v-Myb of E26 leukemia virus up-regulates *bcl-2* and suppresses apoptosis in myeloid cells

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Many oncogenes have been shown to be deregulated transcription factors, yet direct target genes mediating cell transformation remain elusive. Here we describe such a target for v-Myb by exploiting a temperature-sensitive mutant of the E26 avian leukemia virus encoding Myb-Ets. Myeloblasts transformed by the mutant differentiate into macrophages or die by apoptosis when shifted to the nonpermissive temperature as a result of inactivation of v-Myb. During this process mRNA of the antiapoptotic oncoprotein Bcl-2 is down-regulated with kinetics similar to those of *Mim-1*, a differentiation-related protein whose expression is directly regulated by Myb. Forced expression of *bcl-2* rescues the cells from apoptosis, without preventing either their withdrawal from the cell cycle or their differentiation. v-Myb appears to act directly on the *bcl-2* gene, because a *bcl-2* promoter-driven reporter is activated by Myb-Ets and v-Myb-VP16 and requires intact Myb binding sites within the promoter. Surprisingly, inactivation of v-Myb in multipotent progenitors transformed by E26 virus does not induce apoptosis, indicating that *bcl-2* regulation by the oncoprotein is required for the transformation of some cell types but not others.

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The c-Myb protein, which contains three homeo domain-related DNA binding repeats, acts as a sequence-specific transcription factor (for review, see Lüscher and Eisenman 1990; Graf 1992). Inactivation experiments in mice (Mucenski et al. 1991) have shown that it plays a central role in definitive hematopoiesis. Also, its abundant expression in various immature blood cell types suggests that it affects differentiation and/or growth control in a number of different lineages (Westin et al. 1982; Kastan et al. 1989). Myb was identified originally by its occurrence in two acute avian leukemia retrovirus strains, E26 and AMV (Roussel et al. 1979). In the E26 strain, the v-Myb oncoprotein is fused to v-Ets, and this is a prerequisite for the high leukemogenic capacity of the virus (Metz and Graf 1991a). The infection of yolk sac cells from 2-day-old embryos with E26 virus results in the transformation (i.e., induction of proliferation) of two different cell types: myeloblasts, which can differentiate into both macrophages and neutrophil granulocytes; and Myb-Ets-transformed progenitors (MEPs), which have the potential to differentiate into thrombocytes, erythrocytes, eosinophils, and myeloblasts (Graf et al. 1992; Frampton et al. 1995). v-Myb alone is sufficient to transform myeloid cells, because both E26 mutants lacking the *ets* oncogene and the AMV strain are

capable of transforming cells of this lineage (for review, see Lüscher and Eisenman 1990; Graf 1992).

In an attempt to unravel the mechanism of transformation by the E26 virus, our laboratory has developed, through the use of biological screens, a number of temperature-sensitive (*ts*) mutants with lesions in v-Myb. The mutation in the prototypic mutant, *ts21*, corresponds to a threonine to arginine substitution in the third repeat (R3) of the Myb DNA-binding domain (Frykberg et al. 1988). It completely abolishes specific Myb DNA binding activity at 42°C, the nonpermissive temperature (Frampton et al. 1995). *ts21*-transformed myeloblasts shifted to 42°C are arrested in their growth and differentiate into macrophages (Beug et al. 1984, 1987), whereas under the same conditions *ts21*-transformed MEPs are not growth-arrested and differentiate into thrombocytes (Frampton et al. 1995). Using a differential screening technique to search for downstream targets we have identified *mim-1* as a direct target gene of Myb (Ness et al. 1989). However, because *Mim-1* is a protein expressed in the granules of neutrophil granulocytes, it probably plays no role in establishing the transformed phenotype. A number of additional direct v-Myb targets have been identified recently, but none could be linked to the transforming capacity of the oncoprotein (for review, see Frampton et al. 1996). Targets relevant for transformation by v-Myb (as well as for other transcription factor-type oncogenes), therefore, remain to be identified.

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Here we show that v-Myb acts as a survival factor for myeloid cells by directly up-regulating the antiapoptotic *bcl-2* gene, making this gene a first target relevant to transformation by the oncogene.

Results

Myeloblasts transformed by ts21 E26 undergo apoptosis when shifted to 42°C

When *ts21* E26-transformed myeloblasts were shifted from 37°C (the permissive temperature) to 42°C (the non-permissive temperature) for 2 days we observed not only differentiating cells but also cell death (Beug et al. 1984, 1987; J. Frampton and T. Graf, unpubl.). This prompted us to ask whether the cells had died by apoptosis. Indeed, *ts21* myeloblasts shifted to 42°C for 60 hr exhibited fragmented nuclei and chromatin condensations (Fig. 1A)

Figure 1. Induction of apoptosis in *ts21* transformed myeloblasts shifted to 42°C. (A) Giemsa stained cytopins of *ts21* (*Myb^{ts}-Ets*) transformed myeloblasts grown at 37°C or shifted to 42°C for 60 hr. Cells with characteristics of programmed cell death can be seen in the shifted population. (B) Immunofluorescence staining of apoptotic cells in the *ts21* transformed cell sample shifted to 42°C. The same field is viewed with different filters to show staining of all nuclei (Hoechst) and those that are apoptotic (TUNEL). Note that the apoptotic cells can also be recognized by Hoechst staining because of their condensed chromatin. (C) Quantification of the induction of apoptosis in individual myeloblast clones. Six independent clones transformed by either wild-type E26 or *ts21* were grown at 37°C (open bars) or 42°C (solid bars) for 60 hr and the percentage of apoptotic cells determined by TUNEL staining. The average number of positive cells at 37°C compared with 42°C was 11.2% and 58.0% for *ts21* cells and 2.5% and 7.7% for wild-type E26 cells.

