A dominant interfering Myb mutant causes apoptosis in T cells

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The c-Myb transcription factor is required for the production of most hemopoietic lineages, but information is sparse about its mode of action and the key genes it regulates. We have made an inducible dominant interfering Myb protein, by creating a chimera comprising the DNA binding domain of c-Myb, the *Drosophila* Engrailed repressor domain, and a modified estrogen receptor hormone binding domain. When expressed in the murine thymoma cell line EL4, activation of this mutant results in a significant proportion of the cell population undergoing apoptosis, as assessed by nuclear breakdown and DNA fragmentation, but has no apparent effect on cell-cycle progression. The apoptotic phenotype is mirrored during thymopoiesis in transgenic mice expressing dominant interfering Myb mutants; their T cells are fragile both in vivo and in vitro. Induction of the Myb dominant interfering mutant in EL4 cells correlates with down-regulation of bcl-2, but does not affect transcription of other bcl-2 family members; conversely, overexpression of bcl-2 in the transgenic mouse model rescues thymocytes from death. Analysis of the bcl-2 promoter by run-on transcription, bandshifting, and transient expression assays shows that it is a direct target of Myb. These data suggest a new and important role for Myb proteins as regulators of cell survival during hemopoiesis.

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The Myb family of transcription factors comprises three members: c-Myb, an activator expressed almost exclusively in hemopoietic lineages; A-Myb, likewise an activator, but expressed more promiscuously; and B-Myb, also expressed in many tissues, whose function remains controversial (Foos et al. 1992; Tashiro et al. 1995; Thompson and Ramsay 1995) but whose biological properties to date are similar to those of c-Myb (Arsura et al. 1992; Sala and Calabretta 1992). All three proteins share a highly conserved DNA binding domain and recognize the same binding site. Little is known about the targets regulated by Myb proteins. A number of genes whose upstream regions contain Myb binding sites have been described (Thompson and Ramsay 1995), but with a few exceptions it is unclear whether in vivo regulation is occurring.

Previous studies have proposed that c-Myb is an important regulator of the cell cycle. *c-myb* mRNA can be detected at low levels in some nonhemopoietic cell lines, such as chick embryo fibroblasts (CEFs), in a cell cycle-dependent manner, such that *c-myb* mRNA levels are maximal during late G₁ (Thompson et al. 1986). In the T-cell lineage, although there seems to be no cell-cycle regulation of expression in immature thymocytes (Thompson et al. 1986), levels of *c-myb* mRNA peak at the G₁/S transition following mitogenic stimulation of

peripheral T cells (Stern and Smith 1986; Crabtree 1989). Treatment of many hemopoietic cell lines and also of human peripheral blood lymphocytes and bone marrow cells with *c-myb* antisense oligonucleotides blocks proliferation, possibly by inhibiting the G₁/S transition (for review, see Calabretta 1991), although recent data indicate that the effects of the oligonucleotides used may be nonspecific (Burgess et al. 1995). B-Myb expression is also cell cycle-regulated (Lam and Watson 1993; Lam et al. 1995), and may also be involved in G₁/S progression (Lin et al. 1992).

c-Myb plays a vital role during hemopoietic differentiation. Early studies showed that the protein was detectable in most hemopoietic lineages and that its expression declined as cells matured and ceased to divide. Down-regulation of c-Myb may be essential for maturation to occur in some lineages (Graf 1992). Confirmation that c-Myb is required for normal hemopoiesis came with the demonstration that inactivation of the *c-myb* gene by homologous recombination results in the death of $-/-$ mice at d15 in utero, attributable to a failure of hepatic erythropoiesis (Mucenski et al. 1991). All other hemopoietic lineages, with the exception of megakaryocytes, were also gravely affected; progenitor cells were still present, but in greatly reduced numbers. As these cells were still capable of differentiation, it was suggested that c-Myb protein might normally regulate the switch between growth and differentiation by maintaining progenitors in a proliferative state. Loss of c-Myb expression would then result in an inability to prolifer-

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ate, and cells would enter the differentiation pathway instead.

The embryonic mortality of *c-myb*-null mice made it necessary to develop other techniques to study the consequences of loss of c-Myb expression in the blood cell lineages of live mice. We were specifically interested in the function of c-Myb during T-cell differentiation. c-Myb is expressed at high levels in immature thymocytes of the thymic cortex, switched off as cells mature, but becomes active again in mature T cells during their proliferative response to antigen (Stern and Smith 1986). In order to probe the function of c-Myb in T cell development, we created two dominant interfering Myb mutants, one a simple competitive inhibitor comprising the Myb DNA-binding domain (termed MT), and the other an active repressor in which the DNA-binding domain of the protein was fused to the *Drosophila* Engrailed repressor domain (termed MEnT). These mutants were targeted into the T-cell lineage of transgenic mice (Badiani et al. 1994). Expression of either mutant caused a severe block at an early point in thymopoiesis; furthermore, those T cells that escaped the block and reached maturity in the periphery were unable to proliferate properly in *in vitro* assays. The MEnT protein was effective at 50-fold lower expression levels than MT, because of the presence of the active repressor domain.

We wished to extend our studies on the role of c-Myb in T-cell development by identifying the Myb-regulated genes that, when turned off in our transgenic mice, were responsible for the observed phenotype. To simplify analysis, we chose to develop an inducible version of the powerful active repressor MEnT and to study its effects in the murine thymoma line EL4. We show here that expression of the Myb dominant interfering allele leads to apoptosis both in EL4 cells and in our original transgenic mice, and we present data correlating this phenomenon with rapid down-regulation of the *bcl-2* gene. We propose that Myb proteins can function as a safeguard against apoptosis, at least in part via activation of *bcl-2*, and that this role may be of great importance during hemopoiesis.

Results

Construction and expression of an inducible dominant interfering allele of Myb

In order to interfere with the activity of endogenous Myb in EL4 cells, we constructed an inducible version of the Myb–Engrailed chimeric repressor protein MEnT, by adding a modified murine estrogen receptor hormone binding domain (ER HBD) to its carboxyl terminus (Littlewood et al. 1995). The modified ER HBD can be activated only by the estrogen analog 4-hydroxytamoxifen (4-OHT), and proteins fused to it are inert unless 4-OHT is present. The resulting fusion protein, complete with a carboxy-terminal myc 9E10 epitope tag, was designated MERT [Myb–Engrailed–estrogen receptor–tag]. Schematic structures of MERT and the control fusion pro-

teins ERT [Engrailed–estrogen receptor–tag] and RT (estrogen receptor–tag) are shown in Figure 1A.

