NF- κ B1 Can Inhibit v-Abl-Induced Lymphoid Transformation by Functioning as a Negative Regulator of Cyclin D1 Expression

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Mounting evidence implicates deregulated Rel/NF- κ B signaling as a common feature of lymphoid malignancies. Despite the fact that they promote the survival and proliferation of normal lymphocytes, the underlying mechanisms by which various Rel/NF- κ B proteins with different transcriptional regulatory capacities might facilitate transformation remain to be established. Here we show that the proliferation and tumorigenicity of Abelson murine leukemia virus (A-MuLV)-transformed pre-B cells are enhanced in the absence of NF- κ B1 and that this coincides with elevated levels of cyclin D1. Support for a link between cyclin D1 expression and v-Abl transformation came from the finding that proliferation of transformed pre-B cells was reduced in the absence of cyclin D1, while enforced cyclin D1 expression increased the proliferation and tumorigenicity of wild-type transformants. A reduction in endogenous cyclin D1 levels that coincided with NF- κ B1 transgene reversal of enhanced *nfkb1*^{-/-} pre-B-cell transformation, coupled with NF- κ B1 inhibition of v-Abl-induced κ B-dependent murine cyclin D1 transcription, lends support to a model in which v-Abl-induced cyclin D1 transcription in transformed pre-B cells is controlled by Rel/NF- κ B dimers with different activities.

The v-Abl protein, a nonreceptor tyrosine kinase encoded by the Abelson murine leukemia virus (A-MuLV), induces an acute lymphoid leukemia in infected mice and transforms B-cell progenitors and fibroblasts in culture (16, 43). While the division and survival of A-MuLV-transformed pre-B cells are intimately linked to activation by v-Abl of multiple downstream signaling pathways that include JAK/STATs, protein kinase C, phosphatidylinositol 3-kinase, Ras, and Rac (5, 7, 8, 9, 10, 55, 60, 61), the relative importance of each pathway in specific aspects of the transformation process remains unclear. Moreover, only a few of the many genes regulated by these pathways appear critical for the proliferation of v-Abl-transformed cells (61).

The Rel/NF- κ B signaling pathway has recently emerged as a key regulator of B-cell division and survival (22). Rel/NF- κ B transcription factors collectively comprise a family of homo- and heterodimeric proteins composed of related subunits (2, 35, 47). c-Rel, RelA, and RelB each possess carboxyl-terminal transcriptional transactivation domains, while NF- κ B1 (p50) and NF- κ B2 (p52), which lack intrinsic transactivating properties, instead function as homodimeric transcriptional repressors or modulators of their transactivating partners (47). In most cells, Rel/NF- κ B proteins are retained in the cytoplasm in an inactive form through association with inhibitor (I κ B) proteins (57). Diverse stimuli promote the nuclear translocation of Rel/NF- κ B by activating an I κ B-specific kinase complex (IKK) (28), which phosphorylates I κ Bs, targeting them for ubiquitin-dependent, proteasome-mediated degradation (53, 57). Rel/NF- κ B dimers are then translocated to the nucleus and bind

decameric motifs (κ B elements) found in the regulatory regions of many cellular genes (2).

The role of Rel/NF- κ B proteins in lymphocyte transformation mediated by v-Abl remains unclear. Despite the fact that Rel/NF- κ B is crucial for normal lymphocyte survival and division, v-Abl retards the nuclear translocation of Rel/NF- κ B in A-MuLV-transformed pre-B cells (29), implying that these transcription factors may inhibit rather than promote transformation. This finding prompted us to examine in greater detail the specific function served by this signaling pathway in v-Abl-mediated pre-B-cell transformation. Collectively, RelA/NF- κ B1, Rel/NF- κ B1, and NF- κ B1 homodimers comprise the majority of the DNA binding activity for this transcription factor family in primary B-cell progenitors (19, 29). Although the combined absence of different Rel/NF- κ B proteins such as c-Rel and RelA or NF- κ B1 and RelA disrupts B-cell development (18, 26), individually the absence of these Rel/NF- κ B subunits does not appear to perturb B lymphopoiesis (13). Therefore, we decided to assess the role(s) served by NF- κ B1, c-Rel, and RelA in A-MuLV-induced pre-B-cell tumors by examining transformation of mouse bone marrow (BM) cells lacking these individual transcription factors. These experiments showed that v-Abl-transformed *nfkb1*^{-/-} pre-B cells divided faster and were more tumorigenic than wild-type counterparts and that this enhanced transformation coincided with increased cyclin D1 expression. Cyclin D1 involvement in A-MuLV-mediated pre-B-cell transformation was supported by the findings that v-Abl-induced proliferation of cyclin D1-deficient pre-B cells was lower than that of wild-type transformants and that enforced v-Abl and cyclin D1 coexpression increased the division and tumorigenicity of wild-type pre-B cells. A reduction in proliferation and cyclin D1 levels for transformed *nfkb1*^{-/-} pre-B cells expressing an NF- κ B1 transgene, coupled with the ability of NF- κ B1 to inhibit v-Abl-mediated transcription of murine cyclin D1 promoter reporters in a κ B-dependent manner, suggests that NF- κ B1 homodimers

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inhibit cell division in v-Abl-transformed pre-B cells in part through negative regulation of cyclin D1 expression.

MATERIALS AND METHODS

Mice. *c-rel*^{-/-}, *nfkb1*^{-/-}, and *rela*^{-/-} mice (3, 30, 46) were all backcrossed for at least eight generations with C57BL/6 mice. Cyclin D1-deficient (Cyd1^{-/-}) mice (12) were kindly provided by Clive Dickson (International Cancer Research Fund, London, United Kingdom).

Genomic clones, plasmid constructs, and retrovirus expression vectors. The genomic clone encompassing the murine cyclin D1 promoter (48) was generously provided by Clive Dickson. *cyd1-luc* consisted of a 1.52-kb *SacI/EagI* mouse *cyclin D1* promoter fragment subcloned into the promoterless luciferase reporter plasmid pA3luc (59). *cyd1κB_m-luc* is a derivative of *cyd1-luc* in which the NF-κB binding site (5'-GGGGAGTTT-3'; -42 to -33) has been altered by in vitro mutagenesis (25) to 5'-TTCGAGAAAT-3'. Expression plasmids encoding v-Abl, RelA, p50 NF-κB1 (amino acids [aa] 1 to 413), or p105 NF-κB1 were generated by inserting cDNAs for these proteins into the pCAGGS (38) or pEF-BOS vector. pBABEv-Abl and the bicistronic retrovirus pBABEvAbl/Cyclin D1 were generated by first inserting the v-Abl coding region into the pBABE-puro retrovirus vector (36) under the transcriptional control of the long terminal repeat (LTR). For pBABEv-Abl/Cyclin D1, the puromycin resistance gene was replaced with a murine cyclin D1 cDNA. pMSCVIREs-GFP/HA-p105 was generated by inserting N-terminally hemagglutinin (HA)-tagged p105 (21) into pMSCVIREs-GFP (52).

