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# Participation of cyclin A in Myc-induced apoptosis

(cell cycle/oncogene/programmed cell death)

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ABSTRACT **The involvement of c-Myc in cellular proliferation or apoptosis has been linked to differential cyclin gene expression. We observed that in both proliferating cells and cells undergoing apoptosis, cyclin A (but not B, C, D1, and E) mRNA level was elevated in unsynchronized Myc-overexpressing cells when compared with parental Rat1a fibroblasts. We further demonstrated that $Zn^{2+}$-inducible cyclin A expression was sufficient to cause apoptosis. When Myc-induced apoptosis was blocked by coexpression of Bcl-2, the levels of cyclin C, D1, and E mRNAs were also elevated. Thus, while apoptosis induced by c-Myc is associated with an elevated cyclin A mRNA level, protection from apoptosis by coexpressed Bcl-2 is associated with a complementary increase in cyclin C, D1, and E mRNAs.**

c-Myc is a helix–loop–helix leucine-zipper transcriptional factor that participates in opposite cellular fates of proliferation or programmed cell death (apoptosis) (1–4). Under normal cell culture conditions (i.e., high serum), overexpression of c-Myc causes cells to proliferate. Upon serum starvation or growth factor withdrawal, however, certain cells that overexpress c-Myc undergo apoptosis instead of growth arrest (1, 2). Although the domains of c-Myc protein required for neoplastic transformation and apoptosis are the same, the molecular events underlying these divergent cellular fates remain unknown (2–4).

Recent studies on the interleukin 2-dependent BAF-B03 pre-B cell line indicate that c-Myc alters cyclin gene expression and affects the transition of cells into the $G_2$ phase (5). Inducible c-Myc activity in a Myc–estrogen receptor chimeric system augments cyclin A and E gene expression and stimulates mitogenesis in rat fibroblasts (6). We sought to define further the possible connections between c-Myc, cyclin gene expression and cell fates. Specifically, we hypothesized that cell death might result from constitutive imbalanced cyclin gene expression induced by c-Myc in the setting of growth factor withdrawal (1, 2, 7). In addition, because Bcl-2 could counteract c-Myc-induced apoptosis (8, 9), the Bcl-2 effect may also be manifested through altered cyclin gene expression.

## MATERIALS AND METHODS

**Plasmid Constructions.** A 1.1-kb full-length Bcl-2 cDNA *Eco*RI fragment (in pSKII-bcl2, from J. Reed, La Jolla Cancer Research Center, La Jolla, CA) was subcloned into a simian virus 40 early promoter expression vector, pSG5 (Pharmacia), through a unique *Eco*RI site, yielding pSG5-bcl2. A 2.3-kb *Eco*RI fragment containing human cyclin A cDNA (provided by T. Hunter, Salk Institute, San Diego) was subcloned into the *Eco*RI site of pMT-CB6+ vector (provided by F. Rauscher, Wistar Institute, Philadelphia) downstream of a sheep metallothionein promoter and containing a neomycin-resistant marker gene, yielding pMT-CycA.

**Cell Culture.** Rat1a–myc fibroblasts were generated by cotransfection (Lipofectin; BRL) of a Moloney leukemia virus long terminal repeat-driven genomic c-*myc* sequence (pMLVmyc) with a neomycin-resistance marker plasmid (pSV2neo) into Rat1a fibroblasts. Cells that overexpress Myc were selected with the antibiotic G418 [active G418 at 400 μg/ml/Dulbecco's minimal essential medium (DMEM)/10% fetal calf serum (FCS)]. To generate Rat1a–myc/bcl2 cells, Rat1a–myc fibroblasts were transfected with pSG5-bcl2 along with a hygromycin-resistant marker plasmid pHyg. Rat1a–myc/bcl2 cells were selected in hygromycin (80 μg/ml) and G418, and hygromycin/G418 doubly resistant clones (≈125) were pooled for analysis.