characteristic of programmed cell death. This was confirmed by labeling the cells by the TUNEL technique, which visualizes DNA fragments generated by endonucleolytic cleavage (Fig. 1B). To quantify this effect, six randomly chosen clones transformed at 37°C by *ts21* E26 or wild-type E26 were split and either kept at 37°C or shifted to 42°C for 60 hr. As shown in Figure 1C, there was a dramatic increase in the number of apoptotic cells in all *ts21*-transformed clones at 42°C but only a slight increase in the wild-type E26 myeloblasts under the same conditions. In addition, *ts21*-transformed clones already exhibited an elevated level of apoptotic cells at 37°C relative to the wild-type E26 clones, possibly reflecting the fact that the DNA binding capacity of the mutant Myb DNA binding domain is partially impaired at the permissive temperature (Frampton et al. 1995).

These results show that inactivation of the Myb-Ets oncoprotein in transformed myeloblasts induces apoptosis in a large fraction of the cells.

The antiapoptotic effect of ts21 E26 is mediated by v-Myb

The fact that the lesion in *ts21* E26 is located in the DNA binding domain of Myb, completely abolishing the specific DNA binding capacity of v-Myb at 42°C (Frampton et al. 1995), suggests that the observed antiapoptotic effect of Myb-Ets is a result of the Myb portion of the oncoprotein. However, it is possible that the mutation acts indirectly by affecting the v-Ets portion of the molecule, which, like v-Myb, consists of DNA binding and transactivation domains. Earlier work with MEPs transformed by a mutant of E26 with a lesion in the DNA binding domain of Ets (*ts1.1* E26) that renders its DNA-binding capacity temperature-sensitive showed that the cells are induced to differentiate predominantly along the erythroid lineage when shifted to 42°C; however, v-Ets inactivation had no effect on the differentiation of *ts1.1*-transformed myeloid cells. (Golay et al. 1988; Kraut et al. 1994). We, therefore, tested whether programmed cell death would be induced in *ts1.1*-transformed myeloid cells at the nonpermissive temperature. As shown in Figure 2A, of three *ts1.1*-transformed myeloid cell clones tested, none exhibited a significant increase in apoptosis at 42°C, even as late as 80 hr after shift. In contrast and as expected, a sizable proportion of two *ts21*-transformed myeloblast clones grown in parallel and shifted under identical conditions died by apoptosis.

These results support the idea that the antiapoptotic effect of Myb-Ets is confined to the v-Myb portion of the fusion protein. However, they do not rule out the possibility that apoptosis is prevented by an interaction between the two domains within the fusion protein. To examine this possibility we assessed whether myeloid cells transformed by a separately expressed temperature-sensitive v-Myb would undergo apoptosis at 42°C. Because cells transformed by v-Myb have a limited growth potential, we used myeloid cells transformed by a variant of *ts21* E26 in which Myb and Ets are expressed sep-

Figure 2. Requirement of functional v-Myb for protection from apoptosis. (A) Comparison of the number of apoptotic cells in two clones transformed by *ts21* (Myb^{ts}-Ets) and in three myeloblast clones transformed by *ts1.1* (Myb-Ets^{ts}) grown at 37°C (open bars) or 42°C (solid bars) for 72 hr. (B) Comparison of the number of apoptotic cells in four myeloblast clones transformed by *tsMyb/Ets* and in two clones transformed by *Myb/Ets* grown at 37°C (open bars) or 42°C (solid bars) for 72 hr.

arately (*ts Myb/Ets*) (Metz and Graf 1991b). As shown in Figure 2B, of four clones transformed by *ts Myb/Ets*, all showed a dramatic increase in apoptosis at 42°C whereas two clones transformed by wild-type *Myb/Ets* only showed a marginal increase. We conclude that the myeloid survival-inducing function of the *Myb-Ets* oncoprotein is mediated by its v-Myb moiety.

The antiapoptotic effect of v-Myb is cell type-specific

We have shown previously that *ts21* E26-transformed MEPs shifted to the nonpermissive temperature differentiate into mature thrombocytes within 2–3 days (Frampton et al. 1995). To test whether this also leads to an increased rate of apoptosis we shifted eight clones individually transformed by either *ts21* or wild-type E26 to 42°C for 60 hr, or kept them at 37°C. Surprisingly, none of the clones showed an increased rate of apoptosis at the nonpermissive temperature. On the contrary, the majority of the *ts21* clones actually exhibited a slightly decreased rate of apoptosis at 42°C. This effect was mutant-specific as it was not seen with wild-type E26-transformed MEPs (Fig. 3).

Inactivation of v-Myb in transformed myeloblasts down-regulates *bcl-2* expression

To examine whether *bcl-2* expression is affected by v-Myb inactivation, a Northern blot analysis was performed with RNAs from *ts21* and wild-type E26 myeloblasts grown at 37°C or shifted to 42°C. As shown in Figure 4A, *bcl-2* RNA was almost completely down-regulated after a 24-hr shift to 42°C in the *ts21* but not in the wild-type cells, and became reexpressed within only 2 hr when the cells were backshifted to 37°C. The loss of *bcl-2* RNA and its rapid reappearance after backshift was paralleled by the pattern of expression of *mim-1*, a previously identified direct target gene of v-Myb. In the

Figure 3. Lack of induction of apoptosis in *ts21* transformed MEPs shifted to 42°C. The figure shows a comparison of the number of apoptotic cells in eight individual MEP clones transformed by *ts21* or wild-type E26 and grown at 37°C (open bars) or 42°C (solid bars) for 60 hr.

same experiment RNA of *c-fms* (a marker of mature myeloid cells that encodes the CSF-1 receptor) was up-regulated at 42°C and remained unchanged for 2 hr after backshift. In contrast to the results obtained with myeloid cells, no significant change in the expression of *bcl-2* RNA could be observed in *ts21*-transformed MEPs after shift to 42°C (Fig. 4B).

