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# *BCL2*-mediated tumorigenicity of a human T-lymphoid cell line: Synergy with *MYC* and inhibition by *BCL2* antisense

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**ABSTRACT** **A gene-transfer approach was used to explore the function of the *BCL2* (B-cell lymphoma/leukemia 2) gene in a human T-cell line, Jurkat. Though stable introduction of a *BCL2* expression plasmid into Jurkat T cells was by itself insufficient, the combined transfer of *BCL2* and *MYC* genes markedly enhanced the tumorigenicity of these cells in athymic mice. Moreover, a *BCL2* antisense expression plasmid ablated tumor formation by Jurkat cells, providing further evidence that this oncogene contributes to the regulation of the *in vivo* growth of these human T lymphocytes. In addition to their influence on tumor formation, *BCL2* sense and antisense expression plasmids increased and decreased, respectively, the *in vitro* survival of Jurkat T cells in serum-free medium. These observations extend to T cells the finding of synergy of *BCL2* with *MYC* previously reported for B cells and provide evidence that *BCL2* can regulate the growth of human T cells.**

The *BCL2* (B-cell lymphoma/leukemia 2) gene becomes involved in chromosomal translocations in many human B-cell lymphomas (1). These translocations place *BCL2* into juxtaposition with the immunoglobulin heavy-chain enhancer, resulting in deregulated *BCL2* gene expression primarily through transcriptional mechanisms (2, 3). The altered levels of BCL2 proteins found in these cells with *BCL2* rearrangements are thought to contribute to the pathogenesis of these B-cell neoplasms. In this regard, recent gene-transfer experiments have provided direct experimental evidence that *BCL2* can regulate the growth of human and murine B cells both *in vitro* and *in vivo* (4–8). Interestingly, B cells that overexpress *BCL2* exhibit enhanced *in vitro* survival in the absence of growth factors (4, 8).

Though translocations involving *BCL2* have not been described in T-cell leukemias and lymphomas, *BCL2* is transiently expressed in normal human peripheral blood and tonsillar T lymphocytes when these cells are stimulated to proliferate in culture (9). This expression of *BCL2* in normal T cells has suggested that *BCL2* may normally regulate the growth of these lymphocytes, as well as B cells.

To explore the function of *BCL2* in T cells, we stably introduced *BCL2* expression plasmids into a human T-cell leukemia line, Jurkat. In addition, the relatively low levels of BCL2 mRNAs in Jurkat T cells (3) suggested that these cells might be good candidates for an antisense approach aimed at ablating *BCL2* expression, and for this reason we also prepared antisense *BCL2* expression constructs for stable introduction into these cells. The combined results from transfer and expression of *BCL2* sense and *BCL2* antisense plasmids in Jurkat T cells provide evidence that *BCL2* can regulate the *in vitro* survival and *in vivo* tumorigenicity of these human T cells.

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## METHODS

**Cell Cultures.** Jurkat T cells, subclone 32 (10), were maintained at $10^5$–$10^6$ cells per ml in "complete medium" consisting of RPMI 1640 supplemented with 10% (vol/vol) fetal bovine serum, 2 mM glutamine, penicillin (50 units/ml), and kanamycin, neomycin, and streptomycin (each 100 μg/ml) in a humidified atmosphere of 5% $CO_2$/95% air at 37°C. These cells were rid of mycoplasma by four cycles of treatment with BM-CYCLINE (Boehringer Mannheim) and were subsequently confirmed to be free of contamination by 4′,6-diamidino-2-phenylindole (DAPI) staining.

**Plasmid Constructions.** *BCL2* and *MYC* expression plasmids were prepared by standard recombinant DNA methods (11) and are depicted in Fig. 1. For *BCL2*, an ≈1-kilobase (kb)-long *Eco*RI cDNA from the plasmid pB4 (12) was subcloned into the *Xho* I cloning site of the retroviral expression plasmid pBC140 [a gift of B. Cullen (Duke University) and E. Gilboa (Sloan–Kettering Cancer Center)] by blunt-end ligation after filling in the ends of the *BCL2* insert and the linearized expression plasmid with Klenow and T4 DNA polymerases. Recombinant plasmids with the *BCL2* cDNA in the proper orientation (sense) or in reverse orientation (antisense) were identified by restriction mapping.