Cells bearing a $Zn^{2+}$-responsive human cyclin A construct were generated by transfecting the pMTCycA construct containing a neomycin-resistance marker gene into Rat1a fibroblasts. Twenty-four randomly selected single-cell colonies from a total of 96 G418 (400 μg/ml, final active concentration)-resistant clones were expanded and screened for $Zn^{2+}$-induced cyclin A expression by immunoblot analysis. Two out of 24 clones exhibited high cyclin A expression with 50–75 μM $Zn^{2+}$.

For the soft agar assay, $10^5$ cells were mixed with 0.8% agar (SeaKem, FMC) and poured onto a bed of 1.4% agar (SeaKem) in 100 × 20 mm culture plates. Both top and bottom agar were prepared in DMEM/10% FCS. Cells were fed every week with 2 ml of DMEM/10% FCS.

**Protein Analysis.** For immunoblot analysis of c-Myc and Bcl-2, total cell lysates collected from plates of exponentially growing cells at 70% confluency were boiled in 2× Laemmli buffer (10). Polypeptides from one-tenth of total cell lysates from each 100 × 20 mm plate were resolved by SDS/10% PAGE and subjected to immunoblot analysis with either anti-Myc 9E10 monoclonal antibody (1:100 dilution) (2) or anti-Bcl-2 monoclonal antibody (1:100 dilution) (Dako). After incubation of blots with a secondary goat anti-mouse horseradish peroxidase-conjugated antibody (Bio-Rad) (1:10,000 dilution), reactive polypeptides were detected by the enhanced chemiluminescence (ECL) system (Amersham). Luminograms from the same blot developed with either anti-Myc or anti-Bcl-2 primary antibodies were superimposed and shown with molecular weight standards for clarity of presentation.

Expression of $Zn^{2+}$-inducible human cyclin A in cell lines was detected 24 hr after induction with various final concentrations of $ZnSO_4$. Total cell lysates were prepared, and

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Abbreviations: FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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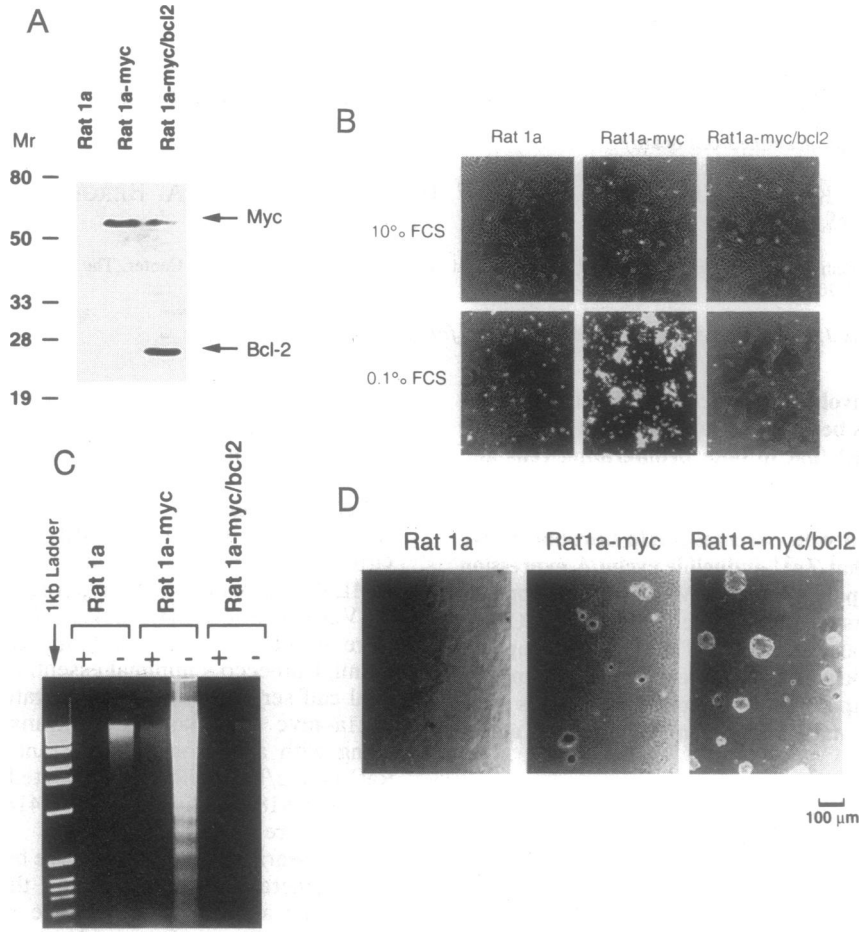