The three fusion proteins MERT, ERT, and RT were stable when translated in rabbit reticulocyte lysate, and the synthetic MERT protein retained the ability to bind to the mimA Myb recognition sequence (Ness et al. 1989) from the chicken *mim-1* promoter (data not shown). MERT was also able to repress v-Myb-mediated transcription in an inducible fashion. Cotransfection into NIH-3T3 cells of an expression vector encoding the MERT allele, a v-myb expression vector, and a CAT reporter gene carrying five copies of the mimA site upstream of a minimal tk promoter resulted in complete repression of CAT gene expression in the presence of 4-OHT (Fig. 1B, cf. lanes 2 and 4). In the absence of 4-OHT, little or no repression was observed (Fig. 1B, lane 3).

A number of stable clones expressing MERT, ERT, and RT were generated by electroporation of EL4 cells, and representative clones were selected for further analysis. Figure 1C shows a Western blot of protein extracts from control untransfected EL4 cells (lane 1), clones derived from cells transfected with the empty vector (MC1; lane 2), RT (R5; lane 3), or ERT (ER1; lane 4) and the two highest expressing clones of MERT (lanes 5 and 6; E16B

Figure 1. Inducible expression system for the dominant interfering Myb mutant, MERT. (A) Schematic representations of the ER HBD fusion proteins utilized in the experiments: Myb DNA binding domain (myb DBD); Engrailed repressor domain (En repressor domain); ER HBD (ER hormone binding domain); myc 9E10 epitope tag (9E10). (B) CAT assay from NIH-3T3 cells transfected with a 5mimA-tk-CAT reporter plasmid along with the plasmids indicated above each lane, using a reporter-effector-repressor ratio of 1 : 5 : 15. A representative CAT assay is shown. (C) Western blot of protein extracts from each cell line, probed with 9E10 antibody. Stably expressed MERT, ERT, and RT proteins and their approximate molecular weights are indicated. (D) Graph showing the fold repression of luciferase activity in the presence of 4-OHT, when each stably transfected EL4 clone was transfected with an SVMim-luciferase reporter gene. Data are standardized for β -galactosidase activity.

and E16C), probed with an antibody against the 9E10 epitope tag. MERT protein was expressed at lower levels than ERT and RT, and all three proteins were of the expected sizes.

To check that the stably transfected MERT proteins were functioning in an appropriate manner and that transcriptional repression was specific, a plasmid containing a luciferase reporter gene under the control of five Myb binding sites, an SV40 enhancer, and a minimal tk promoter was electroporated into all six clones, together with a control β -galactosidase expression plasmid. The MERT proteins, but not the controls, should be targeted to the Myb binding sites and actively repress transcription mediated by the neighboring SV40 enhancer. Levels of luciferase activity were measured in the presence and absence of 4-OHT, and the degree of repression was calculated. Figure 1D shows that, on addition of 4-OHT, luciferase levels dropped five- to sixfold in the MERT clones E16B and E16C, but there was no decline in any of the other clones.

Inhibition of Myb activity does not affect the cell cycle

As Myb proteins have been proposed to be required for cell-cycle progression, we investigated the effects of MERT induction on cell growth and the cell cycle. The MERT lines E16B and E16C, together with the four control lines, were incubated in the presence and absence of 4-OHT for 96 hr. Cells were seeded at 1×10^5 per milliliter, samples were taken from each pair of cultures every 24 hr, and live cells were counted by trypan blue exclusion. Results are shown in Figure 2A. G418 selection of all six clones had slightly reduced their growth rate relative to parental EL4 cells (open bars). Induction of the ER HBD when alone, or fused to the En repressor domain, was not found to affect cell numbers, as both clones produced indistinguishable growth curves whether or not 4-OHT was present, similar to those of the vector alone clone MC1 (Fig. 2A; light gray bars). However, induction of MERT activity in clones E16B and E16C strikingly reduced the number of live cells at 96 hr by ~50% (from 1.8×10^6 to $0.8\text{--}0.9 \times 10^6$; Fig. 2A; cf. -4-OHT with +4-OHT, solid bars).

To determine whether the reduction in cell number was due to a cell-cycle block, samples of cells at each time point were studied by flow cytometry, using propidium iodide to stain their DNA. The resulting cell-cycle profiles of clone E16C at the time points 0, 72, and 96 hr are shown in Figure 2B (similar profiles were obtained with E16B). Comparison of the two sets of profiles ($\pm$ 4-OHT) shows that repression of Myb activity had little or no effect on the cell cycle. Pulsing the cells with bromodeoxyuridine to label DNA at S phase also indicated that there was no block in transit around the cell cycle (data not shown). The most notable observation from the cell cycle profiles is the appearance of a sub- G_1 peak in the presence of 4-OHT; this begins to appear by 72 hr and has increased at 96 hr to include around 30% of cells. As a sub- G_1 peak is indicative of a population of

Figure 2. Inhibition of Myb activity by induction of MERT reduces live cell number but does not affect cell cycle. (A) Graph showing the numbers of live cells counted every 24 hr by trypan blue exclusion over 96-hr treatment without 4-OHT (left graph) or with 4-OHT (right graph). Data from parental EL4 cells (open bars), MC1 control clone (light gray bars), and both high expressors of MERT, E16B (solid bars) and E16C (dark gray bars), are shown; ER1 and R5 clones gave similar results as the MC1 clone. (B) Cell-cycle profiles of propidium iodide-stained nuclei of clone E16C $\pm$ 4-OHT at 0, 72, and 96 hr; similar profiles were obtained with clone E16B: x-axis, DNA content; y-axis, number of events. The percentage of sub- G_1 (apoptotic) cells is indicated on the histograms. The percentages of cells in the different stages of the cell cycle at 96 hr (calculated to exclude sub- G_1 populations) in the absence of 4-OHT, and in the presence of 4-OHT, respectively, were: G_1 63.3 and 62.9; S, 15.0, and 13.3; G_2/M , 21.7 and 23.8.

cells undergoing apoptosis, these data imply that the observed decrease in the live cell number of the induced MERT clones is not caused by a block in the cell cycle, but is instead a result of cell death.