The observed down-regulation of *bcl-2* RNA in *ts21*-transformed myeloblasts within 1 day at 42°C, together with the finding that increased apoptosis became apparent only after 2–3 days, raised the question of whether both processes are related. We therefore examined by Western blotting the change in Bcl-2 protein levels when *ts21*-transformed myeloblasts grown at 37°C were shifted to 42°C for 24 hr. In addition, we probed the blot for MAP kinase as a control for protein levels and for *cdc2* as an indication of the cell cycling status. As shown in Figure 4C, Bcl-2 expression was reduced significantly whereas no change was seen for MAP kinase. In contrast the *cdc2* protein was no longer detectable, reflecting the fact that the cells had exited the cell cycle. This result confirms the known relatively long half-life of Bcl-2 (Kitada et al. 1993) compared with *cdc2* kinase.

We also tested whether the reexpression of *bcl-2* mRNA upon backshift to 37°C occurred in the presence of the protein synthesis inhibitor cycloheximide. Backshifting of *ts21* myeloblasts that had been grown at 42°C for 40 hr was performed for 2 hr in the presence or absence of 5 µg/ml cycloheximide. As can be seen in Figure 4D, the extent of reactivation was little affected by this treatment.

In summary, *bcl-2* mRNA is down-regulated in myeloid cells when v-Myb is inactivated and becomes rapidly reexpressed upon v-Myb reactivation even when de novo protein synthesis is inhibited. In addition, the relatively late onset of apoptosis compared with the reduction in RNA expression correlates with the presence of residual Bcl-2 protein and probably reflects the protein's high stability.

Forced expression of *bcl-2* in *ts21* myeloblasts shifted to 42°C prevents apoptosis but not growth arrest

To test whether apoptosis induced by v-Myb inactiva-

Figure 4. Down-regulation of *bcl-2* RNA expression in *ts21* transformed myeloblasts shifted to 42°C. (A) Northern blot of RNAs from wild-type E26 and *ts21* transformed myeloblasts grown at 37°C, 42°C for 24 hr, or 42°C for 24 hr and then shifted back to 37°C for 2 hr. The blot was probed sequentially with *bcl-2*, *mim-1*, *c-fms*, and β -actin cDNA probes. (B) Northern blot of RNAs from *ts21* transformed MEPs grown as described in A and probed with *bcl-2* and β -actin. Quantification of the signals by scanning of the autoradiograms allowed an approximate comparison of the *bcl-2*/ β -actin RNA ratios. Setting the ratio to unity for the 37°C lanes, then the *ts21* myeloblasts at 42°C exhibit a ratio of <0.15 as compared with 0.8 for *ts21* MEPs under the same condi-

tions. (C) Western blot of total cell extracts derived from 10^6 *ts21* myeloblasts grown at 37°C or at 42°C for 24 hr. The lysates were run on a 10% polyacrylamide gel, blotted, and probed sequentially with antibodies directed against Bcl-2, MAP kinase, and cdc2, by use of the ECL technique. (D) Northern blot of RNAs from *ts21* transformed myeloblasts probed for *bcl-2* and β -actin. The RNAs were prepared from cells grown at 37°C, 42°C for 40 hr, 42°C for 40 hr, and then backshifted to 37°C for 2 hr, or 42°C for 40 hr, and backshifted to 37°C in the presence of 5 μ g/ml cycloheximide (CHX).

tion can be prevented by forced expression of *bcl-2*, we designed a retrovirus that coexpresses the Myb-Ets fusion protein and Bcl-2 from a single bicistronic mRNA (Fig. 5A). We found that ectopic Bcl-2 reduced the degree of apoptosis induced by v-Myb inactivation in all seven clones tested, to a level comparable to clones transformed by wild-type E26 (Fig. 5B). To determine whether the cells still became quiescent, they were labeled with bromodeoxyuridine (BrdU) and their DNA content was analyzed by flow cytometry. As shown in Figure 6, 55% and 63%, respectively, of the *ts21* and *ts21/bcl-2* trans-

formed myeloblasts grown at 37°C incorporated BrdU and >40% of these cells had progressed to G₂/M. In contrast, only 16% and 17.5% respectively of the cells were labeled in the corresponding populations after shift to 42°C and of these <20% had entered G₂/M. The BrdU negative population with low DNA content seen in the *ts21* cells grown at 42°C probably consists of fragmented

Figure 5. Effect of forced Bcl-2 expression on cell survival. (A) Schematic representation of the Myb^{ts}-Ets/Bcl-2 retrovirus. The internal ribosome entry site (IRES), the viral long terminal repeats (LTR), and the second initiating ATG codon are indicated. (B) Comparison of the proportion of apoptotic cells in seven myeloblast clones transformed by *ts21* or *ts21/bcl-2* grown at 37°C (open bars) or 42°C (solid bars) for 60 hr.

Figure 6. Effect of forced Bcl-2 expression on the cell cycle. Analysis of nuclear DNA content and the proportion of cells entering S phase. The diagram shows a flow cytometric analysis of *ts21* or *ts21/bcl-2* transformed myeloblast clones grown at 37°C or at 42°C for 2 days. Cells that incorporated BrdU (included in the last 12 hr of the incubation period) were detected by immunofluorescence (FL1) and total DNA content was monitored by propidium iodide staining (FL2).

nuclei from apoptotic cells because no such population is seen in the *ts21/bcl-2* sample.