FIG. 1. Expression of Myc and Bcl-2 in stably transfected Rat 1a fibroblast. (*A*) Luminogram of immunoblot showing human c-Myc (64 kDa) in Rat1a–myc and Rat1a–myc/bcl2 cells and Bcl-2 (25 kDa) in the Rat1a–myc/bcl2 cells. Molecular weight ($M_r \times 10^{-3}$) markers (Bio-Rad) are indicated at left. (*B*) Phase-contrast micrographs of Rat1a, Rat1a–myc, and Rat1a–myc/bcl2 in either 10% FCS (*Upper*) or at 24 hr after exposure to 0.1% FCS (*Lower*). Rat1a–myc cells exhibit increased number of apoptotic cells that take up the vital dye trypan blue 24 hr after exposure to 0.1% FCS. (*C*) Oligonucleosomal DNA fragments in Rat1a–myc cells 24 hr after 0.1% FCS exposure. The condition of cell culture at time of DNA isolation is denoted as + for 10% FCS or − for 0.1% FCS. The 1-kb DNA molecular weight marker (BRL) is shown at left. (*D*) Soft agar assay for anchorage-independent growth. Rat1a–myc/bcl2 cells displayed more colonies that were larger than either Rat1a–myc or Rat1a after 3 weeks in 10% FCS. Distribution of propidium iodide-stained cells in different phases of the cell cycle as approximated by fluorescence-activated cytometry is as follows: (*i*) Rat1a (10% FCS): $G_0/G_1 = 41\%$, $S = 34\%$, $G_2/M = 25\%$; Rat1a (0.1% FCS): $G_0/G_1 = 58\%$, $S = 23\%$; $G_2/M = 19\%$. (*ii*) Rat1a–myc (10% FCS): $G_0/G_1 = 39\%$, $S = 37\%$, $G_2/M = 24\%$; Rat1a–myc (0.1% FCS): $G_0/G_1 = 59\%$, $S = 23\%$; $G_2/M = 18\%$. (*iii*) Rat1a–myc/bcl2 (10% FCS): $G_0/G_1 = 34\%$, $S = 41\%$, $G_2/M = 25\%$; Rat1a–myc/bcl2 (0.1% FCS): $G_0/G_1 = 43\%$, $S = 32\%$; $G_2/M = 25\%$. The percentage of cells with <2 M DNA content was not reliably resolvable, and therefore these cells are included in the $G_0/G_1$ fraction.

SDS/PAGE analysis was done as described above. Monoclonal anti-human cyclin A antibody (PharMingen) was used at 1:100 dilution as primary antibody. A secondary goat anti-mouse horseradish peroxidase-conjugated antibody (Bio-Rad) (1:10,000 dilution) and the enhanced chemiluminescence (ECL) system (Amersham) were subsequently used for detection of reactive polypeptides.

