosis would be required.

It is notable that Myc overexpression occurs in many neoplasias which are evidently able to escape the programmed cell death pathway. In at least one instance, that of malignant lymphomas derived from the t(14;18) translocation, induction of apoptosis by overexpression of c-Myc may be blocked by the high levels of Bcl-2 expressed as a result of *bcl2* translocation to the immunoglobulin heavy-chain enhancer. In this lymphoma, deregulation of c-*myc* is preceded by deregulation of *bcl2* and genesis of an indolent follicular lymphoma, which then clonally develops into a malignant lymphoma as a single cell acquires a c-*myc* translocation as well (27). It is conceivable that this particular type of lymphoma can develop only if the *bcl2* translocation occurs first, since the c-*myc* translocation may induce the cells to undergo cell death. A similar situation exists in Burkitt's lymphomas, in which Myc is overexpressed as a result of translocation of c-*myc* to an immunoglobulin allele (11, 43), an event which is highly associated with prior infection with Epstein-Barr virus. Interestingly, the Epstein-Barr virus gene *BHRF1* encodes a protein with significant homology to Bcl-2 (9) and may therefore protect cells from apoptosis upon acquiring translocation of a c-*myc* allele. In addition, the latently expressed Epstein-Barr virus protein LMP-1 induces Bcl-2 expression and inhibits apoptosis (16, 18, 19). Finally, the cooperativity of c-*myc* and *bcl2* in immortalization of pre-B cells (48), tumorigenicity of T cells (32), transformation of human B-lymphoblastoid cell lines (29), and transformation of rat embryo fibroblasts with an activated *ras* (33) may result from enhanced survival of clones overexpressing c-*myc*; that is, cells overexpressing c-*myc* which would normally die as a result of the overexpression may instead survive with concomitant coexpression of *bcl2*.

The p53 tumor suppressor gene has also been shown to induce apoptosis, notably when introduced into the myeloid cell line M1 (57) or the EB colon carcinoma cell line (39), neither of which normally expresses p53, suggesting that one mechanism by which p53 may regulate cell growth is by promoting apoptosis. Interestingly, many tumors which possess amplification or deregulation of Myc expression also contain mutant or deleted p53 genes. These include the myeloid tumor cell line HL60, which contains amplified c-*myc* but does not express p53 (12, 55); Burkitt's lymphoma cells, which express unregulated high levels of Myc and possess mutant p53 genes (15, 54); and small-cell lung carcinomas, which exhibit L-*myc* amplification and mutant p53 genes (28, 45). It is possible that in tumors or tumor cell lines in which *myc* is aberrantly expressed, the apoptosis pathway must be disrupted for the cells to survive when the availability of growth factors is limited (e.g., inadequate neovascularization). One mechanism appears to be deregulation and overexpression of the *bcl2* gene. Potentially, p53 mutations or deletions constitute a second mechanism for escaping apoptosis induced by Myc deregulation. This hypothesis would implicate p53 mutation as a requisite predecessor to c-*myc* overexpression to allow for the cells to progress to a malignant state while avoiding apoptosis. It still remains to be determined, however, whether Myc-mediated apoptosis actually involves induction of p53, which would then activate apoptosis and/or cell cycle withdrawal, thereby suppressing tumor development.

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ADDENDUM IN PROOF

While the present paper was submitted, two other independent reports demonstrated that Bcl-2 can block Myc-mediated apoptosis [R. P. Bissonnette, F. Echeverri, A. Mahboubi, and D. R. Green, Nature (London) **359**:552–554, 1992, and A. Fanidi, E. A. Harrington, and G. I. Evan, Nature (London) **359**:554–556, 1992].

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