Retrovirus production and infections. Helper virus-free stocks of A-MuLV, pBABEv-Abl, or pBABEvAbl/Cyclin D1 were obtained from the culture supernatants of high-titer (~2 × 10⁵ fibroblast-transforming units) Ψ2 (34) subclones transfected either with a genomic clone encoding an Abelson provirus (11) or with plasmids for the recombinant v-Abl viruses. Viral titers were determined by measuring the number of transformed foci that arose after infection of BALB/c 3T3 cells. Helper-free MSCVIREs-GFP and MSCVIREs-GFP/HA-p105 viral stocks were generated by transient transfection of BOS23 cells as described previously (52).

Tumorigenicity assays. A total of 10⁶ freshly isolated BM mononuclear cells from wild-type or *nfkb1*^{-/-} mice (Ly5.2⁺), infected in culture for 2 h with equivalent titers of helper virus-free A-MuLV, pBABEv-Abl, or pBABEvAbl/Cyclin D1 virus, were then injected intravenously into lethally irradiated (550 rads given twice, with a 4-h interval between doses) C57BL/6-Ly5.1⁺ mice (8 to 10 weeks old). In each experiment, five recipients were injected with either mock- or virus-infected cells. All mice were monitored daily, and upon detection of a palpable tumor or signs of illness, these animals were sacrificed by asphyxiation with CO₂. Organs were analyzed by histology, while BM, blood, and lymphoid organ suspensions were examined by flow cytometry where possible.

Proliferation and cell division assays. Virus-transformed BM cells (2 × 10⁴) seeded in a 96-well plate in 100 μl of Dulbecco's modified Eagle medium (DMEM)-50 μM 2-mercaptoethanol containing different concentrations (volume/volume) of fetal calf serum (FCS) were cultured for 3 days, after which cellular proliferation was measured by adding 100 μCi of [³H]thymidine for 5 h as described previously (20). The relative rates of cell division were also assessed by using carboxyfluorescein diacetate succinidyl ester (CFSE) or by counting cells. For CFSE, cells were first incubated with 10 μM CFSE in DMEM for 10 min, after which excess CFSE was removed by washing. Cells were then cultured in DMEM-20% FCS-50 μM β-mercaptoethanol (BME), and CFSE intensity was analyzed by FACScan over a 72-h time course.

Nocodazole synchronization. Nocodazole (Sigma-Aldrich) at a concentration of 350 ng/ml was added to suspension cultures (~10⁸ cells) of early-passage A-MuLV-transformed wild-type and *nfkb1*^{-/-} pre-B cells for 24 h, after which time cells were washed extensively in prewarmed growth medium (DMEM-10% FCS-50 μM BME) to remove the drug. Due to extensive cell death (>80%) after this period of nocodazole treatment, the remaining viable cells were purified by flow cytometry using a MoFlo (Becton Dickinson). These cells were then resuspended in fresh growth medium at a density of 10⁶ cells/ml, and replicate cultures were incubated for varying times over a 20-h period. At 3- or 4-h intervals, cultures were pulsed with 10 μM bromodeoxyuridine (BrdU) for 40 min and then fixed in 70% ethanol. After DNA denaturation in 4 N HCl, followed by neutralization with 0.1 M Na₂B₄O₇ (pH 8.5), cells were resuspended in 0.5% Tween 20-1% bovine serum albumin-phosphate-buffered saline, stained with a fluorescein isothiocyanate-conjugated anti-BrdU antibody (Becton Dickinson), and analyzed on a FACScan.

Cell culture. BM cells were flushed from the femurs of 6- to 8-week-old mice, and mononuclear cells were then enriched by using Ficoll-Hypaque PLUS (Amersham Pharmacia Biotech). To quantitate the transformation of B-cell progen-

itors in BM, 10⁶ mononuclear cells were infected with equivalent titers of A-MuLV (expressed in focus-forming units per milliliter) in the presence of 4 μg of Polybrene/ml for 2 h. Cells were then washed and seeded in 0.3% (vol/vol) agarose (SeaPlaque) containing DMEM-20% FCS-50 μM BME as described previously (11). Macroscopic colonies comprising transformed pre-B cells were counted between 12 and 14 days postinfection. Transformed cells from individual colonies were isolated from agarose suspension cultures by using fine glass pipettes and were analyzed directly or cultured in 24-well plates (Costar) without feeder cells in 0.5 ml of DMEM-20% FCS-50 μM BME. Suspension cultures of transformed cells were generated by taking 2 × 10⁷ BM cells, infecting them with helper virus-free stocks of either A-MuLV, pBABEv-Abl, or pBABEvAbl/Cyclin D1 as described above, and culturing them in DMEM-20% FCS-50 μM BME. Nonadherent cells from all infections were passaged every 3 to 5 days for approximately 14 days, after which time >98% of cells were B220⁺ sIgM⁻ and expressed p120^{v-abl}.

Flow cytometry and cell sorting. Primary BM cells stained with fluorochrome-labeled or biotinylated monoclonal antibodies (MAbs) specific for B220, Ly5.2, Thy-1, and immunoglobulin M (IgM) were examined by flow cytometry as previously described (30). Green fluorescent protein (GFP)-positive BM cells infected with either MSCVIREs-GFP or MSCVIREs-GFP/HA-p105 were purified by cell sorting as described previously (30, 52).

Semiquantitative reverse transcription-PCR (RT-PCR). Five micrograms of total RNA isolated from cells by using RNeasy (Promega) was reverse transcribed by using the Ready-To-Go T-Primed First-Strand Kit (Pharmacia). A 167-μl volume of TE (10 mM Tris-Cl-1 mM EDTA [pH 8.0]) was then added to the reaction mixture, and 5 μl of this was used in each PCR. For all PCRs with the exception of hypoxanthine ribosyltransferase (HPRT; 27 cycles), the cDNA was amplified for 30 cycles, with each cycle programmed for denaturation at 94°C for 60 s and annealing at 55°C for 60 s, followed by elongation at 72°C for 60 s. Samples were then fractionated on a 1.5% agarose gel. The oligonucleotides used as forward and reverse primers to amplify the HPRT, *c-myc*, cyclin D1, cyclin D2, and cyclin D3 cDNAs were derived from different exons in order to distinguish cDNA products from amplified genomic DNA. The sequences of the PCR primers were as follows: for murine HPRT, the sense primer was 5'-CAC AGGACTAGAACACCTGC-3' and the antisense primer was 5'-GCTGGTGA AAAGGACCTCT-3' (249-bp PCR product); for murine cyclin D1, the sense primer was 5'-GAGAAGTTGTGCATCTACAC-3' and the antisense primer was 5'-GAAGGGCTTCAACTGTTC-3' (400-bp PCR product); for murine cyclin D2, the sense primer was 5'-CAGCAAAGGAGAAGCTGTC-3' and the antisense primer was 5'-TTCCTCACAGGTCAACATCC-3' (373-bp PCR product); for murine cyclin D3, the sense primer was 5'-ACGACTTCTGGCCTT GATT-3' and the antisense primer was 5'-CTACAGGTGAATGGCTGTGA-3' (407-bp PCR product); and for murine *c-myc*, the sense primer was 5'-CACTC ACCAGCACAACCTACG and the antisense primer was 5'-ATGCCACAGAGT TTCGAAGC-3' (410-bp PCR product).

