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Bax Loss Impairs Myc-Induced Apoptosis and Circumvents the Selection of p53 Mutations during Myc-Mediated Lymphomagenesis

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The ARF and p53 tumor suppressors mediate Myc-induced apoptosis and suppress lymphoma development in Eμ-*myc* transgenic mice. Here we report that the proapoptotic Bcl-2 family member Bax also mediates apoptosis triggered by Myc and inhibits Myc-induced lymphomagenesis. *Bax*-deficient primary pre-B cells are resistant to the apoptotic effects of Myc, and *Bax* loss accelerates lymphoma development in Eμ-*myc* transgenics in a dose-dependent fashion. Eighty percent of lymphomas arising in wild-type Eμ-*myc* transgenics have alterations in the ARF-Mdm2-p53 tumor suppressor pathway characterized by deletions in *ARF*, mutations or deletions of *p53*, and overexpression of Mdm2. The absence of *Bax* did not alter the frequency of biallelic deletion of *ARF* in lymphomas arising in Eμ-*myc* transgenic mice or the rate of tumorigenesis in *ARF*-null mice. Furthermore, Mdm2 was overexpressed at the same frequency in lymphomas irrespective of *Bax* status, suggesting that Bax resides in a pathway separate from ARF and Mdm2. Strikingly, lymphomas from *Bax*-null Eμ-*myc* transgenics lacked *p53* alterations, whereas 27% of the tumors in $Bax^{+/-}$ Eμ-*myc* transgenic mice contained *p53* mutations or deletions. Thus, the loss of *Bax* eliminates the selection of *p53* mutations and deletions, but not ARF deletions or Mdm2 overexpression, during Myc-induced tumorigenesis, formally demonstrating that Myc-induced apoptotic signals through ARF/Mdm2 and p53 must bifurcate: p53 signals through Bax, whereas this is not necessarily the case for ARF and Mdm2.

The oncoprotein c-Myc, paradoxically, is an inducer of both cell proliferation and cell death, and the levels of Myc and/or the conditions in which it is expressed dictate cell fate (2, 7, 40). Most cancer cells that overexpress Myc, by translocation, amplification, or other means, harness the full growth potential of this oncogene by inactivating the apoptotic effectors of Myc, including the tumor suppressors ARF and p53 (5, 50). ARF is a nucleolar protein that binds to and sequesters Mdm2 (55, 59). Mdm2 is a p53 transcription target (3, 61) that inhibits p53's transactivation functions (37) and ubiquitinates p53 (12), leading to p53 degradation (48). Myc activation induces the sustained expression of both ARF and p53, and this triggers apoptosis; as a consequence, primary *ARF*- and *p53*-null hematopoietic and fibroblast cells are impaired in their apoptotic response to Myc (5, 63). Furthermore, deletion of *ARF*, mutation or deletion of *p53*, and Mdm2 overexpression occur in 24, 28, and 48%, respectively, of the lymphomas that arise in Eμ-*myc* transgenic mice (80% overall [5]), and *ARF*- or *p53*-null Eμ-*myc* transgenic mice have a markedly accelerated course of lymphoma (5, 17, 50).

Loss of the antiapoptotic protein Bcl-X_L or Bcl-2 compromises hematopoietic cell survival, whereas loss of ARF or p53 has no effect upon hematopoietic cell development (6, 38, 39, 41, 57). Bax is a proapoptotic Bcl-2 family member whose deletion has modest effects on lymphocyte numbers (22). However, the combined loss of Bax and Bak, another proapoptotic Bcl-2 family member, results in profound defects in both development and lymphocyte homeostasis (24). Bax normally resides in the cytosol of healthy cells, yet it relocalizes and inserts into the outer mitochondrial membrane after stimulation with a variety of apoptotic stimuli (reviewed in reference 9). In turn, this leads to mitochondrial dysfunction with alterations in the permeability transition pore, the release of cytochrome *c*, and the activation of Apaf-1 and caspases, which cleave intracellular targets required for cell survival (9). The balance of proapoptotic and antiapoptotic Bcl-2 family members regulates the susceptibility of cells to apoptosis (reviewed in reference 23). For example, an excess of Bax can overwhelm the cell and trigger an apoptotic response, whereas the antiapoptotic Bcl-2 family members Bcl-2 and Bcl-X_L inhibit the deleterious effects of Bax (23).

Bcl-2 and Bcl-X_L are overexpressed in many human malignancies (reviewed in reference 46), and Bcl-X_L expression is activated by retroviral insertions in some murine T-cell leukemias and lymphomas (41). Bcl-2 and Myc have been shown to cooperate in transformation (8, 56), and Eμ-*myc*/Eμ-*bcl-2* double transgenic mice develop an aggressive and rapid lymphoma composed of primitive lymphoid cells (54). Although the cooperation between Bcl-2 and Myc and the regulation of Bax by Bcl-2 are well documented, the precise role that Bax plays in Myc functions is less clear.

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Bax-deficient mice manifest a modest lymphoid hyperplasia but are not prone to spontaneous tumor development (20, 22). However, mutations that inactivate *Bax* are found in a subset of human colon adenocarcinomas (45) and some human hematopoietic cancer cell lines (4, 30, 31), and *Bax* loss cooperates with simian immunodeficiency virus (SV40) large T antigen in transgenic mouse models of cancer (52, 62). Recently, *bax* has been suggested to be a direct transcriptional target of c-Myc in human tumor cell lines (32). However, it is unclear how Bax influences Myc-induced hematopoietic cell apoptosis and tumorigenesis and whether Bax expression influences the ARF-Mdm2-p53 tumor suppressor pathway. Here we report that, although Myc activation fails to regulate Bax levels in primary murine pre-B cells, *Bax*-deficient cells are markedly resistant to Myc-induced apoptosis. More importantly, *Bax* loss accelerates Myc-induced tumorigenesis in Eμ-*myc* transgenic mice, and the lymphomas arising in *Bax*-null transgenics selectively lack mutations or deletions of *p53*. However, *Bax*-null Eμ-*myc* transgenics still display the same frequency of alterations of ARF and Mdm2, indicating that Bax is not necessarily a target of ARF or Mdm2 even though both can function with p53 in this tumor suppressor pathway. The results support a model whereby Bax functions as a critical downstream effector of the p53 apoptotic pathway, and thus ARF and Mdm2 must have other mediators important for tumorigenesis.