These findings show that the induction of apoptosis in *ts21* myeloblasts at 42°C can be prevented by forced *bcl-2* expression, without affecting the cell's withdrawal from the cell cycle.

ts21/bcl-2 myeloblasts shifted to 42°C differentiate into both macrophage- and granulocyte-like cells

Next we tested whether ectopic *bcl-2* expression has an effect on the temperature-induced differentiation of *ts21/bcl-2* transformed myeloblasts. For this purpose, we analyzed the cell morphology of 11 clones transformed by either *ts21* or *ts21/bcl-2*, kept at 37°C or shifted to 42°C for 3 days. In several of the shifted clones ectopic expression of *bcl-2* led to the formation of granules characteristic of immature avian neutrophil granulocytes (Lucas and Jamroz 1961; Golay et al. 1988). In the same clones similar cells were already detectable at 37°C, although at lower proportions than at 42°C. This is illustrated for clone C10 in the micrograph of Figure 7A and quantified for all clones in Figure 7B. Other clones shifted to 42°C resembled immature macrophages, although their differentiation was less dramatic than that of *ts21* clones shifted to 42°C. This is shown for clone D4 in Figure 7A. When a pool of *ts21/bcl-2* transformed myeloblasts shifted to 42°C was examined for the expression of the Myb target gene *mim-1* they showed a clear decrease of expression with time although this downregulation was less severe than in *ts21* myeloblasts in the absence of ectopically expressed Bcl-2 (Fig. 7C).

These results show that forced *bcl-2* expression in *ts21* myeloblasts shifted to 42°C allows the detection of granulocyte-like cells in addition to the macrophage-like cells that are typically seen when *bcl-2* is not overexpressed. In addition, it appears that *bcl-2* partially impairs macrophage differentiation.

Withdrawal of cMGF from Myb-Ets transformed myeloblasts causes growth arrest but not apoptosis

The proliferation of myelomonocytic cells transformed by the E26 virus is dependent on the presence of chicken myelomonocytic growth factor (cMGF) (Beug et al. 1982; Leutz et al. 1984). To determine whether factor withdrawal has an impact on cell death, *ts21* myeloblasts were thoroughly washed and reseeded at 37°C in the presence or absence of cMGF. As a control, the cells were also shifted to 42°C in the presence of cMGF. Sixty hours later the number of apoptotic cells was determined and the growth rate of the population assessed by thymidine incorporation. As shown in Figure 8A, *ts21* myeloblasts grown at 37°C exhibited no appreciable increase in cell death upon removal of the growth factor. Nevertheless, growth factor withdrawal resulted in a 3- to 4-fold decrease in thymidine incorporation (Fig. 8B). As expected, *ts21* myeloblasts shifted to 42°C showed a significant increase in apoptosis in addition to a decreased level of thymidine incorporation.

Figure 7. Effect of forced Bcl-2 expression on differentiation. (A) Micrographs of cells prepared by cytocentrifugation and Giemsa staining of *ts21/bcl-2* clones grown at 37°C and 42°C. Clones C10 and D4 represent examples that mature predominantly into more granulocytic or monocytic phenotypes, respectively. (B) Quantification of granulocytic differentiation of individual *ts21* or *ts21/bcl-2* transformed myeloblast clones grown for 4 days at 37°C (open bars) or at 42°C (solid bars). (C) Northern blot of 5 µg total RNA derived from *ts21* or *ts21/bcl-2* transformed myeloblast clones grown at 37°C or at 42°C for 1 or 2 days. The blot was hybridized sequentially with probes for *mim-1* and β -actin.

These results show that growth factor depletion of Myb-Ets transformed myeloid cells leads to their withdrawal from the cell cycle without inducing apoptosis.

Myb binds to specific sites on the bcl-2 P2 promoter

The observed rescue by Bcl-2 of *ts21* myeloblasts shifted to 42°C and the rapid reexpression of *bcl-2* RNA during backshift raised the possibility that the *bcl-2* gene is regulated directly by Myb. The human *bcl-2* promoter has been shown to have two major sites of transcription initiation (Seto et al. 1988); an upstream site (P1), which has

Figure 8. Effect of growth factor withdrawal on transformed myeloblasts. Two *ts21* transformed myeloblast clones were grown for 60 hr at 37°C or at 42°C in the presence of cMGF (open and solid bars, respectively) or at 37°C in the absence of cMGF (gray bars). Aliquots of the cells were then stained with Hoechst to determine the proportion of apoptotic cells (A) or labeled for 2 hr with ³H-thymidine (B) to determine their growth potential.

features of a housekeeping-type promoter and which drives expression of RNA starting at exon 1, and a second site (P2), which is located within exon 2. Inspection of the sequences of the P2 promoters of the human (Seto et al. 1988), mouse (Negrini et al. 1987), and chicken *bcl-2* genes (Eguchi et al. 1992; Fig. 9A) revealed a number of perfect matches to the core Myb consensus recognition element (Weston 1992) whereas no such matches were found in the P1 region. To identify sites which are capable of binding to Myb in vitro we performed a DNaseI footprint analysis of a fragment encompassing the P2 promoter with bacterially expressed Myb. As shown in Figure 9B, of the four consensus Myb binding sites (MREs) present in the sequence analyzed (Fig. 9A), three were found to be protected by Myb.