Inhibition of Myb activity causes apoptosis

To demonstrate that MERT induction causes apoptosis, rather than necrosis, we examined the effects of induction in more detail. We first performed a simple dead cell count. Cells were grown in the presence and absence of 4-OHT over a 96-hr time course, and samples were taken every 24 hr and scored for the percentage of dead cells in the culture by trypan blue staining. Figure 3A shows the

Figure 3. Inhibition of Myb activity causes apoptosis. (A) Graph of the percentage of dead cells of all control clones and MERT-expressing clones at 96 hr as counted by trypan blue inclusion, when incubated either without 4-OHT (gray bars) or with 4-OHT (solid bars). (B) DNA harvested from 10^6 cells from each of the clones $\pm$ 4-OHT at 96 hr, electrophoresed through a 1% agarose gel and stained with ethidium bromide. (C) Hoechst 33258 staining of nuclei from MC1 control cells, and both E16B and E16C clones at 96 hr treated either without 4-OHT (upper panels) or with 4-OHT (lower panels).

data at 96-hr. For the control clones, there is no change in cell viability whether or not 4-OHT is present. However, for the MERT clones, the cultures are ~45–55% dead after 96 hr treatment with 4-OHT, and this death is accompanied by DNA laddering, a hallmark of apoptosis

(Fig. 3B). Only the MERT clones induced with 4-OHT show any significant laddering (Fig. 3B; cf. E16B+ and E16C+ lanes with all others); DNA from control clones and uninduced MERT clones is unaffected. Finally, examination of nuclear morphology by staining with the nonintercalating benzimidazole dye Hoechst 33258 demonstrated that after 96 hr the nuclei of the MERT-expressing cells have a bright and irregular appearance when 4-OHT is present, but have normal rounded appearance when it is absent (Fig. 3C). The fragmented morphology of the nuclei suggests that the DNA is being degraded and nuclear collapse is occurring. Together, these data confirm that interfering with the activity of Myb proteins, at least in the EL4 cell line, causes apoptosis.

Interfering with Myb activity enhances apoptosis during thymopoiesis

We next wished to determine whether the apoptosis we observed in EL4 cells upon induction of the MERT allele was recapitulated in the thymocytes of our MT and MEnT transgenic mice. As described above (see the introduction), thymocyte development in these mice is inhibited. Animals heterozygous for either transgene have an essentially normal thymocyte subset profile, as assessed by staining with antibodies against CD4 and CD8, but total thymocyte numbers are reduced by between four- and eightfold relative to nontransgenic controls. Homozygotes are more severely affected, with a 20-fold reduction in thymocyte numbers; a block to differentiation apparently occurs at an early point in thymopoiesis, the transition from CD4⁻ CD8⁻ (double negative, DN) to CD4⁺ CD8⁺ (double positive, DP) cells (Badiani et al. 1994). Peripheral T cells from both heterozygotes and homozygotes are unable to proliferate efficiently in response to antigen stimulation in vitro assays. From our observations in EL4 cells, it seemed possible that the phenotype of these animals might be attributable to an increase in the susceptibility of transgenic T cells to apoptosis. We therefore assayed the fragility of thymocytes and splenic T cells from nontransgenics, or from animals heterozygous for either the MT or MEnT transgene. Heterozygotes were chosen, because their thymocyte subset distribution is very similar to that of nontransgenics, enabling a direct comparison between transgenic and wild-type mice. Thymocytes and T cells were cultured under conditions known to mimic in vivo apoptotic stimuli: growth in simple tissue culture medium, growth in the presence of dexamethasone, and growth following X-irradiation. Results are shown in Figure 4A. When grown for 18 hr without any treatment, an average of ~10% of transgenic thymocytes and T cells were still alive, in contrast with around 40% of the thymocytes and T cells of nontransgenic littermates. Treatment of cells with dexamethasone or X-rays resulted in almost complete death of transgenic cultures, whereas around 5–10% of cells in nontransgenic cultures survived. Therefore, transgenic cells from both lines of mice were

Figure 4. Thymocytes and T cells of transgenic mice expressing a Myb dominant interfering allele have increased susceptibility to apoptotic stimuli. (A) Survival in culture of nontransgenic (open bars), MT (gray bars), and MEnT (solid bars) thymocytes and splenocytes after no treatment, addition of 1 mM dexamethasone, or exposure to 225-rad X-rays. (B) TUNEL assays on nontransgenic, MT, or MEnT thymocytes. Cells were cultured for 5 hr, stained, and assessed by flow cytometry. A representative experiment is shown.

at least four times more fragile than nontransgenic controls when exposed to apoptotic stimuli.

We looked for evidence of increased levels of apoptosis in the thymuses of transgenic animals from the MT and MEnT lines. Although thymic apoptosis is normally rather hard to discern because of efficient clearance of dead and dying cells, better detection is possible if cells are cultured for a short time before analysis (Kishimoto et al. 1995). Accordingly, thymocytes from 3-week-old mice were purified, cultured for 5 hr, labeled by TdT-mediated dUTP-fluorescein end-labeling (TUNEL), and analyzed by flow cytometry. A representative experiment is shown in Figure 4B. When compared with a nontransgenic sibling, thymocytes from heterozygous animals from both transgenic lines show a higher percentage of apoptotic death, as measured by a 2.4-(MT) to 2.9-fold (MEnT) increase in the amount of TUNEL staining. In support of this, TUNEL staining of thymic sections from heterozygous MEnT animals also revealed more cell death in situ than was found in the thymuses of nontransgenic siblings (data not shown). These data show that thymocytes from Myb dominant interfering transgenic animals are dying in vivo in greater numbers than normal.

Bcl-2 expression is repressed by induction of MERT

Having shown that apoptosis in response to Myb shutoff occurs in mice as well as in a cell line, we were interested to discover whether genes known to control apoptosis were differentially regulated in the presence or absence of Myb dominant interfering proteins. For this experiment, we returned to the EL4-derived cell lines.

Cells from the two high MERT expressors E16B and E16C, as well as the MC1 clone, were incubated with and without 4-OHT, and both RNA and protein extracts were harvested every 24 hr. Northern blots were probed for the *bcl-2* family members *bcl-2*, *bax*, *bak*, and *bcl-x*. No changes were detected in levels of *bax* and *bcl-x* mRNA, and *bak* expression was undetectable (data not shown). However, as shown in Figure 5A, *bcl-2* mRNA is down-regulated as early as 24 hr after 4-OHT induction in both MERT clones; levels remain constant in control MC1 cells with and without 4-OHT, and were also unchanged after 96 hr with or without 4-OHT in the control RT and ERT lines (data not shown). The decrease seen in the MERT clones is recapitulated at the protein level, albeit with slower kinetics, as shown in the Western blot in Figure 5B; again levels of Bcl-2 protein in control MC1 cells stay the same. The apparently slow

Figure 5. Down-regulation of Bcl-2 expression correlates with induction of MERT. (A) Northern blot of 20 µg total cellular RNA harvested from E16B, E16C, and control MC1 cells at 24-hr intervals $\pm$ 4-OHT, probed with *bcl-2* (upper panels) and, as a loading control, GAPDH (lower panels). (B) Western blot of protein extracts taken from 10^6 cells from MC1, E16B, and E16C clones every 24 hr $\pm$ 4-OHT and probed with anti-mouse *bcl-2* antibody.

turnover of Bcl-2 protein in MERT cells correlates well with the 48- to 72-hr lag in appearance of the apoptotic phenotype after 4-OHT induction.