For *MYC*, an 8.1-kb *Hin*dIII–*Eco*RI human *MYC* genomic clone from pMC41 (13) was subcloned into the expression plasmid p290 (7) (provided by B. Sugden, University of Wisconsin) by first filling in the ends of the *Eco*RI- and *Bam*HI-cleaved *MYC* and p290 plasmids, respectively, prior to treatment with *Hin*dIII and subsequent ligation by T4 DNA ligase (Fig. 1).

As additional controls for antisense experiments, we also subcloned portions of the human *NRAS* or *FOS* gene in antisense orientation into the expression plasmid pBC-CMV (14). For *NRAS*, a 0.3-kb *Hin*dIII fragment containing the first exon of *NRAS* was removed from pNRsac (ATCC no. 41031) and subcloned into the *Hin*dIII site of a pBC-CMV plasmid that contained a defective *IL2* (interleukin 2) gene (pRB70-1; Fig. 1). For *FOS*, a 5.5-kb *Bam*HI–*Hin*dIII fragment was obtained from pc-FOS (human) (15) (not shown).

Recombinant plasmids were propagated in *Escherichia coli* strain HB101 (for *BCL2*) or DH5, banded twice by CsCl gradient centrifugation, linearized by cleavage with the appropriate restriction endonuclease [*Hpa* I (*BCL2*), *Sal* I (*MYC*), *Pvu* I (*NRAS, FOS*), or *Bam*HI (p290, pBC140)], extracted twice with phenol/chloroform, and sterilized by ethanol precipitation prior to use in gene-transfer experiments.

**Electroporation.** Jurkat cell cultures were initiated at $10^5$ cells per ml 3 days prior to electric-field-mediated DNA transfer (electroporation). One day before electroporation, fresh complete medium, equal to the volume of the cultures, was added. Immediately prior to electroporation, Jurkat cells

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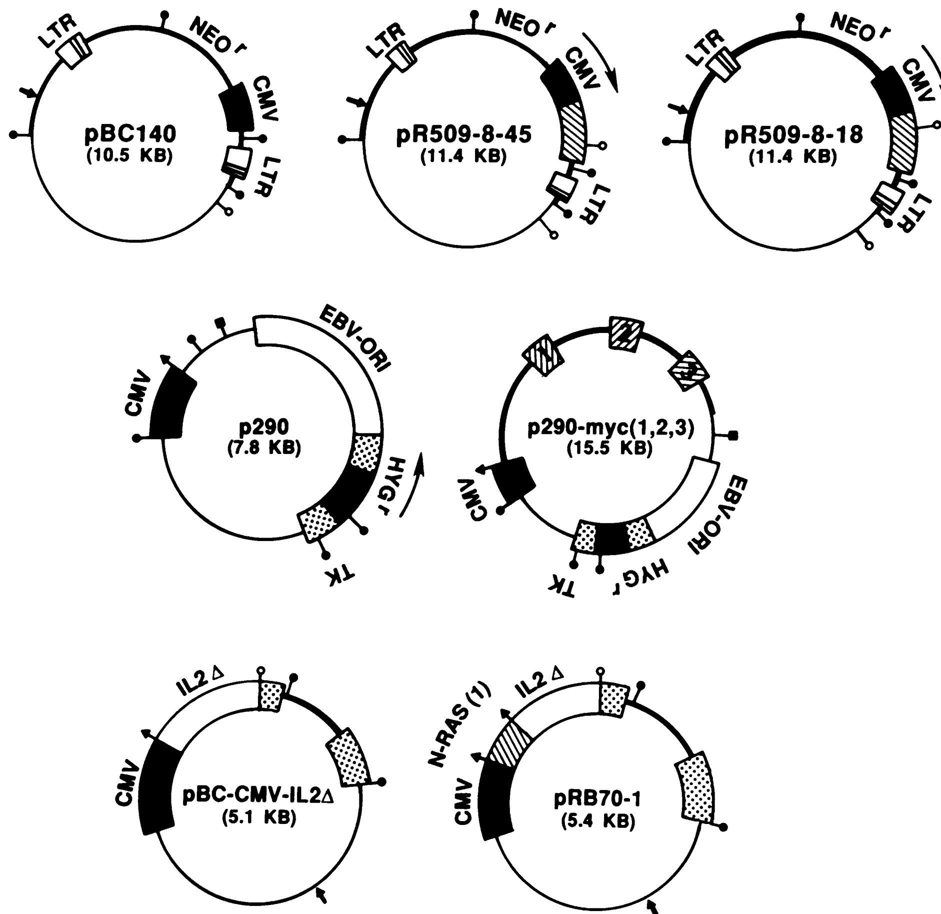