MATERIALS AND METHODS

Transgenic and knockout mice. The inbred C57BL/6 Eμ-*myc* transgenic mouse strain was kindly provided by Alan Harris (Walter & Eliza Hall Institute, Melbourne) and Charles Sidman (University of Cincinnati). *Bax*-null mice have been previously described and were C57BL/6 × 129/svj (22). The *ARF*-null (C57BL/6 × 129/svj) and p53-null (C57BL/6 × 129/svev) mice were generously provided by Charles Sherr and Gerard Grosveld, respectively. Eμ-*myc* transgenics were mated to $Bax^{+/-}$ mice and the F_1 littermates were then mated to each other to obtain $Bax^{+/+}$, $Bax^{+/-}$, and $Bax^{-/-}$ Eμ-*myc* transgenics.

Primary B cells. Primary pre-B cell cultures were generated from the bone marrow of 6- to 8-week-old wild-type, *Bax*-, *ARF*-, *p53*-, and *ARF*/*p53*-double null mice as previously described (5). Briefly, culture of bone marrow in interleukin-7 (IL-7)-containing medium after 12 to 14 days established >98% pure population of pre-B cells as determined by phenotype analysis using B-cell-specific antibodies and fluorescence-activated cell sorting (FACS). The pre-B cells expressed CD19, B220, and CD24 and were negative for surface immunoglobulin M (IgM) and CD43 irrespective of genotype. $IgM^+/CD19^+$ B cells were sorted from spleens from age- and gender-matched mice: one wild-type and two precancerous Eμ-*myc* transgenics. All antibodies used for phenotypic analyses were from PharMingen (San Diego, Calif.) or Southern Biotechnology (Birmingham, Ala.).

Virus infection. Virus was produced and used to infect primary pre-B cells as previously described (5). Briefly, MSCV-Myc-ER-IRES-GFP or control MSCV-IRES-GFP virus was cotransfected with helper virus into 293T cells, and live virus was then collected at intervals, pooled, and filtered (49). Viral stocks, MSCV-Myc-ER-IRES-GFP virus or the MSCV-IRES-GFP control virus, were used to infect primary pre-B cells in the presence of 8 μg of Polybrene/ml. Green fluorescent protein (GFP)-positive infected cells were isolated 3 to 4 days postinfection by sterile sorting with a Cytomation MoFlo cell sorter (Fort Collins, Colo.). GFP-positive cells were expanded in IL-7-containing medium and analyzed for levels of Myc-ER (previously referred to as Myc-ER™ [5, 6]) protein and sensitivity to Myc-induced apoptosis. Myc-ER is a fusion protein of c-Myc linked to a modified estrogen receptor hormone binding domain (25) and is designed to hold Myc-ER in heat shock complexes in the cytosol. Upon addition of 1 μM 4-hydroxytamoxifen (4-HT), which binds to the ER portion of Myc-ER, Myc-ER then translocates to the nucleus and activates transcription (25). As reported elsewhere (5), addition of 4-HT to uninfected or MSCV-IRES-GFP control virus-infected cells had no effect on pre-B cell growth or viability.

Viability and apoptosis assays. Cell viability was determined at specific intervals by trypan blue dye exclusion after the removal of IL-7 or the addition of 1 μM 4-HT (Sigma, St. Louis, Mo.) to the culture medium to activate Myc-ER. For the IL-7 deprivation experiments, wild-type and $Bax^{-/-}$ pre-B cells were washed twice with phosphate-buffered saline and resuspended in medium lacking IL-7 but still containing 10% fetal calf serum. Apoptosis was measured by propidium iodide staining of DNA and quantitation of fragmented (sub-G_1) DNA.

Western blotting. Whole-cell protein extracts from primary pre-B cells or pre-B- or B-cell lymphomas from Eμ-*myc* transgenic mice were isolated as previously described (5, 63). Briefly, ice-cold lysis buffer (50 mM HEPES, pH 7.5; 150 mM NaCl; 1 mM EDTA; 2.5 mM EGTA; 0.1% Tween 20; 1 mM phenylmethylsulfonyl fluoride; 0.4 U of aprotinin/ml; 1 mM NaF; 10 mM β-glycerophosphate; 0.1 mM sodium orthovanadate; 10 μg of leupeptin/ml) was added to cells pellets or small (3- to 5-mm^2) tumor chunks. Samples were then subjected twice to sonication for 8 s and centrifuged (4°C, 7 min, 14,000 rpm) to sediment the undissolved cellular material, and then the protein in the supernatant was quantified by using a Bio-Rad Protein Assay (Hercules, Calif.). Equal amounts of protein (200 μg per lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10%), transferred to nitrocellulose membranes (Protran; Schleicher & Schuell, Dassel, Germany), and blotted with antibodies specific for the $p19^{ARF}$ (44), p53 (Ab-7), and poly(ADP-ribose) polymerase (PARP; Ab-2) (both from, Calbiochem, La Jolla, Calif.), Mdm2 (C-18; Santa Cruz, Inc., Santa Cruz, Calif.), c-Myc (06–340; Upstate Biotechnology, New York, N.Y.), and Bax (13686E; PharMingen, San Diego, Calif.). Bound immunocomplexes were detected by enhanced chemiluminescence (Amersham, Piscataway, N.J.) or Supersignal (Pierce, Rockford, Ill.).

Southern blotting. Genomic DNA was isolated from lymphomas arising in $Bax^{+/-}$ and $Bax^{-/-}$ Eμ-*myc* transgenic mice and digested with *Afl*II or *Bam*HI. Equal amounts of DNA were electrophoretically separated in agarose gels, transferred to nitrocellulose membranes, and then probed with cDNAs coding for *ARF* (exon 1β) (*Afl*II digested) and *p53* (exons 2 to 10) and the joining region of immunoglobulin heavy chain (J_H) (both *Bam*HI digested). Genomic DNA isolated from the spleen of a wild-type littermate was used as a control.

Northern blotting. Total RNA was isolated by using TRIzol Reagent according to the manufacturer's directions (Life Technologies, Grand Island, N.Y.) at intervals (0, 1, 3, or 6 h) from primary pre-B cells after addition of 1 μM 4-HT after a 30-min pretreatment with 10 μg of cycloheximide or vehicle control (100% ethyl alcohol)/ml. Northern blotting with 20 μg of total RNA per lane was performed using conventional techniques and probed with the coding portion of murine *bax* cDNA (kindly provided by John Reed, The Burnham Institute).