Expression of *bcl-2* is associated with rescue of MERT or MEnT-induced apoptosis

We wished to determine whether Bcl-2 could rescue cells expressing Myb dominant interfering proteins from apoptosis, both in the cloned cell lines and in our transgenic mice. For the cell lines, rather than simply overexpressing Bcl-2, we tested whether a Myb protein could override the effects of MERT induction and whether this correlated with a change in endogenous *bcl-2* mRNA levels. The v-Myb oncoprotein was used for the rescue experiment, as it is a stronger transcription activator than its close relative c-Myb. Clone E16C was transfected stably with a vector constitutively expressing v-Myb, or a control empty vector, and doubly transfected clones were isolated. In comparison with the E16C parent clone, the clones transfected with empty vector alone were more susceptible to death when MERT was induced with 4-OHT, probably because double selection with G418 and puromycin had increased their sensitivity to apoptotic stimuli (Fig. 6A; cf. E16C with P1 and P7). However, clones V3 and V4, which constitutively express v-Myb, were partially protected from apoptosis when MERT was induced. After 96 hr, 69% of control P1 and P7 cells were dead, in contrast to 31% (V4) and 41% (V3) of v-Myb expressing cells (Fig. 6A; cf. P1 and P7 with V3 and V4). Although levels had not returned to those seen in the absence of 4-OHT, significant rescue had occurred. Using RNase protection mapping we exam-

ined endogenous *bcl-2* mRNA in the doubly transfected clones. As shown in Fig. 6B, control clone P1 showed a marked reduction in *bcl-2* mRNA levels after 24 hr, and this was still the case 96 hr after MERT induction, in good agreement with our previous data. However, when MERT was induced in the v-Myb expressors, V3 and V4 (Fig. 6B), the level of *bcl-2* mRNA did not decrease, but instead remained similar to that seen in the absence of 4-OHT, even 72 and 96 hr after induction. Therefore, v-Myb can counteract the effects of MERT, and the decrease in apoptosis coincides with rescue of *bcl-2* expression.

A number of transgenic mouse lines have been established in which Bcl-2 is overexpressed in the T cell lineage (Sentman et al. 1991; Strasser et al. 1991). Although T-cell homeostasis is not affected, transgenic thymocytes and splenocytes are extremely resistant to apoptosis under a number of conditions, both in vivo and in vitro. We crossed heterozygous MEnT mice with $E\mu bcl-2-25$ mice (Strasser et al. 1991), which express Bcl-2 from early times in thymopoiesis. To assay for rescue of the MEnT phenotype, we initially counted the total number of thymocytes. In this experiment, MEnT animals had an average thymocyte count of 2.4×10^7 , in contrast to normal or $E\mu bcl-2-25$ mice, whose thymuses contained on average 2.3×10^8 thymocytes. In double MEnT/ $E\mu bcl-2-25$ transgenics, numbers had increased to 5.8×10^7 indicating that introduction of the *bcl-2* transgene had compensated partially for the effects of MEnT on thymocyte number. In a modification of the experiment shown in Figure 4B we also looked, using TUNEL, for a decrease in the proportion of apoptotic cells in double transgenic animals. Figure 6C shows that introduc-

Figure 6. Expression of Bcl-2 rescues apoptosis induced by Myb dominant interfering proteins. (A) Graph of the percentage of dead cells in the E16C parent clone and the doubly transfected clones P1 and P7 (empty vector) and V3 and V4 (v-Myb expressors) at 96 hr, as counted by trypan blue inclusion, either when incubated without 4-OHT (gray bars) or with 4-OHT (solid bars). Clone MC1 results from Fig. 3A are shown for comparison. (B) RNase protection mapping of 20 μ g total cellular RNA harvested from v-Myb expressing (V3 and V4) and control cells (P1), grown either with (+) or without (-) 4-OHT, after the times indicated. Protected fragments correspond to *bcl-2* mRNA (upper band) and GAPDH mRNA (lower band). (C) TUNEL assays on nontransgenic, MEnT and $E\mu bcl-2-25$ (*bcl-2*) transgenic, and MEnT/ $E\mu bcl-2-25$ (MEnT/*bcl-2*) double transgenic thymocytes. Cells were cultured for 5 hr, stained, and assessed by flow cytometry. The histogram depicts the means and standard deviations of results from two to five separate animals.

ing the $\text{E}\mu\text{bcl-2-25}$ transgene into our MEnT lines reduced the number of TUNEL positive thymocytes from an average of 27.7% to 4.0%, a figure close to the 2.4% obtained using $\text{E}\mu\text{bcl-2-25}$ single transgenics. Taken together with the data obtained in cell lines, these results show that an increase in *bcl-2* expression is associated with rescue of MEnT- or MERT-induced apoptosis and provide further evidence that Myb may be an upstream regulator of Bcl-2.

Bcl-2 is a direct target of Myb

Having shown that down-regulation of Bcl-2 appears to be an important factor in MERT- and MEnT-induced apoptosis, we tested whether *bcl-2* transcription was regulated directly by Myb. Using MERT clone E16C, we assessed the speed with which *bcl-2* transcription was inhibited after induction of MERT, by performing nuclear run-on assays. Cells were incubated with or without 4-OHT and nuclei were harvested at 0 and 2 hr after induction. Radiolabeled nascent RNA transcripts were hybridized to *bcl-2* sense and antisense DNA (schematic in Fig. 7A) bound to nitrocellulose filters. Results are shown in Figure 7B. Two hours after incubation with 4-OHT, *bcl-2* sense transcription was reproducibly reduced by half, indicating that inhibition of Myb activity causes a rapid shutdown of *bcl-2* transcription. As observed previously for the human *bcl-2* gene (Young and Korsmeyer 1993), there was some hybridization of an-

tisense RNA, which was more apparent under conditions where sense strand transcription was elevated.