FIG. 1. Plasmids constructed for expression of *BCL2* and *MYC* in lymphoid cell lines. The *BCL2* cDNA pB4 (hatched box) was subcloned in both orientations into a unique *Xho* I site (not shown) 3′ of the cytomegalovirus (CMV) promoter/enhancer (black box) in the retroviral expression plasmid pBC140, creating the *BCL2* sense (pR509-8-45) and *BCL2* antisense (pR509-8-18) plasmids. The human *MYC* gene with its three exons (hatched boxes 1, 2, and 3) was subcloned 3′ of the CMV promoter/enhancer in p290. For the *NRAS* antisense plasmid pRB70-1, the first exon of the human *NRAS* gene (hatched box) was subcloned in reverse orientation between the CMV promoter/enhancer and mutant *IL2* cDNA in pBC-CMV-IL2. The pBC140 constructs contain a G418-resistance gene whose transcription is driven from the 5′ long terminal repeat (LTR). p290 contains a hygromycin-resistance gene (HYG$^R$, black box) regulated by elements from the herpes simplex virus thymidine kinase gene (TK, stippled boxes). pBC-CMV contains 3′ untranslated sequences and an intron from the rat preproinsulin II gene (stippled box). The *Bam*HI ( ), *Eco*RI ( ), *Hin*dIII ( ), *Pvu* I (in p290 and pBC-CMV), *Hpa* I (in pBC140) ( ), and *Sal* I ( ) restriction endonuclease sites are indicated. EBV-ORI, Epstein–Barr virus origin of replication.

were washed twice at 4°C with Hanks' balanced salt solution (GIBCO) and then resuspended at 1–2 × $10^7$ per ml in fresh sterile Dulbecco's phosphate-buffered saline (pH 7.4) (GIBCO) on ice. Linearized plasmid DNAs (10–50 μg in sterile phosphate-buffered saline) were incubated with 0.5 ml of cells for 1 min on ice, and then the cell/DNA mixture was placed into a chamber (cross-sectional area, 1 cm$^2$; length, 0.5 cm) and subjected to the discharge of a bank of capacitors (14 μF) charged to 500 V (apparatus model ZA1000, Precision Design Systems, Madison, WI). After another 10 min on ice, electroporated cells were transferred to fresh complete medium (2 × $10^5$ viable cells per ml) at room temperature for 10 min and then cultured at 37°C for 2 days before addition of Geneticin (GIBCO; 2 mg/ml, actual G418 concentration ≈1 mg/ml) or hygromycin (Calbiochem; 300 μg/ml). Cells (initially $10^4$ per well) were cultured with antibiotic in 0.2 ml per flat-bottom well of 96-well plates. After 3 weeks, microcultures with cellular growth were expanded for further analysis. The efficiency of stable DNA introduction averaged 2.8 × $10^{-5}$ for G418 selections and 1.3 × $10^{-5}$ for hygromycin.

**Cell Viability Assays.** Jurkat cell survival was evaluated in 2-ml cultures that were initiated at $10^6$ cells per ml and that contained RPMI medium without serum. Viable cells were determined by their ability to exclude trypan blue dye and were enumerated in a hemocytometer.