Immunoblotting. Total-cell extracts were generated by lysing cells in phosphate-buffered saline containing 1% NP-40, 0.5% sodium deoxycholate, 0.5% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg of aprotinin/ml. Equivalent amounts of protein determined by the Bradford assay were electrophoresed on sodium dodecyl sulfate-10% polyacrylamide gels and then transferred to nylon membranes as described previously (19). Filters were incubated with either mouse anti-cyclin D1 monoclonal Ig (catalog no. sc-246; Santa Cruz Biotechnology), rabbit anti-cyclin D2 polyclonal Ig (catalog no. sc-593; Santa Cruz Biotechnology), or the mouse anti-HA MAb 12CA5 (Babco, Emeryville, Calif.), and bound antibody was revealed by horseradish peroxidase-conjugated goat anti-mouse (for cyclin D1 and HA-p105) or goat anti-rabbit (for cyclin D2) Ig (Silenus) by using enhanced chemiluminescence (Amersham Pharmacia Biotech).

Luciferase assays. 293T fibroblasts were transiently transfected by electroporating 10⁷ cells (at 500 μF and 280 V), and 48 h later, luciferase assays were performed by using the dual-luciferase reporter system (Promega). All the results shown represent luciferase activities normalized against a control luciferase reporter of *Renilla* luciferase, pRL-TK (Promega). The amounts of plasmid used in the various transfections were as follows: pRL-TK, 0.5 μg; *cyd1-luc*, 1 μg; *cyd1κB_m-luc*, 1 μg; pCAGv-Abl, 10 μg; pEFBOS-RelA, 5 μg; pEFBOS-NF-κB1p50, 10 μg; and pEFBOS-NF-κB1p105, 10 μg. Experiments were performed four times.

RESULTS

Loss of NF-κB1 enhances A-MuLV transformation of pre-B cells. To assess the role(s) served by c-Rel, RelA, and NF-κB1 in v-Abl-induced pre-B-cell transformation, quantitative trans-

formation assays were first performed by infecting equivalent numbers of nucleated wild-type, *c-rel*^{-/-}, *rela*^{-/-}, and *nfkbl*^{-/-} BM cells with A-MuLV and examining transformed colonies in agar. While *c-rel*^{-/-} and *nfkbl*^{-/-} colony numbers were slightly greater than those obtained from normal or *rela*^{-/-} BM (Fig. 1A), the most striking observation was the larger size of the *nfkbl*^{-/-} colonies compared with those of the other genotypes (Fig. 1B), with >5-fold more cells in the *nfkbl*^{-/-} colonies (Fig. 1C).

Although all major hematopoietic populations are present in normal numbers in mice that lack c-Rel, RelA, or NF- κ B1 (10, 30, 46), initially it was unclear if the altered transformation profiles of *c-rel*^{-/-} and *nfkbl*^{-/-} BM might reflect a change in the type or frequency of target cells transformed by A-MuLV. This, however, appeared unlikely. Firstly, the B220⁺ Thy-1^{lo} BM population, which is enriched for A-MuLV targets (49), appeared normal in *c-rel*^{-/-} and *nfkbl*^{-/-} mice (data not shown). Secondly, *c-rel*^{-/-}, *rela*^{-/-}, and *nfkbl*^{-/-} transformants were all found to be B220⁺ sIgM⁻ and to display Ig heavy-chain, but not Ig light-chain, gene rearrangements (data not shown). In addition, B220⁺ BM cells from all mutants were infected by GFP-expressing retroviruses with normal efficiency (data not shown), ruling out differences in infectivity as an explanation for increased numbers of transformants. Finally, enhanced immortalization was ruled out as an explanation for the altered transformation profiles of A-MuLV-transformed *nfkbl*^{-/-} and *c-rel*^{-/-} pre-B cells, since immortal mutant pre-B-cell lines arose from prolonged culturing with a frequency and kinetics similar to those described for wild-type cells (50).

To determine if the altered transformation profile of *c-rel*^{-/-} and *nfkbl*^{-/-} cells in culture coincided with a change in tumorigenicity, equivalent numbers of A-MuLV-infected Ly5.2⁺ wild-type, *c-rel*^{-/-}, or *nfkbl*^{-/-} BM cells were engrafted into lethally irradiated Ly5.1⁺ C57BL/6 mice. Only mice receiving infected BM died or became ill, and these were sacrificed for further analyses. A higher proportion of animals engrafted with infected *nfkbl*^{-/-} cells succumbed to illness (35, 40, and 55% for mice engrafted with wild-type, *c-rel*^{-/-}, and *nfkbl*^{-/-} cells, respectively), and the mean period for disease onset or death was significantly shorter among these recipients (Fig. 2). Sick mice presented with impaired gait, paraplegia, and in many cases a detectable subcutaneous tumor mass, all pathologies symptomatic of A-MuLV-induced disease (17). Histology and flow cytometric analysis revealed that most sick mice had leukemia or evidence of a lymphoid tumor (Table 1); all such tumors were Ly5.2⁺. Whereas tumors arising within 60 days of engraftment were all B220⁺, most *nfkbl*^{-/-} tumors that occurred after this period were Ly5.2⁺ CD4⁺ CD8⁺ thymomas (Table 1). This differs from the profile of wild-type cancers that developed in the same time frame (Table 1). These findings indicate that whereas transformed *nfkbl*^{-/-} cells display increased tumorigenicity, the absence of c-Rel did not appear to have a significant effect on tumorigenicity.

A-MuLV-transformed *nfkbl*^{-/-} pre-B cells exhibit increased cell cycle activity. Since an absence of c-Rel did not alter the tumorigenicity of A-MuLV-infected BM cells, we focused exclusively on the role of NF- κ B1 in transformation. The proliferative capacities of wild-type and *nfkbl*^{-/-} transformants were compared by using A-MuLV-infected BM ex-

FIG. 1. A-MuLV transformation of BM cells from Rel/NF- κ B mutant mice. (A) Focus assays. Equivalent numbers of wild-type (WT), *c-rel*^{-/-}, *rela*^{-/-}, and *nfkbl*^{-/-} BM cells infected with A-MuLV were seeded in agar cultures and incubated for 12 to 14 days; then the number of compact macroscopic colonies was enumerated. No colonies were observed in mock-infected BM cultures. Data are means $\pm$ standard deviations from five experiments. (B) Colonies of A-MuLV-transformed *nfkbl*^{-/-} BM cells are larger than normal. Representative examples of typical A-MuLV-transformed wild-type and *nfkbl*^{-/-} BM colonies after 14 days of incubation are shown (magnification, $\times 20$). v-Abl-transformed wild-type and *nfkbl*^{-/-} colonies were typically 1.5 to 2.0 mm and 3.0 to 3.5 mm in diameter, respectively. (C) A-MuLV-transformed *nfkbl*^{-/-} colonies contain more cells. Individual wild-type or *nfkbl*^{-/-} colonies were picked, and cells were dispersed and enumerated. Results shown are representative of a typical experiment.