RESULTS

Bax loss impairs Myc-induced apoptosis. *Bax*-null mice have modest increases in B- and T-lymphocyte numbers, presumably due to decreased apoptosis (22). To determine whether *Bax*-deficient B lymphocytes were intrinsically more resistant to apoptosis than wild-type lymphocytes, we harvested bone marrow cells and expanded pre-B cells in IL-7-containing medium and measured proliferation rates and viability. The *Bax*-null pre-B cells grew at a rate similar to wild-type pre-B cells (data not shown) and showed no difference in viability (Fig. 1A, open symbols). Moreover, deprivation of IL-7 induced apoptosis in *Bax*-null pre-B cells with kinetics virtually identical to that of wild-type pre-B cells (Fig. 1A, closed symbols). Similar results were obtained when wild-type and $Bax^{-/-}$ thymocytes were deprived of cytokines (22). Therefore, Bax deficiency does not confer a growth or survival advantage to lymphocytes during ex vivo culture.

To address whether Bax influences Myc-induced apoptosis, we infected primary wild-type and *Bax*-deficient pre-B cells with a Myc-ER-GFP (previously referred to as Myc-ER™-GFP [5, 6]) expressing retrovirus or with a control GFP-only expressing retrovirus and then sorted for virus-infected cells by FACS. There was a modest decrease in the viability in wild-type pre-B cells infected with the Myc-ER encoding retrovirus compared to *Bax*-deficient pre-B cells infected with the same virus (Fig. 1B). This is most likely due to the somewhat leaky nature of Myc-ER expression system (5, 63). Activation of Myc-ER with 4-HT led to rapid apoptosis in the wild-type

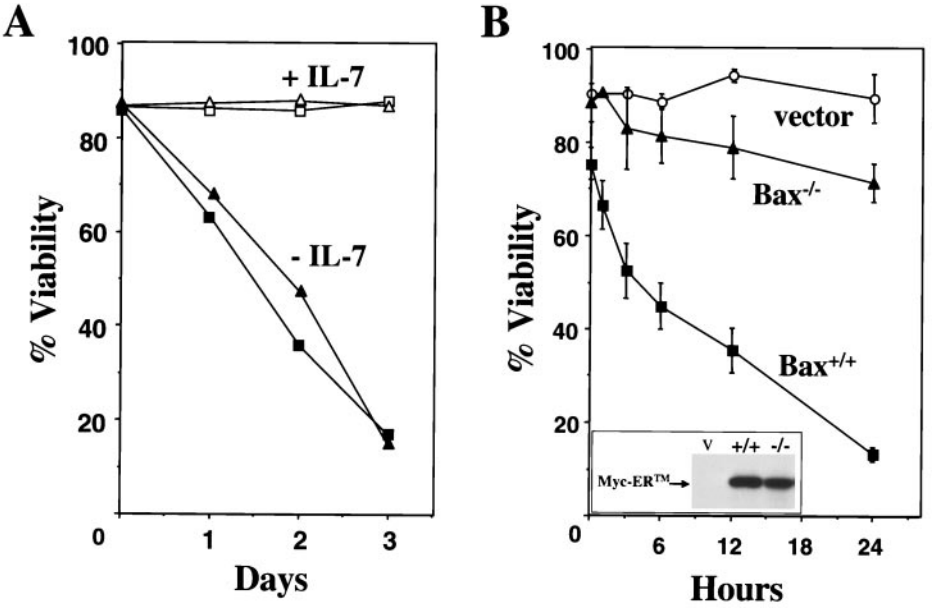

FIG. 1. Bax does not influence cytokine deprivation-induced apoptosis but does mediate c-Myc-induced apoptosis. (A) $Bax^{+/+}$ (squares) and $Bax^{-/-}$ (triangles) pre-B cells were cultured in medium with (open symbols) or without (solid symbols) IL-7, and their viability was determined at intervals by trypan blue dye exclusion. The data are representative of two independent experiments. (B) 4-HT was added to the indicated primary pre-B-cell cultures to activate Myc-ER, and their viability was determined at intervals thereafter by trypan blue dye exclusion. Apoptosis was confirmed by analysis of subdiploid DNA content after staining with propidium iodide. Steady-state levels of apoptosis in the wild-type primary pre-B cells are indicated at the zero hour time point. (Inset) The protein levels of Myc-ER in $Bax^{-/-}$ (−/−) and $Bax^{+/+}$ (+/+) pre-B cells infected with a retrovirus encoding Myc-ER-GFP and pre-B cells infected with the GFP vector control (V) retrovirus were determined by immunoblotting with an antibody for Myc. The data are the mean of three independent experiments, and error bars represent one standard deviation.

pre-B cells cultured in IL-7-containing medium, whereas the *Bax*-null cells were very resistant to Myc-induced apoptosis (Fig. 1B). Few wild-type pre-B cells (<10%) were alive after 24 h of Myc activation, whereas *Bax*-null pre-B cells were greater than 60% viable at this interval. Primary pre-B cells died by apoptosis upon Myc-ER activation, as determined by DNA fragmentation analysis (data not shown) and cleavage of caspase targets such as PARP (Fig. 2A). Thus, Bax is an important mediator of Myc-induced apoptosis in primary pre-B cells.

bax has been suggested to be a direct transcriptional target of c-Myc in immortal human tumor cell lines (32). However, Myc activation in primary pre-B cells was not associated with changes in Bax protein (Fig. 2A) whereas, as expected (5), p53 expression was increased upon Myc activation. Moreover, Bax levels were unaltered in IgM^+ splenic B cells from precancerous Eμ-*myc* transgenic mice compared to wild-type littermate controls (Fig. 2B). Therefore, Myc activation or overexpression does not alter Bax protein levels in B cells ex vivo or in vivo. Only a very slight increase in *bax* RNA levels was observed following Myc activation in pre-B cells, and this was prevented by pretreatment of the cells with cycloheximide (Fig. 2C). Since cycloheximide blocks new protein synthesis, the modest changes in *bax* RNA induced by Myc activation are indirect. Furthermore, the modest upregulation of *bax* RNA by Myc did not result in any increase in Bax protein levels (Fig. 2A).

Immortal cell lines, such as those used by Mitchell et al. (32), generally inactivate *p53* or *ARF* to become established in vitro (11, 19), and *bax* has been reported to be a transcriptional target of p53 (34, 35). Therefore, it was possible that the loss of *p53* and/or *ARF* influences Bax expression independent of Myc and/or could alter the response of Bax to Myc activation. To address this issue, primary pre-B cells lacking *p53* and/or *ARF* were infected with the Myc-ER encoding retrovirus. Loss of *ARF* and/or *p53* did not significantly alter the steady-state levels of Bax protein (Fig. 2D). Moreover, activation of Myc-ER with 4-HT did not result in alteration of Bax protein levels in *ARF*-, *p53*-, or *ARF*/*p53*-double null primary pre-B cells (Fig. 2D). Therefore, loss of p53 and/or ARF does not influence Bax expression, even when Myc is activated.