**Oligonucleosomal DNA Analysis.** To assay for oligonucleosomal DNA produced during apoptosis, Rat1a, Rat1a–myc, and Rat1a–myc/bcl2 cells ($0.5 \times 10^6$ cells per plate) were grown in duplicate 100 × 20 mm plates in DMEM/10% FCS. Twenty-four hours later, one set from each cell line was exposed to 0.1% FCS. After another 24 hr both sets (10% FCS and 0.1% FCS) were processed to harvest low-molecular-weight DNA as described (9, 11). With the Rat1a-InCycA, the cells were expanded in the presence of G418 and then were induced at various $Zn^{2+}$ concentrations for 24 hr in high serum before exposure to low serum with the same $Zn^{2+}$ concentrations. Low-molecular-weight DNA was harvested after 24-hr exposure to 0.1% FCS as described above. Isolated DNAs were electrophoresed in a 1.8% agarose gel and stained with ethidium bromide. One-third of the total DNA isolated from two 100 × 20 mm plates of each cell line is represented in each lane after normalization for differences among samples by the total number of cells per plate at time of harvest.

**RNA Analysis.** Total RNAs from each cell line grown in either high- or low-serum conditions were isolated using the guanidium/phenol/chloroform extraction method. Ten-microgram RNA samples were loaded into a 1.4% formamide-agarose gel, electrophoresed, blotted overnight onto a Nytran membrane filter, and probed with randomly primed (Prime-It kit; Stratagene) [$^{32}$P]dATP-labeled cDNAs from human cyclin A, B, C, D1, and E (provided by T. Hunter and S. Reed) and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (American Tissue Culture Collection) (12, 13).

**Flow Cytometry.** Approximately $1 \times 10^6$ cells were washed with phosphate-buffered saline, fixed overnight in 70% (vol/vol) ethanol, stained with propidium iodide (final concentration was 0.01 μg/ml in phosphate-buffered saline), and analyzed on a FACScan cytometer (Becton Dickinson). Resolution of $G_1$, S, and $G_2/M$ phases was done with the LYSIS II analysis software (Becton Dickinson).

## RESULTS AND DISCUSSION

Cell lines that overexpress c-Myc (Rat1a–myc) and both c-Myc and Bcl-2 (Rat1a–myc/bcl2) were generated from a

parental Rat1a fibroblast cell line. Expression of Myc and Bcl-2 proteins in these cells was detected by immunoblot analysis (Fig. 1*A*). A 64-kDa human c-Myc polypeptide was observed in both Rat1a–myc and Rat1a–myc/bcl2, and a 25-kDa Bcl-2 protein was present only in Rat1a–myc/bcl2 cells. Repeated immunoblots of extracts from Rat1a–myc and Rat1a–myc/bcl2 cells did not show a change in c-Myc protein level in the presence of Bcl-2, suggesting that Bcl-2 does not exert its effect by altering c-Myc level.

Upon serum withdrawal (0.1% fetal calf serum) the parental Rat1a cells displayed rare rounded apoptotic cells (Fig. 1*B*). However, as reported (2), significant numbers of Rat1a–myc cells rounded up and underwent apoptosis when exposed to 0.1% serum (Fig. 1*B*). This observation was further confirmed by the presence of oligonucleosomal DNA fragments (11) (Fig. 1*C*). In contrast, the Rat1a–myc/bcl2 cells did not undergo apoptosis 24 hr after exposure to 0.1% FCS, demonstrating the protective effect of Bcl-2 (Fig. 1 *B* and *C*).

The transformed phenotypes of these cell lines were assayed by culture in soft agar. Rat1a–myc/bcl2 cells displayed increased frequency of colony formation and larger colonies compared with either the Rat1a–myc or the Rat1a parental cell line (Fig. 1*D*). Most Rat1a–myc colonies showed opaque, darkened centers, as compared with the refractile Rat1a–myc/bcl2 colonies after 3 weeks in culture. Histologic sections through these colonies revealed evidence of cell death in the centers of Rat1a–myc colonies but not in Rat1a–myc/bcl2 colonies (data not shown). These observations suggest that serum deprivation *in vitro* may be related to the *in vivo* growth of cells at the center of a tumor mass. In a mass that is sufficiently large, access to nutrients becomes diffusion-limited toward the center of the mass as tumor size increases. Thus, cells at the center of such masses may undergo apoptosis unless additional genetic alterations are sustained, such as the overexpression of Bcl-2 (14, 15).