panded as a suspension culture for 2 weeks, after which time >98% of cells of either genotype were B220⁺ Thy-1⁻ and expressed equivalent levels of p120^{v-ab1} (data not shown). Proliferation of equivalent input numbers of wild-type and *nfkbl*^{-/-} transformants in medium containing different concentrations of serum was assessed after 72 h by [³H]thymidine incorporation (Fig. 3). Serum was essential for expansion of both transformed populations; however, proliferation rates,

FIG. 2. A-MuLV-infected *nfkb1*^{-/-} BM cells display enhanced tumorigenicity. The survival of lethally irradiated C57BL/6-Ly5.1 mice engrafted with mock-infected (20 recipients, comprising 10 wild-type, 5 *nfkb1*^{-/-}, and 5 *c-rel*^{-/-} mice) (triangles) or A-MuLV-infected wild-type (20 recipients (squares), *c-rel*^{-/-} (20 recipients) (diamonds), or *nfkb1*^{-/-} (20 recipients) (circles) BM cells is presented as a Kaplan-Meier plot. After engraftment, irradiated recipients were monitored daily and sacrificed for analysis either upon presenting with symptoms typical of A-MuLV-induced disease, upon detection of a palpable tumor, or before imminent death.

while similar in low levels of FCS (4%, vol/vol), were greater for *nfkb1*^{-/-} cells over a range of higher (6 to 20%, vol/vol) serum concentrations. An increased rate of division for *nfkb1*^{-/-} transformants was supported by CFSE staining and BrdU incorporation (data not shown). The enhanced proliferation of *nfkb1*^{-/-} cells did not appear to reflect a reduced rate of apoptosis, as the proportions of viable and apoptotic wild-type and *nfkb1*^{-/-} transformants were similar throughout the period of culture in complete medium, and upon serum withdrawal, cells of both genotypes underwent death at equivalent rates (unpublished data).

The absence of NF- κ B1 shortens G₁ in v-Abl-transformed pre-B cells. For most cells, doubling times are largely determined by the length of G₁ (33). The fact that interleukin 7 (IL-7) induced equivalent proliferation of wild-type and *nfkb1*^{-/-} BM-derived pre-B cells (data not shown) indicated that it was the loss of NF- κ B1 in conjunction with a v-Abl mitogenic signal that led to accelerated cell cycle progression. Furthermore, the fact that v-Abl activity is required only for progression from G₀/G₁ to S phase (6) pointed to G₁ as the

most likely phase of the cell cycle to be affected in transformed *nfkb1*^{-/-} cells. This was tested by treating v-Abl-transformed wild-type and *nfkb1*^{-/-} pre-B cells with nocodazole, a compound that induces metaphase arrest (31). Upon removal of this agent, cells normally progress synchronously into G₁ and then through to S phase. As the doubling time of wild-type v-Abl transformants was approximately 16 h (unpublished data), both groups of cells were treated with nocodazole for 24 h to ensure that the vast majority were arrested in M phase. After nocodazole removal, viable cells purified by cell sorting were placed in culture, and progression through G₁ into S phase was monitored over time by BrdU incorporation. These results are summarized in Fig. 4. Following a short lag, cells entered G₁, and after 9 h, both populations had begun to enter S phase. At this time point, there were significantly more *nfkb1*^{-/-} cells labeled with BrdU. While BrdU incorporation by *nfkb1*^{-/-} cells peaked after approximately 12 h of culturing in growth medium, maximal BrdU incorporation by the wild-type population occurred later (after approximately 16 h), indicating that entry into S phase by the wild-type transformants

TABLE 1. A-MuLV-infected *nfkb1*^{-/-} BM cells are more tumorigenic.

Disease kinetics	Disease	No. of mice engrafted with:	
		Wild-type BM	<i>nfkb1</i> ^{-/-} BM
Early onset (<60 days)	B-cell leukemia ^a	3	5
	B cytopenia ^b	0	1
	Other ^c	0	1
	Total	3	7
Late onset (>60 days)	B-cell leukemia ^a	1	1 ^f
	Thymoma ^d	1	3
	Dead ^e	1	0
	Other ^c	1	0
	Total	4	4

^a More than 70% of BM cells were B220⁺.
^b Only 11% of cells in the BM were B220⁺.
^c Paraplegia and histological confirmation of tumor cell invasion into surrounding muscles of the vertebrae.
^d Thymomas composed of Ly5.2⁺ CD4⁺ CD8⁺ cells.
^e Mouse found dead with no histological evidence of leukemia or a tumor.
^f Mouse with leukemia that also presented with B220⁺ subcutaneous tumors.

appeared to be slower. By 20 h, BrdU incorporation by both populations was reduced from peak levels as cells entered M phase and subsequently reentered G₁. These findings show that the *nfkb1*^{-/-} transformants synchronously arrested in M phase enter S phase more rapidly than transformed wild-type pre-B cells treated in the same fashion. This suggests that for v-Abl-transformed pre-B cells, G₁ is shortened in the absence of NF-κB1.

Cyclin D1 expression is elevated in v-Abl-transformed

FIG. 3. Enhanced proliferation of A-MuLV-transformed pre-B cells lacking NF-κB1. A-MuLV-infected wild-type (open circles) or *nfkb1*^{-/-} (solid circles) B220⁺ BM cells (2 × 10⁴) were seeded into 96-well plates in the presence of varying concentrations of FCS and were cultured for 72 h. Cultures were then pulsed with 0.1 μCi of [³H]thymidine for 5 h prior to harvesting. Data are means ± standard deviations of results from triplicate wells from three separate experiments. The absence of error bars indicates that the standard deviation is <2,500 cpm.

FIG. 4. The duration of G₁ is reduced in *nfkb1*^{-/-} transformed pre-B cells. Viable v-Abl-transformed wild-type or *nfkb1*^{-/-} pre-B cells previously treated with nocodazole for 24 h to induce M-phase arrest were placed in culture at a density of 5 × 10⁵/ml (a total of 10⁶/well) and incubated for 3, 6, 9, 12, 16, or 20 h. Forty minutes prior to each time point, wild-type (open bars) and *nfkb1*^{-/-} (solid bars) cultures were pulsed with BrdU, harvested, and fixed, and BrdU-positive cells were detected by flow cytometry using an anti-BrdU antibody. Data are means ± standard deviations of results from duplicate wells from two separate experiments.