Myb transactivates a reporter driven by the *bcl-2* P2 promoter, requiring intact *Myb* binding sites

To determine whether Myb transactivates the *bcl-2* promoter we constructed a luciferase reporter driven by the chicken *bcl-2* P2 region and tested its activity in transient transfection assays. For comparison, we tested a reporter driven by the MRE from the *mim-1* gene. Cotransfection of a vector expressing the Myb-Ets fusion protein with the *bcl-2* P2 promoter led to a ~12-fold activation of the promoter, compared with a ~27-fold effect on the *mim-1* promoter (Fig. 10A). In addition, Myb-Ets was significantly more active on the wild-type *bcl-2* P2 promoter than on a mutated version in which each of the four MREs were altered so as to impair binding by Myb (Fig. 10B). As the E26 v-Myb lacking the Ets sequences was found to stimulate transcription from the *bcl-2* P2 promoter only by a factor of 1.5–2, we determined whether the activity of v-Myb could be enhanced by fusing the oncoprotein with the strong VP16 activation domain. This had been found previously to increase

Figure 9. The chicken *bcl-2* promoter and Myb binding sites. (A) Schematic representation of the chicken *bcl-2* promoter showing the first two exons. (NRE) Negative regulatory element; (ORF) open reading frame. The blowup shows the sequence of exon 2 until the translation initiation codon. Consensus Myb recognition elements (Sites 1–4) are indicated by bold letters; sequences that are protected by Myb (see below) are boxed. The location of the mutations introduced within the four potential Myb binding sites is indicated (A to G or T to C). (B) DNase nuclease protection analysis of the chicken *bcl-2* P2 promoter with bacterially expressed Myb. DNA prepared in the absence of Myb (lane 1); with 0.2 µg of Myb (lane 2); with 2 µg of Myb (lane 3). The two regions protected from digestion by DNase are indicated and correspond to the boxed sequences in A.

its activity on the *mim-1* promoter (Frampton et al. 1993). As shown in Figure 10C, v-Myb-VP16 gave a 4- to 5-fold activation of the wild-type *bcl-2* P2 promoter and only a marginal activation of its mutated version.

These observations indicate that the *bcl-2* P2 promoter can be transactivated both by Myb-Ets and by v-Myb-VP16 and that this requires intact Myb binding sites within the promoter.

Discussion

Using a temperature-sensitive mutant of the E26 leukemia virus encoding Myb-Ets we have shown here that a functional form of v-Myb prevents primary chicken myeloblasts from undergoing apoptosis. This is paralleled by the ability of the oncoprotein to maintain high levels of *bcl-2* expression through what appears to be a direct effect on the promoter of the gene. The antiapoptotic properties of Myb-Ets can explain, at least in part, its capacity to transform myelomonocytic cells and makes it the first nuclear oncoprotein for which a transformation relevant target gene has been identified. Using a similar *ts* mutant approach, v-Rel has been shown to rescue T cells from apoptosis, although a direct target responsible for this effect has not been described (White

Figure 10. Transactivation of the *bcl-2* P2 promoter. (A) Transactivation by Myb-Ets. QT6 fibroblasts were cotransfected with either the *bcl-2*/P2 or the *mim-1* luciferase reporter together with either 10 ng of the Myb-Ets expressing plasmid or the corresponding vector DNA (-). (B,C) Requirement for intact Myb binding sites within the *bcl-2* P2 promoter for transactivation by Myb-Ets and Myb-VP16. Transfections were performed with either the *bcl-2*-P2 reporter (Bcl-2) (open bars) or the mutated reporter construct (Bcl-2 mut) (solid bars) together with (B) the Myb-Ets expressing plasmid (10 ng) or with (C) the construct expressing v-Myb-VP16 (2 ng or 5 ng).

et al. 1994). Likewise, the mechanism by which Myc and AP-1 accelerate apoptosis in fibroblasts and in neuronal cells (Evan et al. 1992; Ham et al. 1995) has yet to be elucidated. However, it has been reported recently that apoptosis induced by the tumor suppressor protein p53 is mediated by its direct activation of the death promoting gene *bax* (Miyashita and Reed 1995).

Is the *bcl-2* gene a direct target of v-Myb?

A number of observations suggest that v-Myb directly controls *bcl-2* expression through interaction with the P2 promoter of the gene. P2 is located upstream of the translation initiation site in exon 2; this region also contains a negative regulatory element implicated in stage-specific expression of *bcl-2* in human B cells (Young and Korsmeyer 1993) and confers sensitivity to p53 in human lung and murine M1 myeloid leukemic cells (Miyashita et al. 1994a,b). In the equivalent region of the chicken *bcl-2* gene we now have identified three MREs whose integrity is required for the promoter's full responsiveness to Myb-Ets and v-Myb. In addition, we found that *ts21* myeloblasts shifted back from 42°C to 37°C reaccumulate *bcl-2* RNA within only 2 hr, with kinetics similar to those seen for the Myb-regulated gene *mim-1* (Ness et al. 1989). Finally, and again as for the *mim-1* gene, we observed reexpression of *bcl-2* RNA in the presence of cycloheximide, indicating that de novo protein

synthesis is not required upon reactivation of v-Myb. These results support the idea that v-Myb regulates the *bcl-2* promoter by directly binding to it.

The finding that Myb-Ets is more active in regulating *bcl-2* P2 than v-Myb raises the possibility that the Ets portion of the fusion protein also participates in *bcl-2* activation, such as by binding to other sites of the promoter. Indeed, several GGAA/T Ets core binding sites can be identified in the P2 promoter. However, in myeloid cells transformed by a *ts* mutant of E26 with a lesion in Ets, we observed no effect on apoptosis upon shifting to the nonpermissive temperature. In addition, since Myb-Ets has been found to be more active than v-Myb on promoters not known to contain Ets responsive elements, such as that of the *mim-1* gene (Frampton et al. 1993), the Ets domain of the oncoprotein might not bind to DNA but instead serve to recruit other factors to the transcription activation complex. This would be consistent with the recent identification of nuclear proteins that specifically interact with c-Ets-1 (Sieweke et al. 1996).