**Tumorigenesis.** Jurkat cells were injected subcutaneously into either nonirradiated ($10^6$ viable cells per injection) or irradiated ($10^7$ cells) *nu/nu* athymic mice (Harlan Sprague Dawley). Animals were observed for tumor formation for up to 4 months. Tumors removed from sacrificed mice were subjected to histological and Southern blot analyses, confirming a Jurkat cell origin.

**RNA and Protein Blotting.** Total cellular RNA was isolated from 1–2 × $10^7$ Jurkat cells by the method of Chomczynski and Sacchi (16), except that an additional phenol/chloroform extraction was performed. Samples of RNA (20 μg) were analyzed by Northern blotting as described (17). Relative levels of BCL2 protein were assessed by immunoblot analysis using a rabbit antiserum prepared against a synthetic BCL2 peptide (7, 18).

## RESULTS AND DISCUSSION

**Expression of *BCL2* and *MYC* Plasmids in Jurkat T Cells.** Expression of *BCL2* and the *MYC* protooncogenes correlates with cellular growth in normal and Jurkat T cells (refs. 19–21 and unpublished work). We therefore wished to contrast the actions of these two oncogenes in Jurkat cells. In addition, we were interested in assessing the combined effects of deregulated *BCL2* and *MYC* expression, since previous gene-

transfer experiments in B cells had demonstrated synergistic actions of these oncogenes (4, 6).

For these reasons we stably introduced *BCL2* and *MYC* expression plasmids, both singly and in combination, into Jurkat cells by electroporation. The pBC140 retroviral vector and p290 expression plasmids used for these studies contained a strong promoter/enhancer from cytomegalovirus for driving expression of the inserted oncogenes (Fig. 1). Among antibiotic-resistant Jurkat clones tested, 30–40% contained high levels of plasmid-derived *BCL2* or *MYC* mRNAs (data not shown). This high efficiency suggested that high-level expression of both *BCL2* and *MYC* plasmids could be obtained in the same cell by simultaneously introducing both plasmids and employing a two-antibiotic selection strategy; the use of two antibiotics was made possible by the subcloning of *BCL2* and *MYC* into plasmids that contained a G418-resistance and a hygromycin-resistance gene, respectively (Fig. 1). Indeed, when antibiotic selections were performed sequentially, first with G418 and then with hygromycin, we obtained Jurkat clones at a frequency of $\approx 10^{-5}$ that were capable of long-term growth in the simultaneous presence of G418 and hygromycin.

Several of these doubly resistant clones were then analyzed for *BCL2* and *MYC* expression by RNA blotting (Fig. 2). Because the plasmid-derived *MYC* transcript was nearly the same size as the normal endogenous 2.4-kb *MYC* mRNA, Jurkat cell clones were treated with phorbol 12-myristate 13-acetate for 24 hr (which inhibits expression of the endogenous *MYC* protooncogene; unpublished observation) prior to isolation of RNA. Thus, the levels of *MYC* mRNA seen in the Jurkat clones shown in Fig. 2 reflect plasmid-mediated expression. The retroviral *BCL2* plasmid (pR509-8-45) produced three mRNAs representing the 4.7-kb full-length and a shorter spliced subgenomic transcript, as well as the expected 2-kb mRNA for transcripts initiating within the cytomegalovirus promoter.

As shown, though resistant to both G418 and hygromycin, clones were identified that expressed at high levels either *BCL2* (lanes 3 and 6), *MYC* (lanes 5 and 7), both (lane 1), or neither (data not shown) of these plasmids, probably as a result of random plasmid DNA integration. Jurkat cell lines displaying high levels of *BCL2* and *MYC* expression were subcloned by limiting-dilution, to ensure expression of both plasmids within the same cell. Those Jurkat clones containing high levels of both *BCL2* and *MYC* plasmid-derived mRNAs were then employed in subsequent experiments. For most experiments, we also made use of doubly resistant Jurkat clones that expressed only *BCL2*, only *MYC*, or neither of these plasmids, to control for any effects of the selection procedure.