***nfkb1*^{-/-} pre-B cells.** Among those key regulators of cell division that function in G₁ are the D-type cyclins. The facts that cyclin D1 has been identified as a transcriptional target of NF-κB (NP-κB1/RelA) (23, 24, 27, 56) and its overexpression facilitates fibroblast transformation by v-Abl (1) prompted us to determine whether the enhanced division of v-Abl-transformed *nfkb1*^{-/-} pre-B cells was associated with altered D-type cyclin expression. *cyclin D1*, *cyclin D2*, and *cyclin D3* mRNA levels in IL-7-stimulated or A-MuLV-transformed wild-type and *nfkb1*^{-/-} pre-B cells were examined by semi-quantitative RT-PCR (Fig. 5A). As expected, *cyclin D1* mRNA was not detectable in IL-7-stimulated wild-type or *nfkb1*^{-/-} pre-B cells, while *cyclin D2* and *cyclin D3* expression was equivalent in cells of both genotypes. In contrast, *cyclin D1* was expressed in A-MuLV-transformed wild-type and *nfkb1*^{-/-} lymphocytes, and *cyclin D1* mRNA levels were consistently higher in *nfkb1*^{-/-} transformed cells. This difference was specific for *cyclin D1*, as mRNA levels for *D2* and *D3*, or *c-myc*, a target gene upregulated by v-Abl (60), were equivalent in wild-type and *nfkb1*^{-/-} transformants. Consistent with the mRNA expression studies, cyclin D1 protein levels, but not cyclin D2 protein levels, were ~4-fold higher in v-Abl-transformed *nfkb1*^{-/-} lymphocytes (Fig. 5B).

Proliferation of v-Abl-transformed pre-B cells is reduced in the absence of cyclin D1. Despite the fact that cyclin D1 is not expressed in primary B lineage cells (4, 32, 37), its expression in wild-type and *nfkb1*^{-/-} transformants prompted us to examine whether cyclin D1 contributed to the transformed phenotype. This was assessed by comparing the proliferation of v-Abl-transformed wild-type and *cyd1*^{-/-} pre-B cells in culture. BM cells of both genotypes infected with A-MuLV were

FIG. 5. Cyclin D1 expression induced by v-Abl is enhanced in the absence of NF- κ B1. (A) Cyclin mRNA expression in primary and transformed pre-B cells. Total mRNA was isolated from equivalent numbers of primary wild-type or *nfkb1*^{-/-} pre-B cells cultured in IL-7 for 5 days or BM cells 14 days after infection with A-MuLV. Expression of *cyclin D1*, *cyclin D2*, *cyclin D3*, *c-myc*, and *HPRT* was determined by semiquantitative RT-PCR using primers specific for each of the transcripts. PCR products were fractionated on 1.5% agarose gels. Data shown are representative of two independent experiments for IL-7-stimulated primary pre-B cells and five independent experiments for v-Abl-transformed cells. (B) Western blot analysis of cyclin D1 and cyclin D2 expression in A-MuLV-transformed pre-B cells. Total-cell extracts from equivalent numbers of wild-type and *nfkb1*^{-/-} A-MuLV-transformed pre-B cells 14 days postinfection were subjected to Western blot analysis using antisera specific for cyclin D1 and cyclin D2. Results from two typical experiments are shown.

expanded as suspension cultures for 2 weeks, after which time equivalent input numbers of each were placed in culture and proliferation was measured by counting cells at 24-h intervals over 4 days. These results, summarized in Fig. 6, show that the number of wild-type cells was significantly greater after 96 h. The increased proliferative rate of wild-type transformants was confirmed by pulsing cultures with [³H]thymidine (data not shown). Although the percentage of apoptotic cells is normally quite high in v-Abl-transformed populations prior to immortalization (Y. Nakamura, unpublished data), percentages of apoptotic cells were similar in the two cultures, ruling out differential rates of death as a factor that contributed to this difference in cell numbers. This finding suggests that cyclin D1 expression does contribute to the proliferation of wild-type pre-B cells transformed by v-Abl.

Restoration of normal proliferation in v-Abl-transformed *nfkb1*^{-/-} pre-B cells by enforced NF- κ B1 transgene expression coincides with a reduction in cyclin D1 levels. In an attempt to better define the relationship between the elevated cyclin D1 expression that accompanies the loss of NF- κ B1 and enhanced v-Abl-induced pre-B-cell proliferation, a transgene encoding p105 NF- κ B1 was introduced into *nfkb1*^{-/-} pre-B cells. BM

FIG. 6. An absence of cyclin D1 reduces the proliferation of v-Abl-transformed pre-B cells. Equivalent numbers (input, 10⁶ cells) of wild-type (open circles) or *cyclin D1*^{-/-} (solid circles) B220⁺ BM cells infected 14 days earlier with A-MuLV were placed in culture, and viable cell numbers were determined at 24-h intervals over 96 h. Data are means $\pm$ standard deviations from two experiments performed in quadruplicate.

cells from 5-fluorouracil-treated *nfkb1*^{-/-} (Ly5.2⁺) mice were infected with an MSCV-IRES-GFP retrovirus that either lacked an insert or expressed N-terminal HA-tagged murine p105 NF- κ B1. Lethally irradiated C57BL6 (Ly5.1⁺) mice reconstituted with infected cells were then examined 8 weeks later. Ly5.2⁺ GFP⁺ cells isolated by flow cytometry from control (MSCV-GFP) and test (MSCV-GFP/HA-p105) mice were subjected to Western blot analysis using an anti-HA antibody (Fig. 7A). Proteins of approximately 105 and 50 kDa that corresponded to the NF- κ B1 precursor and mature processed subunit, respectively, were detected only in cells infected with the MSCV-GFP/HA-p105 virus (Fig. 7A, lane 2).

The impact of NF- κ B1 transgene expression on the proliferation of v-Abl-transformed *nfkb1*^{-/-} pre-B cells is summarized in Fig. 7B. Equivalent input numbers of transformed B220⁺ Thy1⁻ pre-B cells derived from suspension cultures of A-MuLV-infected wild-type, *nfkb1*^{-/-}, GFP⁺ *nfkb1*^{-/-}, and GFP⁺ HA-p105 *nfkb1*^{-/-} BM were cultured for 72 h, and proliferation was monitored by tritiated thymidine incorporation. In contrast to the enhanced growth of v-Abl-transformed *nfkb1*^{-/-} pre-B cells, the proliferation of *nfkb1*^{-/-} pre-B transformants expressing HA-p105 was similar to that of A-MuLV-infected wild-type cells. Western blots of *nfkb1*^{-/-} transformants expressing HA-p105 revealed that the reduced proliferation coincided with lower cyclin D1 levels (Fig. 7C).