To determine the effects of c-Myc on cyclin gene expression, relative mRNA levels of cyclins A, B, C, D1, and E in Rat1a and Rat1a–myc cells were determined by Northern analysis of total RNA using cDNAs for human cyclins A, B, C, D1, and E as radiolabeled probes. GAPDH mRNA level was chosen as a control for densitometric normalization. In medium with 10% FCS, only cyclin A mRNA was elevated in unsynchronized c-Myc-overexpressing Rat1a–myc cells as compared with Rat1a fibroblasts (Fig. 2). An independent Rat1a Myc-overexpressing cell line (Myc#2-8) displayed the same changes in cyclin A mRNA level (data not shown). mRNAs for cyclins B, C, D1, and E remained virtually unchanged (Fig. 2).

The pattern of steady-state cyclin mRNA expression was also examined in Rat1a–myc 24 hr after serum deprivation. Cyclin A mRNA level remained elevated, while mRNA levels of cyclins B, C, D1, and E remained unchanged as compared with levels in serum-deprived Rat1a cells (Fig. 2). These results, when considered with the observation that Myc induces the expression of cyclin A and E in proliferating cells (6), suggest a causal link between *myc* expression, differential cyclin gene expression, and cell fate. It is noteworthy, however, that the reported induction of cyclin E and suppression of cyclin D1 mRNA by c-Myc (6) were not observed in work of others (16) or in our study. The basis for these differences is unknown.

Our results (Fig. 2) and those of others (5, 6) show that c-Myc expression augments cyclin A mRNA levels. Thus, we sought to determine directly whether cyclin A could be a mediator of c-Myc-induced apoptosis. We chose to construct inducible cyclin A cell lines because previous attempts to generate cells that constitutively overexpress cyclin A have failed (17). Conditional expression was achieved by placing full-length human cyclin A cDNA (13) under the transcriptional control of the zinc-inducible sheep metallothionein