Myb, cell transformation, and differentiation

v-Myb is known to arrest the differentiation of myeloid cells (Beug et al. 1984, 1987) at a stage where their normal counterparts express *bcl-2* (see below). It is, therefore, difficult to assess the relationship between the differentiation-blocking and the survival-inducing capacity of the oncoprotein. For the reasons outlined above, it is unlikely that Myb targets that mediate the differentiation block active *bcl-2* indirectly. However, it is still possible that Myb concomitantly regulates both *bcl-2* and unknown target genes involved in blocking differentiation, or that it only activates *bcl-2*, which in turn mediates the differentiation block. Our data suggest that a combination of the latter possibilities might be correct because forced expression of *bcl-2* in *ts21* cells shifted to 42°C not only prevented apoptosis but also slightly impaired differentiation into macrophages. This phenomenon deserves further study. The finding that cMGF withdrawal causes growth arrest without inducing apoptosis is consistent with a constitutive up-regulation of *bcl-2* in Myb-Ets-transformed myeloblasts. In contrast, in spontaneously transformed myeloid cell lines such as FDCP-1 or FDCP-2, factor deprivation induces apoptosis (Fairbairn et al. 1993). In addition, it has been shown recently that forced expression of Myb-Ets in FDCP-2 cells (kept in the presence of erythropoietin) prevents them from apoptotic death following withdrawal of IL-3 (Athanasίου et al. 1996).

Although activation and maintenance of *bcl-2* expression appears to be required for the myeloid cell transforming capacity of v-Myb, it is not likely to be sufficient. Thus, the oncoprotein not only blocks differentiation but also facilitates differentiation and induces cell proliferation (for review, see Graf 1992). Several myeloid differentiation specific genes have been described that are directly activated or repressed by v/c-Myb in conjunction with other transcription factors, such as *mim-1*

(Ness et al. 1989), the CD13 aminopeptidase gene (Shapiro 1995), and *c-fms* (Reddy et al. 1994). However, because none of these targets seem to be involved in controlling growth or in blocking differentiation, it is doubtful that they play a role in cell transformation by v-Myb.

The observed capacity of v-Myb to regulate *bcl-2* expression cannot be a general explanation for the transforming capacity of the oncoprotein because *ts21*-transformed MEPs do not undergo apoptosis and retain high levels of *bcl-2* expression. This indicates that *bcl-2* is controlled independently from v-Myb in MEPs and suggests that the mechanism by which the oncogene transforms cells differs according to the cell type involved. In support for this notion is the fact that v-Myb does not transform fibroblasts (or does so only weakly) (Graf 1973; Beug et al. 1984). The diversity of transformation mechanisms by v-Myb probably reflects the requirement to act in concert with other tissue-restricted transcription factors. For example, Myb-Ets regulates the expression of the *mim-1* gene in transformed myeloblasts but not in MEP cells (Ness et al. 1989); this is accounted for by its cooperation with C/EBP β , a protein whose expression in hematopoietic tissues mirrors that of Mim-1 (Burk et al. 1993; Ness et al. 1993).

Myb, Bcl-2, and leukemogenesis

A question raised by our findings is whether the antiapoptotic effect of v-Myb is relevant to the leukemogenic potential of the oncogene. As the predominant type of leukemic cells induced by the E26 virus corresponds to immature erythroid/MEP-type cells, with variable contributions of myeloblasts (Radke et al. 1982; Graf et al. 1992), the answer is not simple. v-Myb of E26, either alone or in combination with a separately expressed v-Ets, is not leukemogenic or only weakly so (Metz and Graf 1991b) and may reflect the factor dependence of both v-Myb and Myb/Ets transformed myeloblasts (Leutz et al. 1984). Indeed, viruses containing E26 v-Myb (in the absence of Ets) together with either an autocrine growth-inducing oncogene, such as *v-erbB* or the cMGF gene, cause acute myeloblastic leukemias (Metz et al. 1991). In addition, the highly mutated v-Myb protein of AMV virus possibly causes monoblastic leukemia because the transformed cells also appear to produce an autocrine growth factor (for review, see Graf 1992). This might make it difficult to determine whether AMV, like E26, protects primary myeloid cells from apoptosis.

Our observations raise the possibility that alterations in the *c-myb* gene, resulting in *bcl-2* up-regulation and prevention of apoptosis, may contribute to the generation of human myeloid leukemias. Although rearrangements, amplifications or overexpressions of *c-myb* have been reported for only a relatively small number of human lymphoid and myeloid malignancies (for review, see Lüscher and Eisenman 1990; Shen-Ong 1990), more subtle alterations such as point mutations might have been missed. It is striking that most human acute and chronic myeloid leukemias in blast crisis express high levels of *bcl-2* (Delia et al. 1992). Whether this is caused by a

deregulated *c-myb* gene or simply reflects their immature stage of differentiation is unclear. In the light of the observed v-Myb-induced protection of myeloid cells from apoptosis under conditions of growth factor deprivation it seems worth reexamining individual cultures of human myeloid leukemias for their response to cytokine starvation.

Does the proposed function of v-Myb reflect the activity of cellular Myb proteins?

Several indirect lines of evidence suggest that v-Myb may mimic an antiapoptotic function of c-Myb in myeloid cells. Thus, immature myelomonocytic cells express high levels of both *c-myb* (Gonda and Metcalf 1984; Kastan et al. 1989) and *bcl-2* (Quéva et al. 1992), and these genes are downregulated as the cells mature (Kastan et al. 1989; Delia et al. 1992). Neutrophils, which are known to have a short life span due to their propensity to die by apoptosis, can be prevented from dying by overexpression of *bcl-2* (Lagasse and Weissman 1994). Similarly, we have observed that the presence or absence of constitutively expressed *bcl-2* in *ts21*-transformed myeloblasts shifted to 42°C results, respectively, in either the formation of immature granulocytes in addition to monocytic cells or to apoptotic death and monocytic cells. As in our system, differentiated macrophages lack *bcl-2* expression. This suggests that cells committed to differentiate into granulocytes die by apoptosis because they lose *bcl-2* and that monocyte/macrophage-type cells survive because they do not require *bcl-2* for survival.