Bax loss accelerates Myc-induced lymphomagenesis. Loss of ARF or p53 impairs Myc-mediated apoptosis and consequently accelerates Myc-induced lymphomagenesis (5, 50, 63). To test the genetic contribution of Bax to Myc-induced lymphomagenesis, we crossed Eμ-*myc* transgenic mice onto the *Bax*-null background. Congenic C57BL/6 Eμ-*myc* transgenic mice were mated to C57BL/6 × 129/svj $Bax^{+/-}$ mice, since $Bax^{-/-}$ males and females have fertility problems (22, 43). F_1 littermates were intercrossed to obtain $Bax^{+/+}$, $Bax^{+/-}$, and $Bax^{-/-}$ Eμ-*myc* transgenic mice, and these transgenics were carefully monitored for disease development. Notably, *Bax* loss accelerated the course of lymphoma development, decreasing the average age of survival from 21.7 weeks in $Bax^{+/+}$ Eμ-*myc* transgenics to 12.6 weeks in *Bax*-null Eμ-*myc* transgenics (Fig. 3). None of the $Bax^{-/-}$ Eμ-*myc* transgenic mice survived past 30 weeks, whereas 22% (12 of 54) of the $Bax^{+/+}$ Eμ-*myc* transgenics lived longer than 30 weeks. *Bax* haploinsufficency also accelerated tumor development, since $Bax^{+/-}$ Eμ-*myc* transgenic mice had a mean life span of 16.0 weeks (Fig. 3).

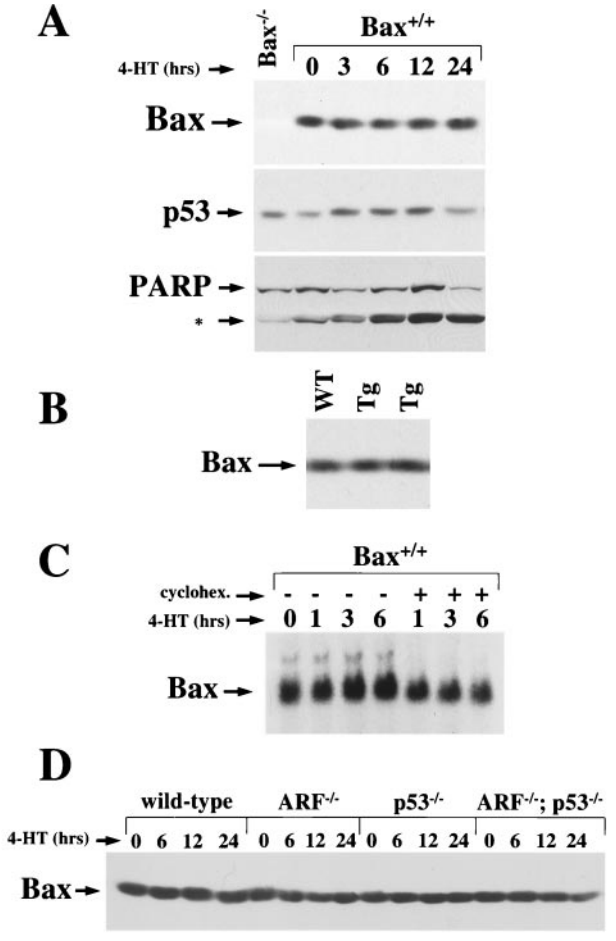

FIG. 2. Myc does not upregulate Bax protein expression in B cells. (A) 4-HT was added to wild-type pre-B-cell cultures infected with the Myc-ER encoding retrovirus to activate Myc-ER. At the indicated intervals cells were collected and protein lysates were made. Equal quantities of protein were assessed by immunoblotting with antibodies specific for Bax, p53, or PARP. The 85- kDa caspase cleavage fragment of PARP is denoted by an asterisk. (B) Equal quantities of protein from FACS sorted $IgM^+/CD19^+$ splenic B cells from one wild-type (WT) and two precancerous Eμ-*myc* transgenics (Tg) were assessed by immunoblotting with an antibody specific for Bax. (C) Total RNA was isolated from Myc-ER-infected wild-type pre-B cells activated with 4-HT for the indicated intervals. Pre-B cells pretreated with cycloheximide are indicated by a plus sign. The expression of *bax* transcripts was assessed by Northern blot analyses utilizing *bax* cDNA. (D) 4-HT was added to wild-type, $ARF^{-/-}$, $p53^{-/-}$, and $ARF^{-/-}p53^{-/-}$ pre-B-cell cultures infected with the Myc-ER-encoding retrovirus to activate Myc-ER. At the indicated intervals, cells were collected and protein lysates were prepared. Equal quantities of protein were evident by immunoblotting with antibodies specific for Bax, and the levels of Myc-ER protein expressed in the four genotypes were equivalent (5).

Therefore, Bax impairs Myc-induced lymphomagenesis. FACS analysis with lymphoid-specific antibodies showed that the *Bax*-null and $Bax^{+/-}$ Eμ-*myc* transgenics develop pre-B- and B-cell lymphoma typical of wild-type Eμ-*myc* transgenics (data not shown) and not the primitive lymphoid tumor described in the Eμ-*myc*/Eμ-*bcl-2* double-transgenic mice (54). Predictably, Southern blot analysis revealed that all but one of the lymphomas that arose in *Bax*-null Eμ-*myc* transgenics were clonal (data not shown), which is characteristic of the lymphomas that develop in Eμ-*myc* transgenics (1).

Inactivation of *p53* or *ARF* occurs in a mutually exclusive fashion in over half of all Eμ-*myc* lymphomas (5). Bax appears to function as a tumor suppressor in some scenarios (45), although *Bax*-null mice do not spontaneously develop cancer (20, 22). If Bax functioned as a bona fide tumor suppressor in Eμ-*myc* transgenic mice, one would predict that *Bax* would be inactivated in a subset of lymphomas and that lymphomas arising in $Bax^{+/-}$ Eμ-*myc* transgenics would suffer inactivating mutations of the remaining wild-type *Bax* allele. However, we failed to detect loss of heterozygosity of *Bax* in the tumors analyzed from $Bax^{+/-}$ Eμ-*myc* transgenics ($n = 15$), nor did we observe deletion of *Bax* in any lymphomas from $Bax^{+/+}$ Eμ-*myc* transgenics ($n > 50$) (data not shown). Furthermore, all tumors derived from wild-type and $Bax^{+/-}$ Eμ-*myc* transgenics expressed Bax protein (Fig. 4A and data not shown). In previous studies *Bax* has been shown to be inactivated in tumor cells by frameshift and missense point mutations (4, 30, 31, 45). However, sequencing full-length *Bax* cDNA derived from 16 tumors from $Bax^{+/+}$ and $Bax^{+/-}$ Eμ-*myc* transgenics failed to reveal any tumor-specific changes in *Bax* sequence. All tissue derived from these mice displayed a single nucleotide change (213T to C) in the BH3 domain of Bax, yet this change is silent and maintains aspartate at codon 71. Thus, inactivating mutations of *Bax* either do not occur or are rare in pre-B- and B-cell lymphomas arising in Eμ-*myc* transgenics, even though Eμ-*myc* transgenics that lack *Bax* develop lymphomas at an accelerated rate. Therefore, Bax does not function as a classic tumor suppressor but rather appears to act as a modifier of lymphoma development in Eμ-*myc* transgenic mice.