***BCL2* Expression Plasmids Influence Jurkat Cell Survival.** Because previous reports had demonstrated that *BCL2* overexpression can enhance the *in vitro* survival of B lymphocytes (4, 8), we explored the effects of *BCL2* sense and

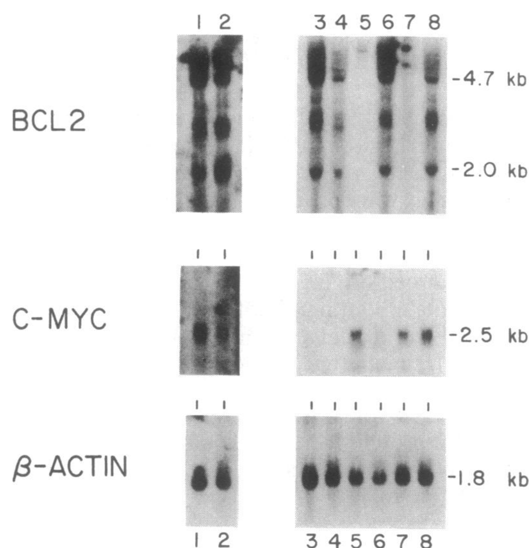


FIG. 2. Expression of *BCL2* and *MYC* plasmids in Jurkat T cells. Jurkat cells were treated for 24 hr with phorbol 12-myristate 13-acetate (50 ng/ml) to inhibit expression of the endogenous *BCL2* and *MYC* genes. Total cellular RNA was then isolated from eight randomly chosen doubly resistant (G418- and hygromycin-resistant) Jurkat clones, and 20 μg per lane was subjected to RNA blot analysis (17). RNA blots were sequentially hybridized with $^{32}$P-labeled probes for *BCL2* (p509-8-45), *MYC* (pRYC7.4), and β-actin (pA1) (3, 17).