Enforced cyclin D1 expression increases the proliferative rate and tumorigenicity of v-Abl-transformed pre-B cells. To determine if elevated cyclin D1 expression alone accounted for the enhanced division and tumorigenicity of v-Abl-transformed *nfkb1*^{-/-} lymphocytes, these properties were compared for wild-type and *nfkb1*^{-/-} BM cells infected with recombinant retroviruses expressing v-Abl or v-Abl plus cyclin D1. As with A-MuLV, 14 days after infection of wild-type or *nfkb1*^{-/-} BM cells with either virus, suspension cultures were composed almost entirely of sIg⁻ B220⁺ cells (data not shown). Wild-type cells transformed by the v-Abl/cyclin D1 virus now proliferated as rapidly as v-Abl-transformed *nfkb1*^{-/-} cells (Fig. 8A). Interestingly, *nfkb1*^{-/-} lymphocytes transformed by the v-Abl/

FIG. 7. The proliferative response of v-Abl-transformed *nfkb1*^{-/-} pre-B cells is restored to that of wild-type transformants by an NF- κ B1 transgene. (A) NF- κ B1 transgene expression in BM cells. BM cells isolated from lethally irradiated Ly5.1⁺ C57BL/6 mice previously reconstituted with Ly5.2 C57BL/6 *nfkb1*^{-/-} BM that had been infected with MSCV-GFP or MSCV-GFP/HA-p105 virus were stained with a biotinylated Ly5.2⁺-specific MAb, and cells were subjected to two-color cell sorting (GFP expression detected as fluorescein isothiocyanate positive). Equivalent numbers (3×10^5) of Ly5.2⁺ GFP⁺ cells infected with these retroviruses were subjected to Western blot analysis and probed with an anti-HA MAb. The precursor (p105) and mature processed (p50) forms of NF- κ B1 are indicated. (B) Enforced NF- κ B1 transgene expression reduces the proliferation of v-Abl-transformed *nfkb1*^{-/-} pre-B cells. Equivalent numbers of wild-type, *nfkb1*^{-/-}, MSCV-GFP *nfkb1*^{-/-}, and MSCV-GFP HA-p105 *nfkb1*^{-/-} B220⁺ BM cells infected 14 days earlier with A-MuLV were placed in culture for 72 h and then pulsed with 0.1 μ Ci of [³H]thymidine prior to harvesting. Data are means $\pm$ standard deviations of results from triplicate wells from three separate experiments. (C) Cyclin D1 expression is reduced in v-Abl-transformed *nfkb1*^{-/-} pre-B cells that express an NF- κ B1 transgene. Total-cell extracts from equivalent numbers of MSCV-GFP *nfkb1*^{-/-} (lane 1) and MSCV-GFP HA-p105 *nfkb1*^{-/-}

cyclin D1 virus proliferated marginally faster than wild-type cells infected by this virus, despite the fact that cyclin D1 levels were equivalent in the two cell types (Fig. 8B). This suggested that altered expression of genes in addition to cyclin D1 contributed to the enhanced proliferation of the *nfkb1*^{-/-} transformants.

Among the mice receiving v-Abl virus-infected wild-type and *nfkb1*^{-/-} BM, the frequency, kinetics, and type of tumors that arose (Fig. 9 and Table 2) were similar to those observed in the respective genotypes for A-MuLV-induced tumors. However, the frequency and onset of tumor formation in mice engrafted with v-Abl/cyclin D1 virus-infected wild-type cells were now similar to those of mice receiving v-Abl/cyclin D1 virus-infected cells, 55 and 65% engrafted with wild-type and *nfkb1*^{-/-} cells, respectively, developed tumors, and these arose with similar kinetics. Whereas all v-Abl/cyclin D1 virus-induced wild-type tumors were B220⁺, interestingly, *nfkb1*^{-/-} cancers induced by coexpression of v-Abl and cyclin D1 comprised B220⁺ leukemias as well as CD4⁺ CD8⁺ thymomas (Table 2). This indicates that elevated cyclin D1 expression, while appearing to contribute to the enhanced tumorigenicity of v-Abl-transformed *nfkb1*^{-/-} BM, did not account for the increased incidence of thymomas.

NF- κ B1 can function as a negative regulator of cyclin D1 transcription. The elevated expression of cyclin D1 in v-Abl-transformed *nfkb1*^{-/-} lymphocytes led us to determine if NF- κ B1 acted to downregulate *cyclin D1* transcription. A comparison of the mouse (48) and human (23) cyclin D1 promoters revealed that the proximal κ B site (-42 to -33; 5'-GGGGA GTTTT-3') and immediate flanking sequence were conserved, but the distal κ B elements found in human *cyclin D1* were absent in the mouse gene (Nakamura, unpublished). The role of NF- κ B1 in v-Abl-induced cyclin D1 transcription was assessed by cotransfecting 293T cells with mouse *cyclin D1* promoter reporter constructs (Fig. 10A) and expression vectors encoding v-Abl and the p50 or p105 form of NF- κ B1. The results are summarized in Fig. 10B. The wild-type promoter reporter, *cyd1-luc*, was induced by RelA (lane 8), a known activator of this gene, albeit to a lesser extent than by v-Abl (lane 7), and the RelA (lane 12) but not the v-Abl (lane 11) upregulation of *cyd1-luc* was completely dependent on the κ B site. This indicated that regulatory sequences in addition to the κ B element contribute to v-Abl-induced *cyclin D1* transcription. In contrast to RelA (lane 9), expression vectors for either the p105 or p50 form of NF- κ B1 inhibited v-Abl-induced *cyclin D1* transcription (compare lanes 16 and 17 with lane 7), and this repression was mediated predominantly through the κ B element (compare lanes 20 and 21 with lane 11). Collectively, these results establish that NF- κ B1 can inhibit v-Abl-induced cyclin D1 transcription.

(lane 2) A-MuLV-transformed pre-B cells 14 days postinfection were subjected to Western blot analysis using antisera specific for cyclin D1 and cyclin D2. Results are representative of those obtained from two experiments.

FIG. 8. Enforced cyclin D1 expression increases the proliferation of v-Abl-transformed pre-B cells. (A) Proliferation of pre-B cells infected with a virus expressing v-Abl or v-Abl plus cyclin D1. Equivalent numbers (input, 10^6 cells) of wild-type (open bars) and *nfkb1*^{-/-} (solid bars) B220⁺ BM cells infected 14 days earlier with a virus expressing either v-Abl or v-Abl plus cyclin D1 were placed in culture, and viable cell numbers were determined after 48 h. Data are means $\pm$ standard deviations from three experiments performed in duplicate. *, $P < 0.01$ by Student's *t* test; **, $P < 0.001$ by Student's *t* test. (B) Cyclin D1 expression in cells infected with the v-Abl/cyclin D1 virus. Equivalent numbers of wild-type or *nfkb1*^{-/-} B220⁺ cells infected as described for panel A were used to determine cyclin D1 mRNA and protein levels. Total RNA was subjected to semiquantitative RT-PCR using primers specific for *cyclin D1*, *cyclin D2*, and *cyclin D3*, and PCR samples were fractionated on a 1.5% agarose gel. Total-protein extracts were subjected to Western blot analysis using cyclin D1-specific antisera.