Bax loss selectively bypasses p53 mutations that arise during Myc-induced lymphomagenesis. *ARF*-null animals are tumor prone and spontaneously develop sarcomas and lymphomas within 8 months of age (19). ARF is upregulated after Myc activation, and loss of *ARF* impairs Myc-induced apoptosis and accelerates lymphomagenesis in Eμ-*myc* transgenic mice (5, 50). To determine whether the loss of *Bax* influences tumorigenesis initiated in *ARF*-deficient mice, we crossed *ARF*- and *Bax*-deficient mice, and F_1 mice were then mated to generate *ARF*/*Bax*-double null mice. The $ARF^{-/-}Bax^{-/-}$ mice were monitored for tumor development and compared with the tumor latency in $ARF^{-/-}Bax^{+/+}$ littermates. We found that Bax deficiency does not alter the survival of *ARF*-null mice, since the average age of survival for $Bax^{+/+}$ $ARF^{-/-}$ (48.9 weeks, $n = 37$) and $Bax^{-/-}$ $ARF^{-/-}$ (47.6 weeks, $n = 39$) mice was essentially equivalent. Therefore, loss of Bax does not influence the overall survival of *ARF*-null mice. At face value this could suggest that Bax is in the same pathway as ARF. On the other hand, the results could indicate that ARF and Bax exist in separate pathways that function independently of each other. In support of the latter concept, biallelic deletion of *ARF* occurred in lymphomas from both $Bax^{-/-}$ Eμ-*myc* transgenics (12%) and $Bax^{+/-}$ Eμ-*myc* transgenic mice (20%) (Fig. 5; Table 1), indicating that *Bax* loss does not prevent inactivation of ARF. The percentage of tumors with *ARF* deletions was slightly lower than previously reported (5), but this could be due to experimental variation or (more likely) to the different background of this cross from the one previously reported. Indeed, genetic background effects are evident when the dif-

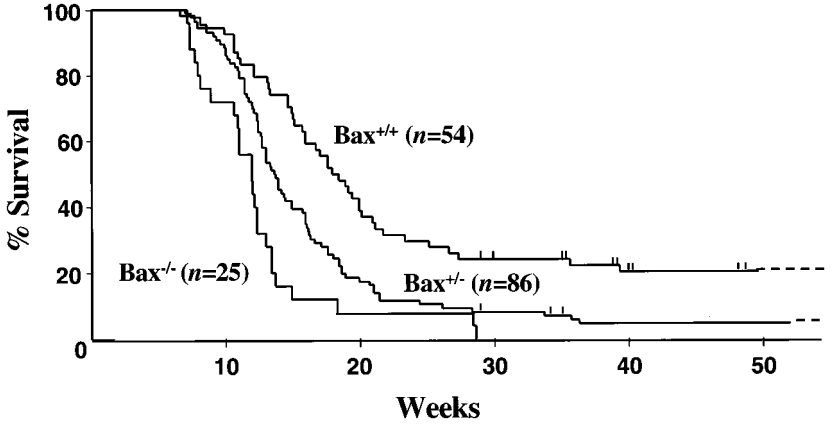

FIG. 3. Myc-induced lymphomagenesis is accelerated by *Bax* loss. The genotypes of the mice are indicated next to the Kaplan-Meier survival curves, and the numbers of mice in each group are denoted by the *n* values. Vertical lines indicate ages of surviving mice: 0 $Bax^{-/-}$, 3 $Bax^{+/-}$, and 10 $Bax^{+/+}$ mice. The average life spans of $Bax^{+/+}$, $Bax^{+/-}$, and $Bax^{-/-}$ Eμ-*myc* transgenics were 21.7, 16.0, and 12.6 weeks, respectively. Pre-B- and/or B-cell lymphoma was documented in all of the animals.

ferences in average survival of wild-type Eμ-*myc* transgenics, 22 weeks in the *Bax* background (Fig. 3) and 33 weeks in the *ARF* background, are compared (5).

The frequency of *p53* alterations in $Bax^{+/-}$ Eμ-*myc* transgenics was similar to that reported for wild-type Eμ-*myc* transgenic mice (5), since approximately one-quarter of the lymphomas arising in $Bax^{+/-}$ Eμ-*myc* transgenics sustained mutations or deletions in *p53* (Fig. 4B and 5A; Table 1). As a consequence of *p53* mutations, these tumors (CM201, CM271, and CM696) displayed high levels of p53 protein and concomitant increases in ARF protein (Fig. 4B and data not shown), presumably due to the loss of feedback control of ARF expression by p53 (47). One tumor (CM622) from a $Bax^{+/-}$ Eμ-*myc* transgenic had deleted both alleles of *p53*, as determined by Southern blot analysis (Fig. 5A). Strikingly, not a single lymphoma arising in *Bax*-null Eμ-*myc* transgenics contained mutant *p53*, nor was *p53* deleted in any of these tumors (Fig. 4B and 5B; Table 1). Therefore, *Bax* loss selectively circumvents the requirement for *p53* mutations and deletions during Myc-induced tumorigenesis but does not alter the frequency of alterations in *ARF*.