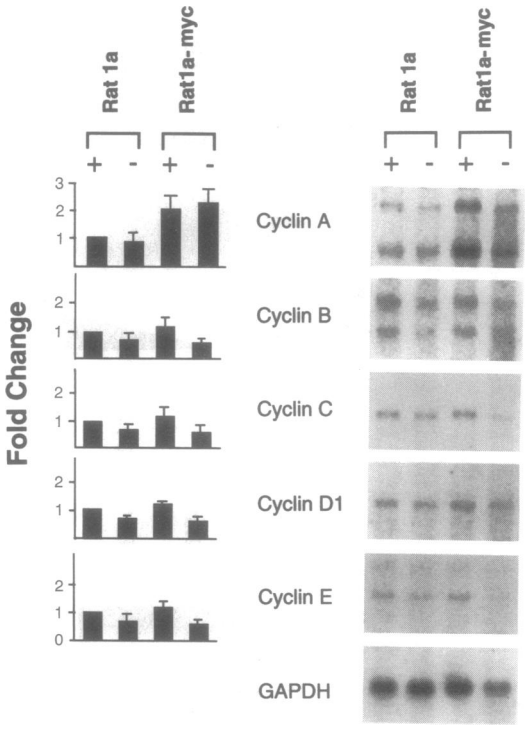


FIG. 2. Expression of cyclins A, B, C, D1, and E mRNAs in Rat1a and Rat1a–myc in 10% FCS (+) and 24 hr after exposure to 0.1% FCS (−). Human cDNA probes are indicated for each panel. GAPDH mRNA levels are included to control for sample loading. Total RNAs from each cell line grown in either high- or low-serum conditions were isolated. RNA samples (10 μg) were loaded onto a 1.4% formamide-agarose gel, electrophoresed, blotted onto Nytran membrane filter, and probed with randomly primed [$^{32}$P]dATP-labeled cDNAs. Estimated mRNA sizes are as follows: cyclin A (3.4 kb and 1.8 kb), cyclin B (2.5 kb and 1.8 kb), cyclin C (4.1 kb), cyclin D1 (4.3 kb), cyclin E (3.8 kb), and GAPDH (1.4 kb). Cyclin mRNA levels were normalized to the GAPDH mRNA levels. Relative fold-change in each cyclin mRNA level (graphs shown at left) was determined by normalizing to the level found in Rat1a cells exposed to 10% FCS. Bar height represents the average value (with SEs shown) derived from three independent experiments.

promoter (18). Stably transfected and conditionally overexpressing cyclin A cell lines were generated. Three independent cell lines [Rat1a-InCycA (or A3), A12, and A13] as determined by Southern blot analysis of total genomic DNA probed with human cyclin A (data not shown) were chosen for further analysis. Fig. 3*A* demonstrates the inducibility of cyclin A protein expression in the Rat1a-InCycA (or A3) cell line by $Zn^{2+}$ (15). Longer exposures of the luminogram did show barely detectable expression of cyclin A in the absence of $Zn^{2+}$, suggesting some leakiness in this system (data not shown). The A12 cell line shows weakly inducible cyclin A expression as compared with the A13 line (Fig. 3*D*, *Inset*).

All three cell lines [Rat1a-InCycA (or A3), A12, and A13] were characterized after exposure to 50 μM $Zn^{2+}$ (Fig. 3 *A* and *D*). About 30–40% of Rat1a-InCycA (or A3) or A13 cells and 10–20% of A12 cells rounded up, and the majority of these was not viable as determined by trypan blue dye uptake after 24 hr of exposure to 50 μM $Zn^{2+}$ and 0.1% serum. In contrast, neither the Rat1a-InCycA (or A3) unexposed to $Zn^{2+}$ nor the parental Rat1a cells displayed these changes (Fig. 3*B*). Fig. 3*D* shows the fold change of $Zn^{2+}$-induced cell death among the Rat1a-InCycA (or A3), A12, and A13 cells 24 hr after exposure to 0.1% FCS. When normalized to the Rat1a cell line that showed no $Zn^{2+}$-inducible cyclin A expression, we observed ≈1.6- to 2-fold increase in the number of dead cells caused by cyclin A expression in A13