The observation that a c-Myb expressing murine retrovirus transforms myelomonocytic cells kept at high cell densities (Ferraro et al. 1995) is also compatible with a myeloid cell survival-inducing function of the protein. In addition, c-Myb overexpression in the avian myeloid line BM2 has been shown to prevent phorbol ester-induced apoptosis (Smarda and Lipsick 1994), although in another study, using the murine M1 myeloid cell line, c-Myb was found to accelerate TGF- β -induced apoptosis (Selvakumaran et al. 1994). Finally, the observation that expression of a dominant negative Myb/Engrailed chimera dramatically increases apoptosis in T-cells (Taylor et al., this issue) indicates that c-Myb has a survival-inducing function in this cell type.

Neither the overexpression nor the dominant-interfering approaches exclude the possibility that other Myb family members play a role in regulating apoptosis. In particular, because B-Myb, like c-Myb, is also expressed in myeloblasts (Golay et al. 1991) and its ectopic expression accelerates TGF- β induced apoptosis in the M1 cell line (Bies and Wolff 1995), it might likewise be involved in the regulation of programmed cell death. To resolve the question of whether c-Myb indeed promotes the survival and/or life expectancy of myeloid cells under physiological conditions, it will be crucial to reexamine myelopoiesis in both knockout and transgenic mice.

Our inability to show a significant activity of c-Myb on the *bcl-2* P2 promoter construct (J. Frampton, unpubl.)

seems to contradict the proposal that, like v-Myb, it regulates *bcl-2* expression. However, because c-Myb has been shown to transactivate the *bcl-2* P2 promoter in 3T3 cells (D. Taylor, P. Badiani, and K. Weston, submitted), it is possible that the QT6 cell line used in our experiments lacks a coactivator necessary for regulation of the gene. That v-Myb is more active on the *bcl-2* P2 promoter than c-Myb reflects findings made earlier with the *mim-1* promoter (Quéva et al. 1992) and might be due to the lack of the negative regulatory domain present in its cellular counterpart (Sakura et al. 1989; Dubendorff et al. 1992). Our results, therefore, suggest that v-Myb-encoding retroviruses have evolved to efficiently evade a cellular mechanism that down-regulates *bcl-2* expression in myeloid cells as a consequence of c-Myb inactivation. In this view, *ts* mutants of E26 with a lesion in v-Myb might serve as a simple model to study how c-Myb regulates the *bcl-2* gene.

Materials and methods

Viruses and virus constructs

The *ts21* wild-type E26 have been described previously (Beug et al. 1984). The viruses expressing Myb and Ets as separate proteins also have been described (Metz and Graf 1991b). To achieve expression of Bcl-2 in myeloid cells transformed by the *ts21* virus we constructed a retrovirus that produces a bicistronic mRNA encoding Myb^{ts}-Ets and Bcl-2. The basic retroviral structure was derived from pMI3, which contains the *gag-myb-ets* sequences between AEV LTRs (Introna et al. 1990). A *PvuII* fragment from the plasmid pCITE2a (Novagen) containing the EMC virus IRES (Jang et al. 1988) and a multiple cloning site was inserted immediately 3' to the termination codon of the fusion protein at a unique *KpnI* site. To generate a Bcl-2 expressing virus a 0.7-kb *EcoRI*-*SspI* fragment from the chicken *bcl-2* cDNA was subcloned from the plasmid pBI-31 (Cazals-Hatem et al. 1992) into pBluescript-KS between the *EcoRI* site and a blunt-ended *BamHI* site. This was subsequently shuttled into the polylinker 3' of the IRES element between the *NcoI* and *XbaI* sites, using the *NcoI* at the *bcl-2* ATG codon.

Constructs used in transient transfection assays

The *bcl-2*/P2 reporter plasmid was derived from the pXP1 promoterless luciferase vector (deWet et al. 1987) into which we cloned a PCR amplified fragment of the chicken *bcl-2* gene from residues -303 to -1 (Eguchi et al. 1992). The 5' and 3' oligonucleotides used to PCR amplify this region were 5'-GAG-GAAGCGTTGCTCAAGT-3' and 5'-GGTTTCCGAGGG-GAAGCA-3'. An equivalent fragment mutated at the four consensus MREs was inserted to generate the *bcl-2*/P2 Mut reporter. Mutation was achieved by PCR using the oligonucleotides 5'-GCAACAGTCATAATCCAGTCAAGGTGTGG-3' (sites 1+2), 5'-TAAATTAAGAACGGCGACAGT-3' (site 3) and 5'-AAGCAGCCGACGGTAATAAAA-3' (site 4) in which the underlined residues are the mutated sites in the MREs (bold-face). The *mim-1*-luciferase control reporter construct has been described (Ness et al. 1989).

Most of the plasmids encoding the Myb activator proteins have been described previously: Myb-Ets (pMI3; Introna et al. 1990), Myb (pMI10; Introna et al. 1990), and MybVP16 (Frampton et al. 1993). A vector control was constructed from pMI3 by

removal of all sequences between the retroviral LTRs by digestion with *SstI* followed by religation (pMI3ΔX; F. Lim, unpubl.).