Normally, Mdm2 protein is expressed at very low levels. However, half of all lymphomas arising in Eμ-*myc* transgenics overexpress Mdm2 protein, and this also occurs in tumors

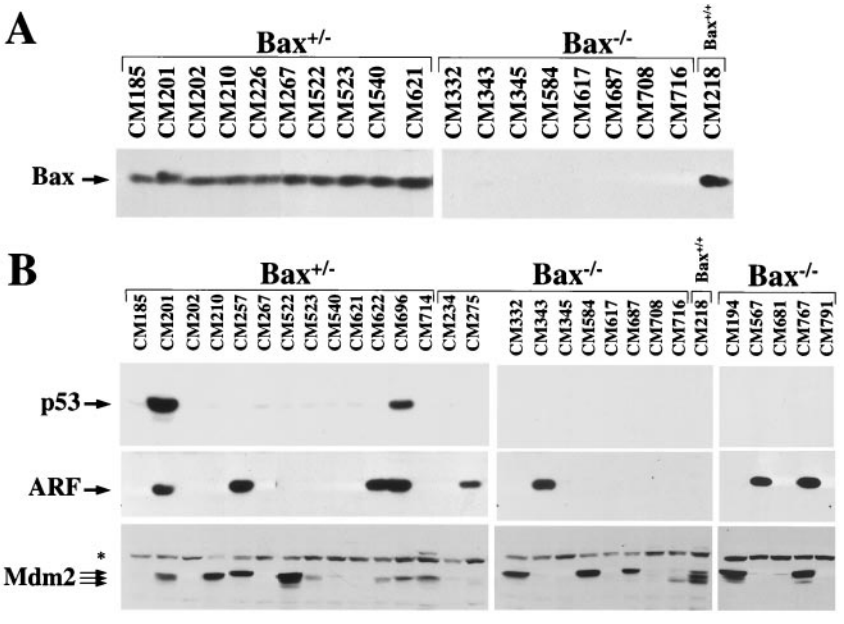

FIG. 4. Western blot analysis of lymphomas arising in $Bax^{+/-}$ and $Bax^{-/-}$ Eμ-myc transgenic mice. Levels of Bax (A), p53 (B, top), $p19^{ARF}$ (B, middle), and Mdm2 (B, bottom) protein in whole-cell extracts of tumors from $Bax^{+/-}$ and $Bax^{-/-}$ Eμ-*myc* transgenic mice were assessed by immunoblotting with antibodies specific for each protein. Protein extracts from a tumor arising in a $Bax^{+/+}$ Eμ-*myc* transgenic mouse that contains the p92, p90, and p85 Mdm2 isoforms were run to show the location of these isoforms in panel B and as a blotting control for Bax expression in panel A. The asterisk in panel B marks the position of a nonspecific background band detected with the Mdm2 antibody.

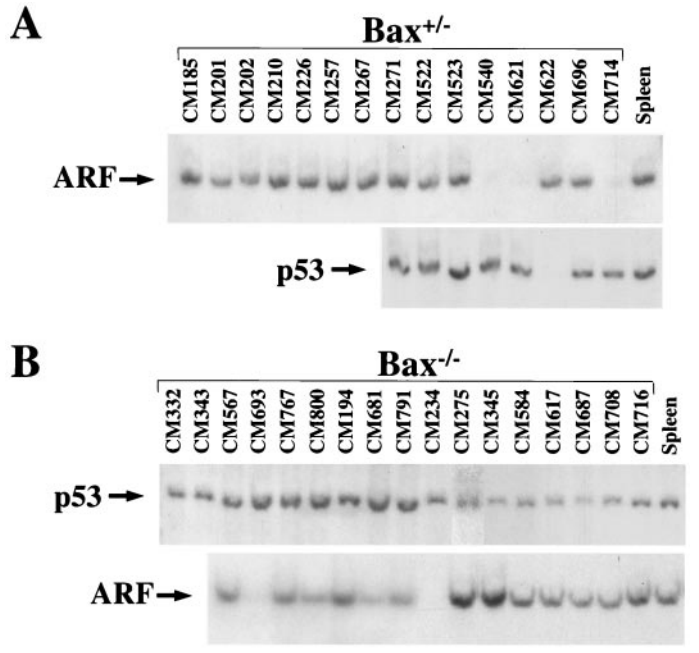

FIG. 5. Southern blot analysis of $Bax^{+/-}$ and $Bax^{-/-}$ Eμ-myc lymphomas. *Afl*II and *Bam*HI restriction fragments containing *ARF* exon 1β and *p53* exons 2 to 10, respectively, from genomic DNA isolated from lymphomas arising in $Bax^{+/-}$ (A) and $Bax^{-/-}$ (B) Eμ-*myc* transgenic mice. Genomic DNA from the spleen of a wild-type littermate was used as a control in both panels A and B. Lack of a band denotes biallelic deletion of that gene.

bearing deletions of *ARF* or mutations or deletions of *p53* (5). Mdm2 was also overexpressed in approximately half (56%) of all lymphomas arising in *Bax*-null and $Bax^{+/-}$ Eμ-*myc* transgenics, regardless of their *ARF* or *p53* status (Fig. 4B and Table 1). The frequency of Mdm2 overexpression in tumors lacking alterations in *ARF* or *p53* was somewhat higher in $Bax^{-/-}$ (35%) and $Bax^{+/-}$ (27%) Eμ-*myc* transgenics (Table 1) compared to lymphomas arising in wild-type (16%) Eμ-*myc* transgenic mice (5). This difference may be due to the lack of p53 alterations in *Bax*-deficient Eμ-*myc* transgenic mice. Overall, lymphomas from $Bax^{-/-}$ Eμ-*myc* transgenics showed a frequency of alterations in the ARF-Mdm2-p53 pathway (71%) similar to that of $Bax^{+/-}$ (80%) (Table 1) and wild-type (80%) (5) Eμ-*myc* transgenics. Therefore, *Bax* loss selectively eliminates the requirement for *p53* mutations or deletions during lymphomagensis, without significantly influencing the frequency of alterations in *ARF* and Mdm2 (Fig. 6).

DISCUSSION

B cells lacking mediators of Myc-induced apoptosis have an accelerated course of lymphoma development in Eμ-*myc* transgenic mice (5, 17, 50). For example, deleting *ARF* or *p53*, both mediators of Myc-induced apoptosis, accelerates lymphomagenesis in Eμ-*myc* transgenic mice (5, 50). Here, by using in vivo models, we extend these observations to the proapoptotic Bcl-2 family member Bax and show that intersecting apoptotic pathways play a crucial role in Myc-induced lymphomagenesis. Loss of *Bax* in pre-B cells confers resistance to Myc-induced apoptosis and accelerates pre-B- and B-cell lymphoma development in Eμ-*myc* transgenic mice. This is consistent with the finding that *Bax*-null mouse embryo fibroblasts (MEFs) are more resistant to Myc-induced apoptosis (32). Therefore, Bax is a mediator of Myc-induced apoptosis and inhibits Myc-initiated tumorigenesis.