The *bcl-2* gene has two promoters, P1 and P2, which are 1.5 kb and 200 bp, respectively, upstream of the initiating ATG. The P2 promoter is rarely used (Young and Korsmeyer 1993). We searched the sequence of the 2-kb region containing these promoters and found one perfect match, CAACGG, to the Myb binding consensus (Fig. 7A). As shown in Figure 7C, a labeled oligonucleotide containing this site and its surrounding sequence forms a complex in a bandshift assay with reticulocyte lysate programmed to express Myb protein (lane 1), but does not recognize any bands in unprogrammed lysate (lane 5). This complex contains Myb, as it can be abolished with a 50-fold molar excess of a cold oligonucleotide carrying a high affinity Myb binding site, mimA (lane 2; Ness et al. 1989), but not with excess cold mutant mimA (lane 3). An identical probe in which the myb site is mutated to CCACGG is unable to bind Myb (bcl-2M; lane 4). To test the importance of this Myb binding site for transcription of the *bcl-2* gene, we constructed a luciferase reporter vector, in which luciferase transcription was driven by a 450-bp fragment of *bcl-2* 5' sequence (Fig. 7A) containing the P2 promoter. We transfected this reporter construct into EL4 cells, and found that the *bcl-2* promoter fragment reproducibly activated expression of the luciferase gene to an average level of 5.3-fold over baseline (Fig. 7D). In contrast, when the Myb consensus was mutated from CAACGG to CCACGG, mak-

Figure 7. Direct regulation of *bcl-2* by myb. (A) The murine *bcl-2* promoter. (Shaded boxes) Introns. The P1 and P2 promoters and the myb binding site are indicated. Sense and antisense DNA probes and the *bcl-2* promoter fragment used for luciferase assays are also shown. (B) Nuclear run-on assays using nuclei from E16C cells harvested prior to induction with 4-OHT ($t=0$) and after 2-hr treatment $\pm$ 4-OHT. Labeled RNA transcripts were hybridized to the sense and antisense *bcl-2* probes shown above (both in an M13 vector background) with M13 ssDNA and GAPDH as controls. "Sense" and "antisense" refer to the detected *bcl-2* transcripts. (C) Myb protein can bind the *bcl-2* promoter. (Lanes 1–3) *bcl-2* probe; (lane 4) *bcl-2M* probe. Lanes 1–4 contain MT-programmed lysate, and lane 5 blank lysate; 100 ng cold competitor oligonucleotide was added where shown. Free probe is shown at a shorter exposure, as otherwise the retarded complex is obscured. (D) Luciferase assays of the *bcl-2* promoter in EL4 cells. Luciferase activity is shown in the actual units derived from scintillation counting. Assays were performed in duplicate; the average and standard deviation of

ing it unable to bind Myb protein, we saw a marked decrease in luciferase activity to an average level of 1.8-fold over baseline (Fig. 7D). Similar effects were observed when the luciferase reporter was driven by a 4.5-kb fragment of *bcl-2* 5' sequence containing both P1 and P2 promoters; mutation of the Myb consensus resulted in the loss of half of the activity of the promoter (data not shown). Taken together, these data show that the Myb consensus in the *bcl-2* promoter is a bona fide Myb binding site, and that this site is an important positive regulatory element in EL4 cells.

Discussion

We have stably expressed an inducible dominant interfering Myb mutant, MERT, in the murine thymoma line EL4. Between 48 and 72 hr after induction, a significant number of cells begin to apoptose, as assessed by cell counts, nuclear morphology, and DNA fragmentation. In transgenic mice expressing similar dominant interfering Myb proteins in their T-cell lineages, transgenic thymocytes and T cells are more responsive to apoptotic triggers, such as dexamethasone and X-rays. The mice also show increased apoptosis during thymopoiesis, as detected by TUNEL staining. In EL4 cells, induction of apoptosis is correlated with down-regulation of *bcl-2* expression; only low levels of *bcl-2* mRNA remain 24 hr after induction, and *bcl-2* protein is also switched off, although with slower kinetics. Expression of exogenous v-Myb partially rescues cells from both down-regulation of Bcl-2 and apoptosis; furthermore, Bcl-2 expression in the dominant interfering transgenic lines also rescues thymocytes from cell death. Run-on assays and analysis of the *bcl-2* promoter suggest strongly that down-regulation may be attributable to Myb regulating *bcl-2* transcription directly.

Are Myb proteins survival factors?

It is formally possible that the apoptosis caused by our dominant interfering alleles is not an indicator that wild-type Myb proteins have antiapoptotic functions, but is induced as a consequence of the inability of the cells to grow properly in the absence of Myb activity. We do not consider this likely for a number of reasons: First, Myb proteins are clearly not essential for the growth and differentiation of many cell types both inside and outside the hemopoietic system (e.g., see Mucenski et al. 1991); second, the direct regulation by Myb of the survival gene *bcl-2* characterizes Myb as an upstream component of a well-established antiapoptotic pathway; third, we and others have shown that overexpression of Myb proteins can protect cells against apoptosis (Smarda and Lipsick 1994; Bies et al. 1996; P. Badiani and K. Weston, unpubl.). Taken together, these data argue strongly that Myb proteins have an important regulatory role in the response of certain cell types to apoptotic stimuli.

The apoptosis observed in EL4 cells on MERT induction is mediated via the Myb DNA binding domain, because control proteins lacking this region are inert. How-

ever, MERT can potentially interfere with the activity of A-, B-, and c-Myb, which all recognize the same binding site. Although A-Myb is not expressed during murine thymopoiesis (Mettus et al. 1994; Sitzmann et al. 1995), the target genes down-regulated by MERT binding may be regulated by B- or c-Myb, or both. B-*myb* is expressed in EL4 cells, although at a lower level than c-*myb* (data not shown), and in vivo, the expression patterns of B- and c-*myb* overlap during T-cell development and activation (Stern and Smith 1986; Golay et al. 1991). Current data suggest that B-Myb appears to fulfill a biologically similar role to c-Myb, inhibiting growth when it is absent, and stimulating proliferation when it is deregulated (Lin et al. 1992; Sala and Calabretta 1992). However, further experiments are required to determine whether c- and B-Myb share the ability to protect T cells from apoptosis.