QT6 quail fibroblasts were transfected with reporter and activator constructs by calcium phosphate coprecipitation. Cells (3×10^5) were transfected with 500 ng of the reporter and variable amounts of the activator. Additionally 250 ng of an RSV LTR- β -galactosidase construct was included as an internal control. Cell extracts were made and assayed for luciferase and β -galactosidase activities after 2 days. All values were normalized for β -galactosidase activity.

DNAseI protection assay for in vitro DNA-protein binding

A *HindIII*-*XbaI* fragment derived from the *bcl-2*/P2-luciferase construct was labeled by fill-in with Klenow polymerase in the presence of [α -³²P]dCTP. Further digestion was performed with *BamHI* to generate a 480-bp fragment labeled at the promoter-proximal end. DNAseI footprinting was carried out using standard procedures (Sambrook et al. 1989).

The Myb protein used in the footprinting analysis was obtained following transformation of bacterial strain BL21(DE3) with the pET-Myb5' construct (F. Lim, unpubl.), containing c-Myb coding sequences up to the carboxyl terminus of the DNA binding domain. Colonies were grown to exponential phase and then induced for 3 hr by the addition of 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG). To prepare proteins the bacteria were pelleted, resuspended, and sonicated in one volume of 10 mM HEPES at pH 7.9 and 100 mM KCl. After centrifugation, the pellet material was resuspended in one volume of STED (40 mM Tris-HCl at pH 7.4, 0.25 mM EDTA, 1 mM DTT, and 25% sucrose). Proteins in the pellet, which consisted of up to 50% of Myb, were denatured by adding urea to a concentration of 4 M at 4°C for 30 min. The solubilized denatured proteins were renatured by dialysis against STED and 1 M urea and then STED.

Cell culture and transformation assays

Chicken cells were obtained from 2-day-old blastoderms of a White Leghorn flock (Lohmann, Cuxhaven). Blastoderm medium consisted of DMEM, 10% fetal calf serum, 2.5% chicken serum, 0.2% NaHCO₃, 56 μ g/ml conalbumin, 80 mM β -mercaptoethanol, and 0.9 μ g/ml insulin. Unless otherwise indicated, all cells were maintained at 37°C in 5% CO₂. Production of virus stocks and the generation of transformed myeloblasts and MEPs by infection of 2-day-old chick embryos has been described previously (Graf et al. 1992). Myeloblast colonies were grown in DMEM containing 10 mM HEPES, 8% fetal calf serum, 2% chicken serum, and 10 units/ml cMGF (Leutz et al. 1984), whereas MEPs were maintained in blastoderm medium (see above).

Cytological and flow cytometric characterization of cells

To visualize the morphology of individual transformed colonies, cells were subjected to cytocentrifugation and stained with a version of the May Grünwald-Giemsa protocol (Diff Quik, Harleco). To determine the extent of apoptosis cells were pelleted and resuspended in 100 ng/ml Hoechst 33258 in PBS. Staining of apoptotic cells with dUTP-FITC was done on cytospin preparations using a modification (Sgonc et al. 1994) of the TUNEL method (Gavrieli et al. 1992). Proliferation of cells was determined by incorporation of ³H-thymidine into newly synthesized DNA as described previously (Beug et al. 1984). To determine DNA content, cells were fixed in 70% ethanol, pelleted, and resuspended in 50 mM sodium citrate, 0.1 mg/ml RNaseA, and 0.01 mg/ml propidium iodide. Following incuba-

tion at 37°C for 2 hr the stained cells were analyzed on a FAC-Scan flow cytometer (Becton Dickinson) using Consort software.

To measure the proportion of cells undergoing S phase, cells were labeled for a defined time with 20 μ M BrdU and then pelleted and fixed in 70% ethanol for 15 min at room temperature. The genomic DNA was denatured by incubation of the fixed cells in 0.5 ml 1 N HCl and 0.5% Tween 20 at 37°C for 15 min. After washing twice in 1 ml ice-cold PBS and 1% BSA the cell pellet was resuspended in 50 μ l anti-BUDR monoclonal antibody (Partec) diluted 1:50 in PBS and 0.1% Triton X-100. After 20 min on ice followed by one wash in PBS and 1% BSA, bound anti-BUDR was detected by incubating the cells in 50 μ l 1:40 diluted goat antimouse Ig-FITC for 30 min on ice. The cells were washed and then analyzed by flow cytometry in the presence of propidium iodide as described above.

Isolation and analysis of RNA

RNA was isolated and processed as described previously (Chomczynski and Sacchi 1987). Total RNA was fractionated on 0.8% agarose-MOPS-formaldehyde gels, blotted onto Nylon membrane (Hybond-N, Amersham) and probed under high stringency conditions with 32 P-labeled, randomly primed fragments as described in standard protocols (Sambrook et al. 1989).

The probes used were as follows: Chicken *bcl-2* cDNA was a 2.2-kb *Eco*RI fragment from clone pIBI-31 (Eguchi et al. 1992); chicken *mim-1* cDNA was a 1-kb *Eco*RI fragment from clone pMim1 (Ness et al. 1989); chicken *c-fms* (kind gift of U. Fuhrmann, European Molecular Biology Laboratory, Heidelberg, Germany); and chicken β -actin cDNA was a 1-kb *Hind*III fragment.

Western blotting

A crude protein lysate was prepared from 1×10^6 cells by incubation for 30 mins on ice in lysis buffer (250 mM NaCl, 50 mM Tris-HCl at pH 7.4, 1 mM EDTA, 0.1% Triton X-100, and protease inhibitors). After clearing by centrifugation the supernatant was adjusted to $1 \times$ protein gel sample buffer, electrophoresed on a 10% SDS-polyacrylamide gel and transferred to Immobilon membrane (Amersham). Blocking of unspecific reactions, antibody binding, and development of the blot by the ECL technique were as described by the manufacturer.

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