DISCUSSION

v-Abl transformation of murine pre-B cells serves as an important model for understanding the molecular mechanisms by which Abl oncoproteins induce cancer. To date, the specific roles of the various signal transduction pathways activated by v-Abl during lymphoid oncogenesis, plus the identities of critical downstream target genes that function as effectors of the transformation process, remain poorly defined. Here we show that *cyclin D1* is a bona fide transcriptional target induced by v-Abl in pre-B cells and propose that modulation of its expression levels by Rel/NF- κ B dimers with different transcriptional activities influences the division rate and tumorigenic properties of transformed cells.

The data presented here for transformed pre-B cells confirm the recent finding made for fibroblasts (7) that the *cyclin D1* gene is located downstream of signaling pathways activated by

v-Abl. *cyclin D1*, although not expressed in primary B lineage cells (4, 32, 37), is induced in other cell types by the same signaling pathways that are activated in normal pre-B and B cells by growth factors and mitogens. The difference in *cyclin D1* regulation between IL-7-stimulated primary pre-B cells and v-Abl-transformed pre-B cells indicates that induction of cyclin D1 in transformed pre-B cells requires a v-Abl-specific signal or the combined dysregulation by v-Abl of multiple pathways utilized in both primary and transformed cells.

Transcriptional induction of human *cyclin D1* in fibroblasts by growth factors and certain oncogenes depends in part on NF- κ B (the NF- κ B1/RelA dimer) acting through multiple κ B elements in the promoter (23, 24, 27, 56). Using mouse *cyclin D1* promoter reporters, we show that v-Abl induction of *cyclin D1* transcription is mediated in part through the conserved proximal κ B site and that this transcription is differentially modulated by various Rel/NF- κ B family members. Although RelA alone was able to upregulate cyclin D1 transcription, transactivating NF- κ B-like dimers appear to be only one type among an unknown number of transcription factors that v-Abl regulates to induce *cyclin D1* transcription. This conclusion comes from the findings that promoter activities induced by v-Abl and those induced by v-Abl plus RelA are equivalent and that v-Abl-induced *cyclin D1* transcription is reduced but not abolished when the κ B site is mutated. In contrast, NF- κ B1, a nontransactivating family member, was found to inhibit v-Abl-induced *cyclin D1* transcription. Support for the notion that NF- κ B1 downregulation of *cyclin D1* promoter reporter activity has physiological significance comes from the observations that cyclin D1 expression in v-Abl-induced pre-B cells is higher in the absence of NF- κ B1 and that this expression pattern is reversed upon reintroduction of an NF- κ B1 p105 transgene. While these findings indicate that NF- κ B1 downregulates v-Abl-induced *cyclin D1* transcription, an inability to obtain *nfkb1*^{-/-} pre-B cells that express only an NF- κ B1 p50-encoded transgene (Nakamura, unpublished) prevented us from determining if the inhibition of *cyclin D1* transcription induced by v-Abl is mediated through the p50 or p105 form of NF- κ B1. p105, while serving as a precursor for p50 (47), also functions as an I κ B protein (57). Consequently, increased cyclin D1 expression in v-Abl-transformed *nfkb1*^{-/-} pre-B cells could conceivably arise from an absence of I κ B function that leads to increased nuclear levels of RelA or c-Rel dimers. Alternatively, v-Abl-dependent transcription may be inhibited directly by p50 homodimers. Because the p50 subunit lacks any intrinsic transactivating capability (2), transcriptional repression mediated by p50 homodimers is thought to result from their occupancy of κ B sites in promoters, preventing the binding of transactivating dimers. If such a mechanism does account for NF- κ B1 inhibition of *cyclin D1* transcription, our data suggest that v-Abl-induced *cyclin D1* transcription might arise in part from NF- κ B-like transactivating complexes displacing p50 homodimers binding to the κ B site. While this is consistent with v-Abl, like BCR-Abl (42), being able to induce the nuclear translocation of Rel/NF- κ B in fibroblasts (data not shown), increased NF- κ B activity in v-Abl-transformed pre-B cells may result from a mechanism other than nuclear import, given that v-Abl negatively regulates nuclear Rel/NF- κ B levels in these cells (29). Nevertheless, even in the absence of precise knowledge of a mechanism by which v-Abl could increase

FIG. 9. Enforced cyclin D1 expression enhances the tumorigenicity of v-Abl-transformed BM cells. Lethally irradiated C57BL/6-Ly5.1⁺ mice were engrafted with equivalent numbers of mock-infected (20 recipients, comprising 10 wild-type, 5 *nfκb1*^{-/-}, and 5 *c-rel*^{-/-} mice) (triangles), v-Abl virus-infected (open symbols), or v-Abl/cyclin D1 virus-infected (closed symbols) wild-type (20 recipients) (squares) or *nfκb1*^{-/-} (20 recipients) (circles) BM cells. Engrafted recipients were sacrificed for analysis upon presenting with symptoms of A-MuLV-induced disease, upon detection of a palpable tumor, or before imminent death.

NF-κB activity in pre-B cells, the data presented here support the model of competition for occupancy of the κB site by nontransactivating and transactivating Rel/NF-κB family members as one means of regulating cyclin D1 transcription. The latter mechanism is of considerable interest, since, despite a large body of data showing that overexpression of p50 homodimers can inhibit NF-κB-dependent transcription, there is little evidence to support such a role for p50 homodimers expressed at physiological levels.

The increased rate of v-Abl-induced pre-B-cell division arising from the loss of NF-κB1 coincides with a reduction in the period required for these cells to transit G₁. Since cyclin D1 overexpression reduces G₁ in fibroblasts (39), and we observed a direct correlation between rates of cell division and varying cyclin D1 levels in v-Abl-transformed pre-B cells, we propose that the reduced length of G₁ in *nfκb1*^{-/-} transformants can be accounted for in part by the increase in cyclin D1 levels. The mechanism by which increased cyclin D1 expression could alter G₁ in v-Abl-transformed pre-B cells is unknown. Despite the facts that cyclin D1 is not expressed in primary B lineage cells (4, 32, 37) and cyclin D1 overexpression does not alter normal pre-B-cell division (4), cyclin D1 function is not redundant in

TABLE 2. Coexpression of v-Abl and cyclin D1 enhances v-Abl-dependent tumorigenesis

Disease kinetics	Disease	No. of mice engrafted with:			
		Wild-type BM		<i>nfκb1</i> ^{-/-} BM	
		v-Abl	v-Abl/ cyclin D1	v-Abl	v-Abl/ cyclin D1
Early onset (<60 days)	B-cell leukemia ^a	2	5	4	6
	Thymoma ^b	0	0	0	1
	Dead ^c	0	1	2	1
	Total	2	6	6	8
Late onset (≥60 days)	B-cell leukemia ^a	3	3	2	3
	Thymoma ^b	0	0	1	2
	Dead ^c	1	2	1	0
	Other ^d	0	0	0	1
	Total	4	5	4	6

^a More than 70% of BM cells were B220⁺ sIgM⁻.
^b Ly5.2⁺ *nfκb1*^{-/-} CD4⁺ CD8⁺ thymomas.
^c All but one of this group of mice had histological evidence of tumor cells in the BM, spleen, or vertebrae.
^d Mouse presented with paraplegia. Histology revealed tumor infiltration in the spinal column.