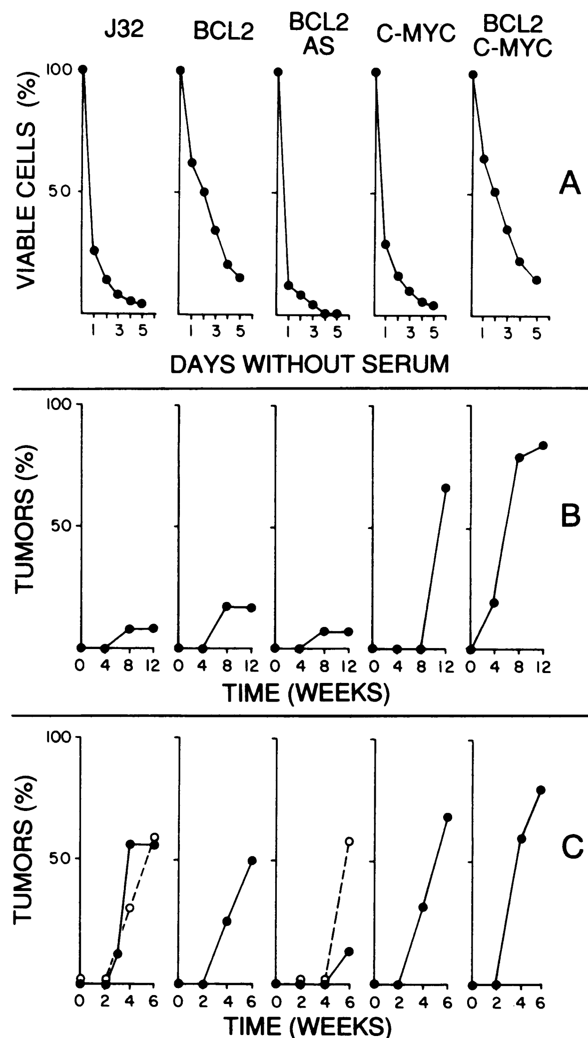


FIG. 3. Effects of *BCL2* and *MYC* expression plasmids on *in vitro* survival and *in vivo* tumor formation by Jurkat T cells. Results are shown for Jurkat cells without expression plasmid (J32), with *BCL2* sense plasmid, with *BCL2* antisense (AS) plasmid, with *MYC* plasmid, or with both *BCL2* and *MYC* plasmids. (*A*) Survival of Jurkat T cells was measured at various times after seeding into serum-free medium at $10^6$ cells per ml. Data are means (SE ≤ 10%) of three independent determinations using at least two independent clones. (*B*) Jurkat T cells ($10^6$) were injected subcutaneously into nonirradiated athymic mice, and the animals were observed for appearance of palpable tumors. (*C*) For antisense experiments, Jurkat T cells ($10^7$) were injected into irradiated (300 rads; 1 rad = 0.01 Gy) athymic mice to assess tumorigenicity. Results for cells expressing the *FOS* antisense and *RAS* antisense (○----○) plasmids are shown in the J32 and BCL2 AS panels, respective. Tumorigenicity data are derived from 6–12 injections of each type of Jurkat cell line and represent the percentage of injections that resulted in tumors.

antisense expression plasmids on Jurkat T-cell survival in serum-free cultures. Jurkat cells were washed three times to remove serum and then placed into culture without serum for various times before enumeration of viable cells. As shown in Fig. 3A, control Jurkat T cells (including those containing pBC140 or p290 plasmids and selected for G418 or hygromycin resistance, respectively) rapidly died in the absence of serum. Within 1 day of serum withdrawal, for example, only ≈25% of the input cells remained viable. In contrast, Jurkat T cells that expressed high levels of the *BCL2* sense plasmid (pR509-8-45) remained ≈60% viable 1 day after withdrawal of serum. Thus, consistent with previous reports (4, 8), *BCL2* enhanced the *in vitro* survival of these human T cells. When Jurkat cells that expressed the *BCL2* antisense plasmid (pR509-8-18) were examined, we observed diminished *in vitro* survival in the absence of serum, with only ≈10% of the cells remaining viable at 1 day (Fig. 3A).

Jurkat T cells expressing the *MYC* plasmid (p290-MYC) behaved the same as control Jurkat cells with regard to their survival in the absence of serum. Similarly, Jurkat cells that expressed both the *BCL2* and the *MYC* plasmids exhibited no greater survival in the absence of serum than did Jurkat cells expressing *BCL2* alone. The effects of *BCL2* expression plasmids on Jurkat cell survival therefore appeared to be specific, since *MYC* plasmids failed to enhance the viability of these T cells. In contrast to cells in serum-free conditions, no differences in the viability of the various Jurkat clones were seen when 5% (vol/vol) serum was used. Also, no obvious effects of *BCL2* and *MYC* expression plasmids were noted on the *in vitro* growth and proliferation of Jurkat cell clones in reduced concentrations of serum (1%, 2%, 5%; data not shown).

**Effects of *BCL2* and *MYC* on Tumor Formation by Jurkat T Cells.** Injections of various Jurkat clones into athymic mice revealed synergy of *BCL2* and *MYC* with regard to tumor formation. As shown in Fig. 3B, control Jurkat cells and those expressing only *BCL2* plasmids formed tumors inefficiently in mice (10–20%). Jurkat cells expressing the *MYC* plasmid formed tumors more efficiently (≈65% of injections), but with a long latency of ≈12 weeks. By comparison, Jurkat cell clones expressing both *BCL2* and *MYC* formed palpable subcutaneous tumors with somewhat higher incidence (≈85%) but with a much shorter latency (Fig. 3B). These tumors not only formed more than twice as rapidly but also grew to larger sizes in animals. Use of clones resistant to both G418 and hygromycin but lacking expression of either *BCL2* or *MYC* (Fig. 2) controlled for the effects of antibiotic selections and verified the importance of simultaneous deregulated expression of *BCL2* and *MYC* for enhanced tumorigenesis (data not shown). Those tumors that resulted rapidly from injections of cells coexpressing *BCL2* and *MYC* did not differ histologically from those arising more slowly from control cells, except that the former exhibited a higher mitotic index (data not shown).