The balance of proapoptotic and antiapoptotic Bcl-2 family members regulates the susceptibility of cells to apoptosis (reviewed in reference 23). An excess of Bax induces cell death, whereas overexpression of Bcl-2 or Bcl-X_L suppresses apoptosis induced by a variety of apoptotic stimuli. In immortal human cells *bax* has been reported to be a transcriptional target of both p53 (34, 35) and Myc (32), yet we failed to detect any direct or significant increase in Bax expression upon Myc activation in primary murine pre-B cells. Nevertheless, activation of p53 can induce *bax* and suppress *bcl-2* expression in certain cell types (33–35). Myc, on the other hand, upregulates p53 and ARF (5, 63) and suppresses Bcl-X_L expression, and the latter is independent of either p53 or ARF in primary murine hematopoietic cells (6). Thus, Myc activation alone is sufficient to alter the ratio of pro- and antiapoptotic Bcl-2 family members with the net result being an excess of Bax, which leads to apoptosis. Bax loss short-circuits this response and confers survival to cells overexpressing Myc.

p53 is a mediator of Myc-induced apoptosis (5, 63), and Bax plays an important role in p53-dependent apoptosis in some cell types in vitro (28, 35) and in vivo (52, 62). This study confirms and extends these results by linking Myc with p53 and Bax in vivo. Lymphomas arising in *Bax*-deficient Eμ-*myc* transgenic mice lack *p53* mutations and deletions, whereas 28% of tumors from wild-type Eμ-*myc* transgenics (5) and 27% of lymphomas from $Bax^{+/-}$ Eμ-*myc* transgenic mice sustain mutations or deletions in *p53*. Therefore, under selective pressure from Myc, *Bax*-deficient B cells differ from their wild-type counterparts by sustaining wild-type *p53* expression. These re-

TABLE 1. p53, ARF, and Mdm2 protein expression in lymphomas from $Bax^{+/-}$ and $Bax^{-/-}$ Eμ-*myc* transgenics

Eμ-*myc* transgenic (no. of tumors analyzed)	Finding (% tumors analyzed)	Mouse	Expression of:		
			p53	p19ARF	Mdm2
Bax$^{+/-}$ (15)	ARF inactivation (20)				
		CM540	Basal	Undetected (deleted)	Low
		CM621	Basal	Undetected (deleted)	Undetected
		CM714	Low	Undetected (deleted)	Moderately overexpressed
	p53 inactivation (26)				
		CM201	Mutant	Overexpressed	Overexpressed
		CM271	Mutant	Overexpressed	Overexpressed
		CM622	Deleted	Overexpressed	Moderately overexpressed
		CM696	Mutant	Overexpressed	Moderately overexpressed
	ARF overexpressed, p53 wild type (7)				
		CM257	Low	Overexpressed	Overexpressed
	Mdm2 overexpression only (27)				
		CM210	Basal	Undetected	Overexpressed
		CM226	Low	Undetected	Moderately overexpressed
		CM522	Basal	Undetected	Overexpressed
		CM523	Basal	Undetected	Moderately overexpressed
	No detectable alteration (20)				
		CM185	Basal	Undetected	Undetected
		CM202	Low	Undetected	Undetected
		CM267	Basal	Undetected	Undetected
Bax$^{-/-}$ (17)	ARF inactivation (12)				
		CM234	Basal	Undetected (deleted)	Low
		CM693	Basal	Undetected (deleted)	Overexpressed
	p53 inactivation (0)				
	ARF overexpressed, p53 wild type (24)				
		CM275	Low	Overexpressed	Low
		CM343	Low	Overexpressed	Undetected
		CM567	Low	Overexpressed	Low
		CM767	Low	Overexpressed	Overexpressed
	Mdm2 overexpression only (35)				
		CM194	Basal	Undetected	Overexpressed
		CM332	Basal	Undetected	Overexpressed
		CM584	Basal	Undetected	Overexpressed
		CM687	Basal	Undetected	Overexpressed
		CM716	Basal	Undetected	Moderately overexpressed
		CM800	Basal	Undetected	Moderately overexpressed
	No detectable alteration (29)				
		CM345	Basal	Undetected	Undetected
		CM617	Basal	Undetected	Undetected
		CM681	Basal	Undetected	Low
		CM708	Basal	Undetected	Undetected
		CM791	Basal	Undetected	Undetected

sults imply that during transformation mutation of *p53* is unnecessary when *Bax* is absent, supporting the observations that Bax is downstream from p53 (10). This is consistent with the observation that there is no cooperative effect on the rate of tumorigenesis in mice lacking both *p53* and *Bax* compared to mice deficient in *p53* alone (20). Moreover, when the statuses of *ARF* and *p53* were analyzed in lymphomas that arise in Eμ-*myc* transgenic mice, biallelic deletion of *ARF* or *p53* deletion or mutation occurred in a mutually exclusive fashion (5). Thus, although ARF and p53 can function in the same tumor suppressor pathway, our results demonstrate that this pathway must bifurcate, since only Bax and p53 are in the same Myc-induced pathway (Fig. 6). Indeed, this study provides formal genetic proof that ARF, Mdm2, and p53 have different targets.

One of the most intriguing outcomes of these studies is the finding that that *Bax* loss influences *p53* status independent of ARF (Fig. 6). *Bax* deficiency did not affect the frequency of *ARF* deletions in Eμ-*myc* lymphomas nor tumor latency in

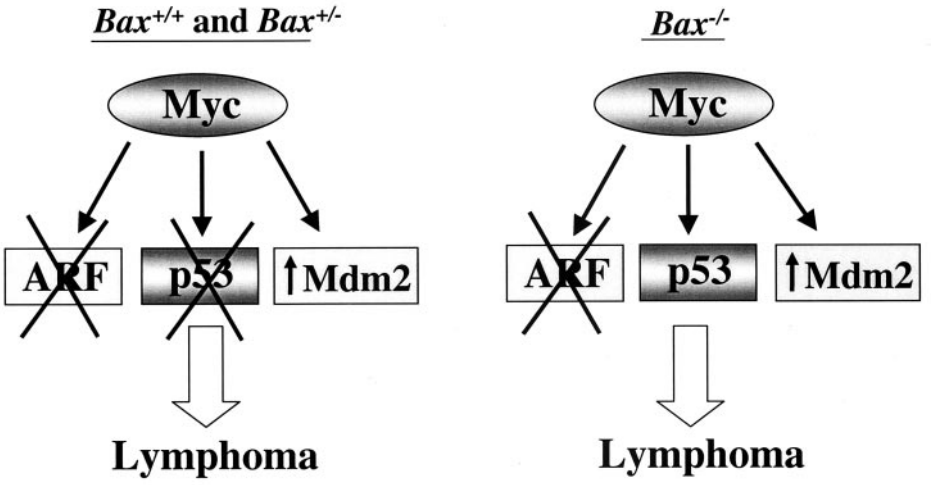

FIG. 6. Schematic describing the outcome of molecular events that occur in lymphomas in $Bax^{+/+}$, $Bax^{+/-}$, and $Bax^{-/-}$ Eμ-*myc* transgenic mice. On the left, the majority of lymphomas that arise in $Bax^{+/+}$ and $Bax^{+/-}$ Eμ-*myc* transgenic mice have alterations in *ARF* (deletion, X) or *p53* (mutation or deletion, X) and/or Mdm2 (overexpression, ↑) (Fig. 4 and 5 and Table 1; see also reference 5). On right, a similar frequency of alterations in *ARF* and Mdm2 still occurs in lymphomas from *Bax*-null Eμ-*myc* transgenic mice; however, no *p53* mutations or deletions are observed in these lymphomas (Fig. 4 and 5, Table 1). Therefore, Myc targets ARF and Mdm2 but not p53 in the absence of *Bax* during Myc-induced lymphomagenesis.