FIG. 10. NF-κB1 represses v-Abl-induced cyclin D1 transcription. (A) Schematic representation of the mouse cyclin D1 promoter and the luciferase reporter plasmids. Numbers in parentheses indicate the positions of the restriction enzyme sites in the murine *cyclin D1* promoter relative to the transcription start site determined by Wood et al. (59). The open box represents the conserved NF-κB binding site κB1 (5'-GGGGAGTTT-3'; -42 to -33), whereas the corresponding symbol with an "X" represents the mutated motif κB1_m (5'TTCGAGAAAT-3'). Wavy and straight arrows represent the transcription initiation and translation start sites, respectively. The firefly luciferase gene is shown as LUC. Plasmid nomenclature is given to the right of each construct. (B) v-Abl-dependent cyclin D1 transcription is repressed by NF-κB1. 293T cells were transiently transfected with 1 μg of pluc3 (lanes 1 to 5), cyd1-luc (lanes 6 to 9 and 14 to 17), or cyd1κB_m-luc (lanes 10 to 13 and 18 to 21) together with the expression plasmid pEF-BOS containing no insert (lanes 1, 6, and 10), v-Abl (lanes 2, 7, 9, 11, 13, 16, 17, 20, and 21), RelA (lanes 3, 8, 9, 12, and 13), p50 NF-κB1 (lanes 4, 14, 16, 18, and 20), or p105 NF-κB1 (lanes 5, 15, 17, 19, and 21). The relative luciferase units for each set of transfections were normalized by cotransfection with the *Renilla* luciferase vector pRL-TK. Results are the mean percentages of luciferase activity ± standard deviations obtained for four separate sets of transfections.

v-Abl-transformed pre-B cells. Cyclin D1-deficient pre-B cells infected with A-MuLV proliferate somewhat more slowly than wild-type transformants, while enforced overexpression of cyclin D1 enhances v-Abl-induced wild-type pre-B-cell proliferation. One plausible model that may account for these findings is that a crucial G₁ substrate for cyclin D1, absent or in limiting amounts in normal B lineage cells, is altered in v-Abl-transformed pre-B cells. Cyclin-dependent kinase (cdk) inhibitors such as INK4 proteins are frequently upregulated in v-Abl-transformed pre-B cells (40), so conceivably the ability of elevated cyclin D1 levels to increase the division rate of v-Abl-transformed pre-B cells could involve cyclin D1 competing with cdk inhibitors for limiting substrates such as cdk4.

The increased division of *nfkb1*^{-/-} transformants is likely to

involve the altered expression of a gene(s) in addition to cyclin D1, as the proliferation of *nfkb1*^{-/-} pre-B cells infected with the v-Abl/cyclin D1 virus is slightly greater than that of wild-type cells transformed by this retrovirus, despite the fact that cyclin D1 levels are equivalent in both cell populations. The involvement of other NF-κB1-regulated genes in v-Abl-mediated oncogenesis is also emphasized by a greater frequency of T lymphomas on the *nfkb1*^{-/-} background which are clearly not a result of increased cyclin D1 expression. As lymphopoiesis seems normal in *nfkb1*^{-/-} mice (46), it appears unlikely that in the absence of NF-κB1, v-Abl drives development of the B220⁺ target cell population normally infected by A-MuLV toward the T lineage. Instead, the emergence of *nfkb1*^{-/-} T lymphomas may be due to v-Abl transforming a T

lineage progenitor. In *nfkb1*^{-/-} mice, increases in the frequency of such a progenitor, a greater percentage of such cells in cycle, or heightened susceptibility to infection are all plausible explanations that could account for the difference in the incidences of wild-type and *nfkb1*^{-/-} T-cell lymphomas. The identities of the other NF- κ B1-regulated genes are unknown. *c-myc*, for example, which is regulated by the Rel/NF- κ B pathway (41), is an essential downstream target of v-Abl in the transformation of fibroblasts (45, 60) that cooperates with cyclin D1 to promote the formation of lymphoid tumors (4). Its expression, however, is the same in wild-type and *nfkb1*^{-/-} transformed cells (Fig. 5), a finding consistent with the fact that *c-myc* expression is not altered by the loss of NF- κ B1 (20).

Despite the fact that cyclin D1 facilitates v-Abl-transformed pre-B-cell division, in precrisis cultures of *nfkb1*^{-/-} pre-B transformants, cyclin D1 levels were higher during early passages (2 to 3 weeks) than in cells propagated for 6 weeks (data not shown), suggesting that expression of cyclin D1 may be more important early in the transformation process. Particularly in view of the fact that the division of these cells did not decrease as cyclin D1 levels dropped, additional genetic changes that accompany immortalization in culture probably obviate the role served by cyclin D1. This is consistent with the fact that cyclin D1 is expressed in only a minor proportion of immortal A-MuLV-transformed pre-B-cell lines (4). Since increased cyclin D1 expression does not appear to influence the incidence or kinetics of pre-B-cell immortalization in culture, we speculate that an increased rate of division might lead to a more rapid expansion of cells *in vivo*, which in turn could result in a higher incidence of malignant cells emerging (17, 58).

Finally, a growing body of evidence indicates that abnormalities in Rel/NF- κ B signaling are a common feature of human cancers (15, 41). While the overexpression of NF- κ B1 (p50) in a range of tumors (41) indirectly suggests that NF- κ B1 may promote the oncogenic process, we show here that loss of NF- κ B1 instead enhances v-Abl transformation of lymphoid cells. This finding in a murine lymphoma model raises the possibility that the genesis of some human cancers may involve *nfkb1*^{-/-} loss-of-function mutations. However, since mice homozygous for the *nfkb1*^{-/-} null mutation do not have an increased incidence of spontaneous tumors (14), any loss of NF- κ B1 function linked with cancer would point to a role in facilitating rather than initiating oncogenesis. Ultimately, whether tumorigenesis is promoted by enhanced or reduced NF- κ B1 activity may depend on the cellular context in which the oncogenic events occur. To date, various studies have demonstrated that inhibiting Rel/NF- κ B activity enhances radiation- or cytotoxic compound-induced death of a range of tumors in culture (51, 54). Our results showing that NF- κ B1 acts to inhibit v-Abl-induced B-cell transformation indicate that blocking NF- κ B1 activity in certain cancers, particularly those in which cyclin D1 acts to promote or initiate tumorigenesis, may instead be detrimental. Together with the recent finding that RelA may be important for p53-dependent apoptosis (44), this highlights the need to understand in greater detail the impacts of modulating the expression of these transcription factors on the division and survival of different tumors and how these in turn might influence any future Rel/NF- κ B-based cancer therapy.

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