Our findings with Jurkat T cells are very similar to previous observations obtained from use of human B cells immortalized by Epstein–Barr virus (6). As with Jurkat T cells, introduction of *BCL2* alone failed to influence the tumorigenicity of these B cells, but when *BCL2* was expressed together with *MYC*, a higher incidence and shorter latency of tumor formation was noted relative to B cells expressing *MYC* alone.

**Investigations of Tumor Formation by Jurkat Cells Expressing *BCL2* Antisense Plasmids.** As an alternative approach for exploring the functions of *BCL2* in Jurkat T cells, we also injected into athymic mice Jurkat T-cell clones that contained the *BCL2* antisense expression plasmid pR509-8-18 (Fig. 1). Two independent G418-resistant clones that accumulated high levels of full-length 4.7-kb and subgenomic 2-kb antisense transcripts were identified by RNA blotting (Fig. 4A *Center*, lanes 3 and 7). As controls, we also prepared antisense *NRAS* (Fig. 1) and *FOS* (not shown) expression plasmids, stably introduced these into Jurkat cells, and identified G418-resistant clones that accumulated high levels of ≈1.5-kb *NRAS* (Fig. 4A *Right*, lane 2) or ≈4-kb *FOS* (*Left*, lane 1) antisense mRNAs.

Tumorigenicity studies with these antisense-expressing T cells were then performed. For these studies we injected large numbers ($10^7$) of Jurkat cells into irradiated athymic mice. Under these circumstances, about half of the injections of control Jurkat cells resulted in tumors within 6 weeks (Fig. 3C). In contrast, injections of cells expressing *BCL2* antisense mRNA produced tumors much less frequently (<15%). This inhibitory effect of *BCL2* antisense expression was specific, since Jurkat cell clones producing high levels of *RAS* or *FOS* antisense mRNA (Fig. 4A) displayed less or no alteration, respectively, in their tumorigenicity (Fig. 3C).

In an attempt to evaluate the mechanisms of tumor inhibition by *BCL2* antisense plasmids, we measured relative levels of BCL2 protein in these Jurkat cells by immunoblot analysis (7, 18). Jurkat cell clones that expressed high levels of the *BCL2* sense plasmid (pR509-8-45) contained abundant