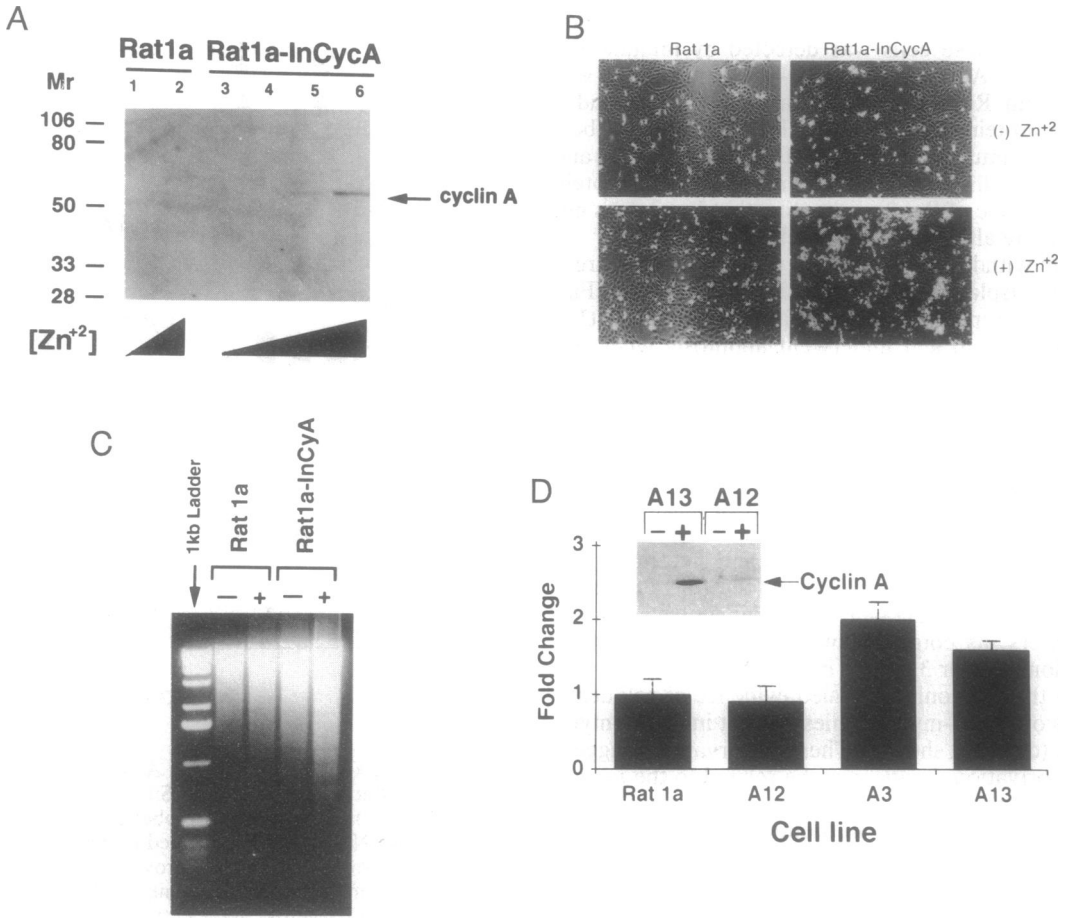


FIG. 3. Characterization of inducible cyclin A-expressing cells. (*A*) Human cyclin A expression in Rat1a-InCycA (or A3) was induced by $Zn^{2+}$ (lanes 3–6 correspond to 0, 25, 50, and 75 $\mu$M $Zn^{2+}$, respectively). Endogenous rat cyclin A protein was not detected by the anti-human cyclin A antibody in Rat1a cells without (lanes 1) or with (lane 2) exposure to 75 $\mu$M $Zn^{2+}$. (*B*) Phase-contrast photomicrographs of Rat1a and Rat1a-InCycA (or A3) with or without 50 $\mu$M $Zn^{2+}$. Both Rat1a and Rat1a-InCycA (or A3) were exposed to $Zn^{2+}$ in 10% FCS 24 hr before exposure to 0.1% FCS and $Zn^{2+}$. The photomicrographs were taken 24 hr after exposure to 0.1% FCS. (*C*) DNA laddering in Rat1a or Rat1a-InCycA (or A3) 24 hr after exposure to 0.1% FCS in the absence (−) or presence (+) of 50 $\mu$M $Zn^{2+}$. (*D*) $Zn^{2+}$-induced apoptosis in A12, Rat1a-InCycA (or A3), and A13 cell lines. Fold change represents the ratio of the number of $Zn^{2+}$-induced apoptotic cells to the number found in the absence of $Zn^{2+}$ normalized to the parental Rat1a cells. Bar height represents an average value (with SE) derived from three experiments. (*Inset*) Immunoblot with anti-human cyclin A antibody showing $Zn^{2+}$ (50 $\mu$M for 24 hr)-induced cyclin A expression level for A13 and A12 cell lines.

and Rat1a-InCycA (or A3), respectively. The A12 cells, which express cyclin A weakly as compared with A13 cells (Fig. 3*D*, *Inset*), exhibit undetectable change in cell death with exposure to $Zn^{2+}$ when compared with the parental Rat1a cell line.