Myb, apoptosis, and the cell cycle

We have shown here that inhibition of Myb activity reduces the apparent growth of EL4 cells and that this is due to apoptosis. Additionally, we found that the thymocytes and splenocytes of mice transgenic for Myb dominant interfering mutants have decreased survival in culture and are more sensitive to apoptotic triggers, and that all stages of T-cell maturity are more prone to apoptosis. In our experiments in EL4 cells, MERT induction had little or no effect on cell-cycle control; relative numbers of cells in each stage of the cell cycle remained the same, and they were cycling normally. These data are contrary to previous reports implicating Myb proteins as regulators of the G₁/S transition of the cell cycle (see the introduction). As it might be expected that a transformed cell line such as EL4 might have accumulated mutations resulting in a deregulated cell cycle, we do not exclude the possibility that Myb may have a dual function in preventing cell death and regulating cell growth. In support of this notion, forced expression of both c- and B-Myb has been reported to enhance TGF- β -induced apoptosis in M1 cells (Selvakumaran et al. 1994; Bies and Wolff 1995), but to maintain proliferation and protect these cells against IL-6-induced death and differentiation (Bies et al. 1996). Intriguingly, in this paper we were able to rescue MERT transgenic thymocytes from stress-induced apoptosis with a *bcl-2* transgene, but total thymocyte numbers did not return to wild-type levels, suggesting that inhibition of Myb might affect either other survival genes (see below) or perhaps other cellular processes, such as the cell cycle.

*Myb and the *bcl-2* family*

We have shown that apoptosis in response to MERT induction in EL4 cells is temporally linked to down-regulation of the *bcl-2* gene, a fundamental component of survival pathways in many cell types, including T cells (Craig 1995). Little is known about transcriptional regulation of *bcl-2*. Most transcripts originate from the distal

P1 promoter, which has no detectable TATA or CAAT boxes, but has GC-rich regions containing multiple Sp1 binding sites and start sites; the proximal P2 promoter is apparently almost inactive. Although no detailed studies of the *bcl-2* promoter have been undertaken in T cells, analysis of the upstream region of the human *bcl-2* gene in a number of B-cell lines has shown that the region between the P1 and P2 promoters, highly conserved between mouse and man, contains a number of DNase hypersensitive sites and a negative regulatory element (Young and Korsmeyer 1993); the region 5' to the P1 promoter is also DNase hypersensitive and contains negative elements (Young and Korsmeyer 1993; Chen and Boxer 1995). Our data suggest that Myb proteins are direct regulators of *bcl-2*, binding to a site in the conserved regulatory region flanked by the P1 and P2 promoters. The apoptosis caused by induction of MERT correlates with a rapid decrease in *bcl-2* transcription, and mutation of the Myb site in the *bcl-2* promoter greatly reduces its activity in EL4 cells, implying that the factor binding there, presumably a Myb protein, is important for *bcl-2* transcription. In support of this conclusion, J. Frampton, T. Ramqvist, and T. Graf (in prep.) have observed a similar regulation of *bcl-2* by chicken Myb-Ets in transformed avian myeloblasts; they also show that v-Myb can bind to and activate transcription from Myb binding sites in the chicken and human *bcl-2* promoters.

Transcriptional activation of *bcl-2* by Myb proteins requires that the expression patterns of the genes coincide. B-myb mRNA is detected in thymus and spleen (Golay et al. 1991; Tashiro et al. 1995), but more detailed expression studies have not been performed. c-myb, like *bcl-2*, is expressed at high levels in hemopoietic stem cells in the bone marrow (Hockenbery et al. 1991; Orlie et al. 1995). Detailed analysis of the subset-specific expression of Myb proteins in the thymus has not been undertaken, but the highest levels of c-myb are found in immature cortical thymocytes, which may be either DN or DP (Sheiness and Gardinier 1984). *bcl-2* is expressed in DN thymocytes, is down-regulated as cells become DP and undergo selection, but is then up-regulated in positively selected DP cells (Linette et al. 1994). After the DP stage, *bcl-2* mRNA is found in medullary thymocytes (Veis et al. 1993), and is also present in peripheral resting T cells; c-myb is not expressed in either of these populations. However, both *bcl-2* and c-myb are up-regulated upon antigenic stimulation (Stern and Smith 1986; Veis et al. 1993). Therefore, there are stages of T-cell development where both *bcl-2* and c-myb are expressed, making the regulation of one by the other feasible. Interestingly, the late DN stage and peripheral T-cell activation are both points at which there is a sustained burst of proliferation. Perhaps c-Myb is the regulatory factor that maintains *bcl-2* expression at these times, thereby preventing death by neglect, which can occur when growth factor levels cannot meet the demands of a rapidly expanding population.

A second *bcl-2* family member, *bcl-x_L*, is important during murine T-cell development (Gonzalez Garcia et al. 1995). *bcl-x_L* is expressed at high levels in the bone

marrow, in DP thymocytes, and activated peripheral T lymphocytes (Fang et al. 1994). It is absent from DN and SP thymocytes and resting mature T cells, places where *bcl-2* is expressed, implying that these genes are regulated differentially. Although we observe no change in levels of *bcl-x* mRNA in response to MERT induction in EL4 cells, we are investigating the possibility that, in vivo, Myb may be involved in regulating *bcl-x_L* in specific thymocyte subsets for which EL4 cells may not be representative.

Other potential Myb-regulated targets in apoptosis

Down-regulation of *bcl-2* expression may not be wholly responsible for the induction of apoptosis caused by Myb dominant interfering proteins, because increasing *bcl-2* expression achieved only partial rescue of the apoptotic phenotype. c-Myb has been proposed to regulate three other genes with known roles in apoptosis. The first, c-myc, has multiple Myb consensus sequences in its promoter (Nakagoshi et al. 1992), but these are not required for activation by c-Myb (Graf 1992), and the biological relevance of the interaction remains unclear. This target cannot account for the effects we see when Myb activity is ablated in EL4 cells because we do not detect any changes in endogenous c-myc mRNA levels when MERT is induced (data not shown). Up-regulation of c-Myc by c-Myb might also be expected to induce apoptosis (Evan et al. 1992) rather than protect against it, and so we do not consider it to be a likely downstream effector of c-Myb in this context. The two other genes, IGF-1 and the IGF-1 receptor (Reiss et al. 1991), have been shown to be involved in rescue from Myc-induced death (Harrington et al. 1994); we have found that addition of exogenous IGF-1 partially rescues the apoptotic phenotype caused by induction of MERT in the EL4 cells (data not shown), making it possible that Myb is required to maintain the expression of this survival factor.