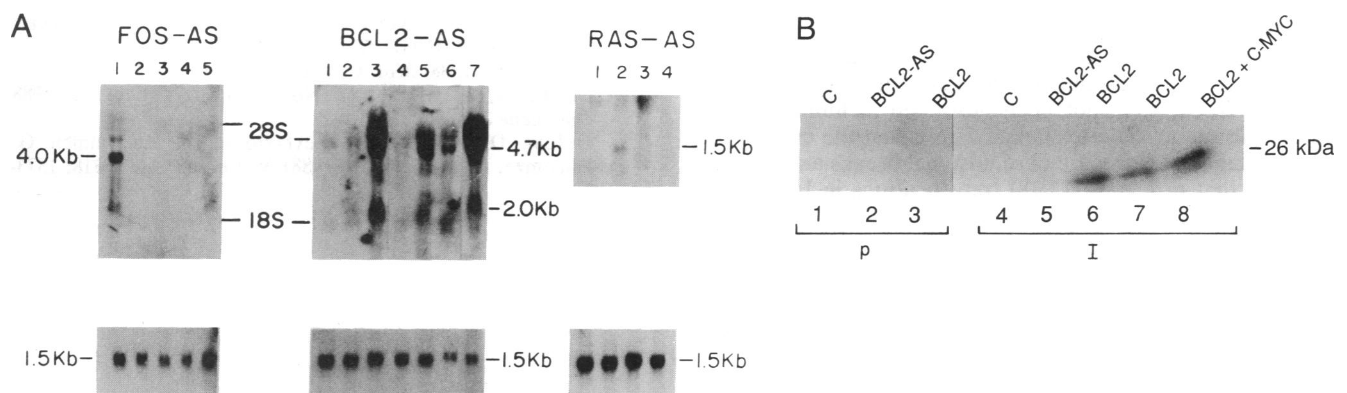


FIG. 4. Expression of antisense (AS) plasmids in Jurkat T cells. (*A*) RNA blot analysis was performed for randomly chosen antibiotic-resistant Jurkat clones, essentially as described (17). Blots were hybridized with $^{32}$P-labeled probes specific for *FOS* (*Left*), *BCL2* (*Center*), or *NRAS* (*Right*). All RNA blots were subsequently rehybridized with a $^{32}$P-labeled *HLA* class I probe to control for amounts of RNA per lane, revealing the expected ≈1.5-kbp mRNA in all lanes. Positions of 28S and 18S rRNAs are indicated. (*B*) Immunoblot analysis was performed for control (C) Jurkat T cells or for selected clones that contained high levels of *BCL2* sense, *BCL2* antisense (AS), or the combination of *BCL2* sense and *MYC* plasmid-derived mRNAs. Equal amounts of protein (100 μg per lane) were transferred from SDS/12% polyacrylamide gels to nitrocellulose, and the resultant blots were incubated with either preimmune (P) or immune (I) serum from rabbits injected with a BCL2 peptide (18). Antibodies were detected with $^{125}$I-labeled protein A as described (7, 18).

amounts of the 26-kDa BCL2 $\alpha$ protein (Fig. 4*B*). Unfortunately, the level of sensitivity provided by use of this BCL2 antiserum was insufficient for detection of the small amounts of BCL2 protein that we anticipate are present in normal Jurkat T cells, based on RNA blot data (3). Thus, we were unable to assess adequately the effects of *BCL2* antisense transcripts on BCL2 protein production (Fig. 4*B*). Nevertheless, the vast excess of *BCL2* antisense transcripts (Fig. 4*A Center*, lanes 3 and 7) suggests that nearly all endogenous *BCL2* mRNAs should be involved in heteroduplexes with antisense mRNA. Moreover, since the antisense-mediated inhibition of tumorigenesis was far more potent for *BCL2* than for *FOS* or *NRAS* (Fig. 3*C*), it seems likely that a principal component of the observed antisense effect was specific for *BCL2* and not attributable to nonspecific mechanisms.

## CONCLUSIONS

Using a gene-transfer approach, we have obtained evidence that *BCL2* plays a critical role in regulating the survival and tumorigenicity of a human T-cell leukemia line, Jurkat. Support for the importance of *BCL2* in influencing tumor formation by Jurkat cells in athymic mice came from two observations: (*i*) *BCL2* plasmids, when expressed in combination with *MYC*, enhanced tumor formation by these T cells (Fig. 3*B*) and (*ii*) *BCL2* antisense plasmids markedly reduced tumorigenesis. The mechanisms responsible for the effects on tumor formation mediated by *BCL2* in Jurkat cells remain unknown but may be related to the ability of this oncogene to influence the viability of these T cells (Fig. 3*A*).

The demonstration of synergy of *BCL2* and *MYC* with regard to tumor formation was of particular interest, since occasional cases of low-grade human B-cell lymphomas with *BCL2* gene rearrangements have been reported to develop a second chromosomal translocation involving *MYC* in association with clinical and histological tumor progression (22, 23). Previous gene-transfer studies in human and murine B cells have revealed cooperation of *BCL2* and *MYC* in tumorigenicity assays (4, 6). The enhanced tumor formation observed when *BCL2* and *MYC* plasmids were expressed together in Jurkat cells indicates that these two oncogenes can also act synergistically in at least some human T cells. Our findings thus provide additional direct evidence that the combined deregulation of *BCL2* and *MYC* gene expression represents one mechanism of lymphoid tumor progression (22, 23), and extend to T cells the phenomenon of *BCL2* and *MYC* oncogene cooperation.

Given that our findings suggest similar functional roles for *BCL2* in B cells and T cells, it is intriguing that translocations involving *BCL2* have not been described in human T-cell tumors. Some possible explanations are that the conditions contributing to the specific chromosomal breaks and recombinations that produce t(14;18) translocations in B cells do not exist in T cells or that *BCL2* translocations occur at a stage of T-cell differentiation when *BCL2* fails to confer a selective growth advantage.

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