Apoptotic death was further confirmed by the DNA laddering assay in which more oligonucleosomal DNA is observed when the Rat1a-InCycA (or A3) is exposed to 50 $\mu$M $Zn^{2+}$ in comparison with the control parental Rat1a cells (Fig. 3*C*). The diminished degree of DNA laddering, which contrasts with the sharp delineation of laddering between Rat1a and Rat1a–myc (Fig. 1*C*), may, in part, be due to the inhibitory effect of $Zn^{2+}$ on the nucleases responsible for DNA laddering (19).

Because Bcl-2 does not affect c-Myc expression level (Fig. 1*A*) but cooperates with c-Myc in transformation (Fig. 1*D*) and oncogenesis (20), we hypothesized that it might block c-Myc-induced apoptosis by a complementary pathway. Such a pathway could be manifested by Bcl-2-induced alteration of cyclin gene expression pattern complementary to that altered by c-Myc. In high serum, mRNAs for cyclins C, D1, and E were found to be elevated in Rat1a–myc/bcl2 cells as compared with the Rat1a–myc cells (Fig. 4). Moreover, mRNAs for cyclins A–E in Rat1a–myc/bcl2 exposed to low serum remained elevated (Fig. 4). Although these results may reflect the direct effect of Bcl-2 on cyclin mRNA levels, it is also possible that protection against cell death by Bcl-2 indirectly augments the mRNA levels of multiple cyclins.

Our results suggest that c-Myc and cyclins are involved at an important nodal point shared by pathways regulating cellular proliferation and apoptosis (21). We observed that the induction of apoptosis by overexpression of c-Myc in Rat1a cells and serum deprivation is manifested by differential expression of cyclin mRNAs. We further determined that deregulated expression of cyclin A is sufficient for the induction of apoptosis in cells exposed to low serum. Indeed, cyclin A-dependent protein kinases are found to be activated during apoptosis of S phase-arrested HeLa cells (22). Furthermore, another study (23) suggests that overexpression of cyclin A alone in BHK cells causes "mitotic catastrophes" with chromosomal fragmentation that resembles apoptosis. This effect of cyclin A occurs synergistically with the coexpression of *cdc2*. Work by Shi *et al.* (24) further suggests that premature activation of $p34^{cdc2}$ kinase is required for apoptosis. These observations strongly implicate altered cyclin gene expression as a mechanism for inducing programmed cell death. In this context, c-Myc-induced apoptosis can be viewed as an abortive pathway that may be activated by conflicting cellular signals, such as when excess cyclin A expression is induced in cells that lack extracellular mitogenic signals.

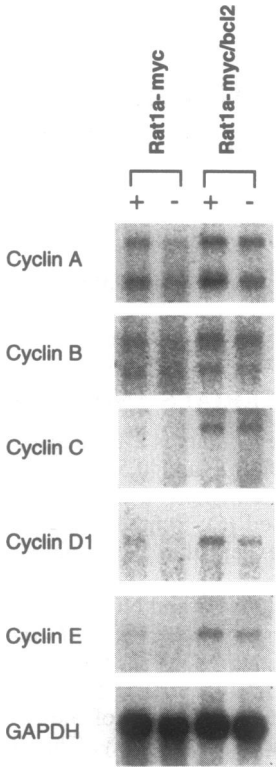


FIG. 4. Expression of cyclin A, B, C, D1, and E mRNA in Rat1a–myc and Rat1a–myc/bcl2. Setup and conditions are as described for Fig. 2 + and − denote exposure of cells to 10% and 0.1% FCS, respectively. Note that the Northern blots were done separately from those of Fig. 2, and film exposure times differ.

**Note Added in Proof.** After submission of our work, cyclin A expression was reported to be adhesion-dependent in NRK and NIH 3T3 fibroblasts (25). Our $Zn^{2+}$-inducible cyclin A-expressing cells display anchorage-independent growth in the presence of $Zn^{2+}$ (A.T.H. and C.V.D., unpublished observations). These observations suggest that Myc-induced cellular transformation (anchorage-independent growth) may be imparted by deregulated cyclin A expression.

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