T cell-mediated cytotoxicity is partially attributable to the interaction between a cell surface molecule, Fas, and its ligand, FasL; this interaction kills target cells and also eliminates activated T cells by induction of apoptosis (Nagata and Golstein 1995). When Fas or FasL are absent or mutated, as in the *lpr* and *gld* mouse mutants, a population of almost inert T cells builds up, which are resistant to Fas-mediated modes of cell death. This resistance is augmented markedly by introduction of a *bcl-2* transgene (Strasser et al. 1995), implying that Bcl-2 and Fas regulate distinct pathways to lymphocyte apoptosis. These data are particularly relevant to our studies, as levels of c-myb mRNA are strikingly high in *lpr* and *gld* T cells (Mountz et al. 1984), and therefore endogenous Bcl-2 might be expected to be similarly abundant. Possibly, in *lpr* cells, the level of a Bax family member is also high, and so Bcl-2 is inactivated. We intend to investigate whether c-Myb is playing a protective role in *lpr* and *gld* T cells and, if so, whether it is by a Bcl-2 dependent or independent mechanism. Interestingly, we detect an up-regulation of Fas in the MERT clones in the presence of 4-OHT (data not shown).

c-Myb and cancer

The notion that Myb proteins may act as antiapoptotic factors has implications for the role of *c-Myb* in oncogenesis. *v-Myb* causes rapid-onset myeloid and B lymphocytic tumors in mice and chickens (Weston 1990). Presumably these tumors arise through some other mechanism than aberrant protection from apoptosis, as such tumors would be expected to be fairly indolent, like the follicular lymphomas associated with *bcl-2* deregulation via the t(14; 18) translocation (Korsmeyer 1992). Nevertheless, a role for *c-Myb* as a survival factor in the maintenance of these and other tumors, including human cancers, is possible. A frequent chromosomal abnormality in acute lymphoblastic leukemias (ALLs) and non-Hodgkins lymphomas (NHLs) is a 6q- deletion (Bloomfield et al. 1983), close to the *c-Myb* locus at 6q23.3–6q24. Although rearrangement of *c-Myb* has been detected in only a few human tumors (Alitalo et al. 1984; Pelicci et al. 1984), significant up-regulation of transcription occurs in many cases (Barletta et al. 1987; Ohyashiki et al. 1988; Tesch et al. 1992). Combining these data and our results, it is feasible that overexpression of *c-Myb* may protect an abnormal cell from apoptosis, thereby allowing it to accumulate other mutations resulting in full-blown malignancy. We are currently investigating this possibility and also are examining the correlation between *c-myb* and *bcl-2* expression in tumor cell lines.

In summary, then, we have shown a new biological role for Myb proteins, as protective factors against apoptosis during thymopoiesis and T-cell growth. Further, we have established a link between Myb and Bcl-2, and have presented evidence that the *bcl-2* promoter is a direct target of Myb. In the light of these data, a reevaluation of the wider role of Myb proteins during hemopoiesis seems necessary, as hemopoietic cells must choose between self-renewal, differentiation, or death at all stages of their development, it seems feasible to suggest that the antiapoptotic function of the Myb proteins might be vital to these decisions. Future experiments will be directed toward resolving this issue.

Materials and methods*Plasmid construction*

Plasmid DNA manipulations and preparations were by standard methods. All constructs were checked by restriction mapping, and in-frame fusions and PCR products were verified by double-stranded sequencing by standard techniques. Details of plasmid constructions are available on request.

ER HBD fusion proteins MERT: A fragment comprising nucleotides 841–1797 (amino acids 281–599) of the murine estrogen receptor was generated by PCR from pBSKSMER/G525R (Littlewood et al. 1995) and inserted into pT7βMEnT (Badiani et al. 1994) 5' to the myc 9E10 epitope tag. Then the MERT fusion gene was excised from pT7βMERT and ligated into pMCEF[−] (R. Marais, unpubl.), a modified expression vector derived from pMC1neo (Stratagene) that uses the strong EF-1α promoter to drive high levels of expression of the inserted genes. ERT:

The 2-kb ERT fragment of pMCEF[−]MERT was inserted into pMCEF[−] to generate pMCEF[−]ERT. RT: A pT7βMRT construct was made by inserting the ER HBD fragment 5' of the myc tag of pT7βMT (Badiani et al. 1994). The MRT fusion gene was excised and inserted into pMCEF[−]. The 1-kb RT portion was excised and inserted into pMCEF[−] to generate pMCEF[−]RT. Plasmids pSCDMEnT, 5mim/CAT, and IEβgal are described in Badiani et al. (1994), and pMTV in Weston and Bishop (1989).

Luciferase reporter gene constructs 5mim/SV/luc: Five copies of the mimA site (Ness et al. 1989) were cloned upstream of the SV40 enhancer and a minimal tk promoter in pGL2Basic (Promega). Nco/luc was made by inserting the 450-bp NcoI–KpnI fragment of *bcl-2* genomic DNA (Negrini et al. 1987) into pGL2Basic. NcoM/luc was identical except for a 1-bp change in the Myb consensus binding site at position 1632 (CAACGG to CCACGG), introduced by PCR mutagenesis.

Run-on assays A 1.47-kb BamHI fragment spanning the murine *bcl-2* P2 promoter region (nucleotides 910–2375; Negrini et al. 1987) was cloned into Phagescript SK (Stratagene) such that single-stranded phage carried the sense orientation. For the antisense orientation, an 815-bp HindIII–BamHI fragment (nucleotides 1560–2375) was cloned in the opposite orientation in the same vector.

Cell culture

NIH-3T3 cells were grown at 37°C, 10% CO₂ in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% fetal calf serum (FCS) (Gibco). EL4 cells were maintained at 37°C, 5% CO₂ in Iscove's Modified Dulbecco's Medium (Gibco) and 8% FCS. Stable transfectants of EL4 cells were produced by electroporation using a BioRad Gene Pulser and were selected with 750 μg/ml G418 (Gibco). The selecting medium was changed every 2 days, and colonies that were resistant to G418 were picked and cloned by limiting dilution. Doubly selected cells were selected and maintained in G418 and 4 μg/ml puromycin (Sigma).

To induce the ER HBD fusion proteins, the cells were incubated with 10^{−7} M 4-OHT (gift of M. Parker) or 10^{−6} M 4-OHT (Semat Technical UK Ltd.) at a starting density of 1 × 10⁵/ml. For time course counting analyses, samples were taken at times indicated, washed once in PBS, and counted by trypan blue exclusion. Experiments were repeated a minimum of five times, and histograms show the mean values and standard deviations. Northern and Western time course studies were performed in the same way (starting density of 1 × 10⁵/ml), taking samples (2 × 10⁶ cells for RNA or 1 × 10⁶ cells for protein) every 24 hr.