ARF-null mice, suggesting that Bax and ARF are not in the same pathway, or that Bax resides in a position in the pathway that does not influence ARF. However, further analysis has demonstrated that *Bax* loss does affect the tumor spectrum in *ARF*-null mice (C. M. Eischen and J. L. Cleveland, unpublished data). Thus, there is cooperativity at least at some level, and ARF and Bax must function in separate pathways.

Even though ARF and p53 function in the same tumor suppressor pathway, p53 has been reported to function independently of ARF in certain situations and vice versa. For example, gamma irradiation-induced apoptosis is p53 dependent and still occurs in *ARF*-null cells (19), and ARF can still induce cell cycle arrest in fibroblasts lacking *p53* and *Mdm2* (58). Our data from *Bax*-null Eμ-*myc* transgenic mice reveals a role for Bax that is dependent on p53 but independent of ARF. Myc thus affects the p53 pathway in two ways: by upstream activation through ARF and by downstream activation of Bax. However, the loss of *Bax* does seem to disrupt the ARF-Mdm2-p53 pathway by other means. The increased percentage of lymphomas in *Bax*-null Eμ-*myc* transgenics that overexpressed ARF (Table 1) suggests that Bax expression could influence the ARF-Mdm2-p53 pathway by somehow targeting proteins that regulate ARF expression, such as Bmi-1 (16), Dmp-1 (13), JunD (60), Tbx2 (15), and Twist (27). Alternatively, Bax status could disrupt the delicate feedback control mechanisms that regulate the expression of ARF, Mdm2, and p53 (47, 53).

Mdm2 is a negative regulator of p53, and we therefore predicted that, since the loss of *Bax* bypassed requirements for inactivating *p53*, then the frequency of Mdm2 overexpression would be decreased as well. Surprisingly, Mdm2 was overexpressed in 50% (16 of 32) of all of the lymphomas analyzed, as shown in wild-type Eμ-*myc* transgenics (5), regardless of *Bax* status. Mdm2 has many targets other than p53 (e.g., E2F-1, DP-1, p300, and pRb) (reviewed in reference 36) and appears to function independently of the p53 pathway in certain scenarios. For example, Mdm2 is overexpressed in a third of all lymphomas that had mutated or deleted *p53* (5), and haploinsufficiency of *Mdm2* alters the tumor spectrum in *p53*-null mice (29). Moreover, Mdm2 transgene overexpression resulted in altered mammary gland development (26) and tumorigenesis (18) in $p53^{-/-}$ mice. Therefore, selection for Mdm2 overexpression in Eμ-*myc* lymphomas is not influenced by Bax status and is also not necessarily linked to alterations in *p53* or *ARF* (Fig. 6).

Bax does not function as a classic tumor suppressor in Eμ-*myc*-induced lymphomas, as do ARF and p53 (reviewed in reference 51). However, Bax tumor suppressor function has been reported in some scenarios. First, a *Bax* frameshift mutation occurs in a subset of colon adenocarcinomas (45), and cells with this mutation display a survival advantage when transplanted into nude mice (14). Second, *Bax* deficiency accelerates brain and breast tumor development in SV40 large T antigen-transgenic mice (52, 62). Finally, there is increased foci formation in transformation assays by using *Bax*-deficient MEFs (28). In contrast, *Bax* is not mutated in any of the $Bax^{+/+}$ or $Bax^{+/-}$ Eμ-*myc* tumors analyzed. Additionally, there was no loss of heterozygosity of *Bax* in mammary carcinomas from $Bax^{+/-}$ C3 (1)/SV40 large T antigen-transgenic mice (52) or in lymphomas from $Bax^{+/-}$ Eμ-*myc* transgenics (Fig. 4A). These latter findings are consistent with the observation that *Bax* is infrequently mutated in most types of human B-cell lymphoma (42). Moreover, *Bax*-null mice do not spontaneously develop cancer (22), and loss of *Bax* did not influence the survival of *ARF*-null mice. Therefore, Bax may function as a classic tumor suppressor under very specific circumstances but acts as a modifier in most situations, such as Myc-induced lymphomas. Our observations also indicate that targets in addition to Bax must contribute to p53-dependent tumor suppression.

It has been known for over a decade that Bcl-2 and Myc can cooperate in transformation. The malignancy in Eμ-*myc*/Eμ-*bcl-2* double-transgenic mice is composed of primitive lymphoid cells (54), rather than the pre-B and/or mature B cells typical of Eμ-*myc* lymphomas (1). Although loss of *Bax* also accelerates lymphomagenesis in Eμ-*myc* transgenics, flow cytometric analysis clearly indicates that these lymphomas are of pre-B- and/or mature B-cell origin. Thus, overexpression of Bcl-2 blocks Myc-induced apoptosis and alters the differentiation of the lymphoid cell, while *Bax* loss alters the sensitivity to Myc-induced apoptosis without overtly affecting B-cell differentiation. Thus, Bcl-2 and Bax appear to provide different developmental roles when B-cell precursors are forced to proliferate by Myc expression. Although Bcl-2 can inhibit the apoptotic effects of Bax (reviewed in reference 23), Bcl-2 and Bax can regulate apoptosis independently of each other (21). Moreover, Bcl-2 is overexpressed at the same frequency in $Bax^{-/-}$ and $Bax^{+/+}$ Eμ-*myc* lymphomas (Eischen and Cleveland, unpublished). Therefore, it will be interesting to evaluate the status of *p53*, *ARF*, and Mdm2 in the tumors from Eμ-*myc*/Eμ-*bcl-2* double transgenics in order to determine whether Bcl-2 and Bax function in common or distinct pathway(s) in relationship to p53.

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