Transient expression assays

NIH-3T3 Assays used lipofectAMINE (Gibco), and conditions were optimized according to the manufacturer's instructions; 1.25 × 10⁵ cells were seeded in each well of a six-well plate 18 hr before lipofection; 0.5 μg DNA total was used per well. For CAT assays, extracts were taken 48 hr post-lipofection by three cycles of freeze-thawing in 0.25 M Tris-HCl at pH 7.5 followed by centrifugation and were assayed for CAT and β-galactosidase activity as described previously (Badiani et al. 1994). Experiments were repeated three times.

EL4 For luciferase assays, 20 μg of luciferase reporter was electroporated into EL4 cells together with 5 μg IEβgal. Cells were

harvested 24 hr postelectroporation, washed once in PBS, and then lysed in cell culture lysis reagent (Promega). Luciferase assays were performed using the Luciferase Assay System (Promega) according to manufacturer's instructions and standardized by levels of β -galactosidase expression. Assays were repeated a minimum of three times in duplicate.

RNA and protein analysis

For the preparation of RNA samples, at least 2×10^6 cells were lysed in RNazol B according to the manufacturer's protocol; 20 μ g RNA was loaded per lane for Northern analysis. For *bcl-2* expression, a full-length murine *bcl-2* cDNA fragment was labeled by hexanucleotide priming. Blots were stripped and re-probed with a labeled DNA fragment hybridizing to murine GAPDH as a loading control. For RNAse protection mapping, 20 μ g total RNA was hybridized by standard methods to cRNA probes against *bcl-2* and GAPDH mRNA and was digested with 10 units RNAse ONE (Promega) according to the manufacturer's instructions. Digested products were visualized by PAGE and autoradiography. For the *bcl-2* probe, nucleotides 2165–2374 of murine *bcl-2* genomic DNA were cloned into pT7 β Sal (Badiani et al. 1994), such that the SP6 promoter was proximal to nucleotide 2374. For the GAPDH probe: the 5'-most 250 bp of murine GAPDH cDNA were cloned into pGEM2. Vectors were cut with *PvuII* and *Bst*II, respectively, and radiolabeled cRNA probes were generated by SP6 transcription as described (Melton et al. 1984). The *bcl-2* probe is 237 nucleotides long, and protects 209 nucleotides. The GAPDH probe is 160 nucleotides long and protects 153 nucleotides.

Protein extracts for Western blotting were made by standard methods, using 1×10^6 cells per sample. Samples were run on a 12% SDS polyacrylamide gel. Bcl-2 protein was detected using an antibody to amino acids 41–54, DHA7 (kind gift of Gerard Evan), a second-layer peroxidase-conjugated goat antirabbit immunoglobulin antibody (DAKO), and ECL reagents (Amersham). MERT, ERT, and RT fusion proteins were detected using monoclonal antibody 9E10 (Evan et al. 1985).

Bandshift assays

Reticulocyte lysate programmed with pT7 β MT to express the murine c-Myb DNA binding domain was prepared as described (Badiani et al. 1994). Double-stranded oligonucleotide probes were made by end-repair in the presence of [α - 32 P]dCTP. The *bcl-2* probe is CTCATGCCAACGGGGAACACCAGAA (nucleotides 1625–1650; Negrini et al. 1987), and the *bcl-2M* probe has CAACGG changed to CCACGG (myb site and mutated myb site underlined). WT cold competitor is the mimA site (Ness et al. 1989) CTAGGACATTATAACGGTTTTT-TAGTCTAG; mutant cold competitor has TAACGG changed to TCACGG; bandshifts were performed as described (Krieg et al. 1995), except that the *bcl-2* and *bcl-2M* probes were used at ~ 2 ng per reaction.

Cell-cycle analysis

We collected 10^6 cells at each time point and washed them once in PBS. The cells were then fixed in 1 ml ice-cold 70% ethanol/30% PBS for at least 30 min at 4°C. Cells were centrifuged and resuspended in 1 ml PBS containing 40 μ g/ml propidium iodide and 2 μ g/ml RNAse A. After incubation at 37°C for 30 min, cells were analyzed on a Becton Dickinson FACScan. Data were processed using LYSYS II software.

Apoptosis studies

For Hoechst 33258 staining, cells were fixed in 70% ethanol/30% PBS as above, then centrifuged and resuspended in 1 mg/ml Hoechst 33258. Following incubation in the dark for 15 min at 4°C, cells were examined under a fluorescence microscope. Apoptotic DNA fragments were isolated as described (Herrmann et al. 1994).

Mice

The derivation of the MEnT and MT transgenic mice has been described (Badiani et al. 1994). *E μ bcl-2-25* mice (Strasser et al. 1990) were a kind gift of Suzanne Cory. Thymocytes and splenocytes were prepared from 3- to 4-week-old mice by standard methods (Coligan et al. 1991). For survival assays, purified thymocytes and splenocytes were resuspended at 5×10^6 cells/ml in RPMI supplemented with 5% heat-inactivated FCS and either treated with 225 rads X-rays, 1 mM dexamethasone, or nothing. Cells were cultured for 18 hr in 5% CO₂ at 37°C, harvested, and counted by trypan blue exclusion. Experiments were performed in triplicate, and histograms depict the mean and standard deviations of a minimum of three separate experiments. For TUNEL assays, thymocytes were purified and resuspended at 2.5×10^6 cells/ml in RPMI containing 10% FCS and 5×10^{-5} M 2-mercaptoethanol. After 5 hr in culture, TUNEL assays were performed using an in situ cell death detection kit (Boehringer) according to the manufacturer's instructions. Cells were analyzed on a Becton Dickinson FACScan using LYSYS II software. TUNEL assays were performed on at least four 3-week-old animals from each transgenic line, together with four nontransgenic age-matched controls. Assays on MEnT/*bcl-2* double transgenics were performed twice.

Nuclear run-on assays

RNA isolation and transcript labeling were performed as described in Roberts and Bentley (1992). Hybridizations were carried out for 36 hr at 65°C in 1 ml 10 mM Tris at pH 7.5, 250 mg/ml RNA, 0.02% BSA, Ficoll 400, PVP360, 0.5% nonfat dry milk, 0.3 M NaCl, 1% SDS, 10 mM EDTA, using 1×10^7 cpm of labeled RNA. Filters were washed for 30 min at 65°C in $2 \times$ SSC, 0.1% SDS, and for another 30 min at 65°C in $0.2 \times$ SSC, 0.1% SDS. Filters were then treated with 10 mg/ml RNAse A in 0.3 M NaCl, 10 mM Tris at pH 7.5, 5 mM EDTA at 37°C for 30 min, and washed for 15 min at 65°C in $1 \times$ SSC prior to autoradiography. The experiment was performed four times.

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