The *v-abl* tyrosine kinase negatively regulates NF- κ B/Rel factors and blocks κ gene transcription in pre-B lymphocytes

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Transformation of B-lineage precursors by the Abelson murine leukemia virus appears to arrest development at the pre-B stage. Abelson-transformed pre-B cell lines generally retain transcriptionally inactive, unrearranged immunoglobulin κ alleles. We demonstrate that nontransformed pre-B cells expanded from the mouse bone marrow efficiently transcribe unrearranged κ alleles. In addition, they contain activated complexes of the NF- κ B/Rel transcription factor family, in contrast with their Abelson-transformed counterparts. Using conditionally transformed pre-B cell lines, we show that the *v-abl* viral transforming protein, a tyrosine kinase, blocks germ-line κ gene transcription and negatively regulates NF- κ B/Rel activity. An active *v-abl* kinase specifically inhibits the NF- κ B/Rel-dependent κ intron enhancer, which is implicated in promoting both transcription and rearrangement of the κ locus. *v-abl* inhibits the activated state of NF- κ B/Rel complexes in a pre-B cell via a post-translational mechanism that results in increased stability of the inhibitory subunit I κ B α . This analysis suggests a molecular pathway by which *v-abl* inhibits κ locus transcription and rearrangement.

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Murine B lymphocytes develop by executing a temporally ordered set of immunoglobulin gene rearrangement events (for review, see Blackwell and Alt 1989). The heavy-chain locus is preferentially activated for rearrangement in progenitor B cells (pro-B). Productive assembly of heavy-chain gene segments leads to the generation of precursor (pre-B) cells that express μ protein primarily in the cytoplasm. A small fraction of μ protein associates with surrogate light chains to form a pre-B cell receptor that is localized to the cell surface (for review, see Melchers et al. 1993) and is needed for further development (Ehlich et al. 1993). Pre-B cells require stromal cell contact and IL-7 for their development and proliferation. To differentiate into antigen receptor-expressing B lymphocytes, pre-B cells must activate rearrangement of the light-chain loci (κ or λ). The vast majority of murine B cells (95%) rearrange and express the κ locus.

Transcriptional activation and rearrangement of the κ light-chain locus represent pivotal regulated events in

the B-cell developmental pathway. It has been proposed that transcriptional activation of unrearranged (germ-line) segments precedes and is required for their rearrangement (Blackwell and Alt 1989). This model is supported by observations correlating germ-line transcription of the J-C region of the κ locus with its rearrangement in developing fetal liver B lymphocytes as well as in transformed pre-B cell lines (Schlissel and Baltimore 1989; Lennon and Perry 1990). Transcription of the κ locus is controlled by two enhancers, the intron enhancer ($E_{i\kappa}$) and the 3' enhancer ($E_{3'\kappa}$), which flank the constant region exon (for review, see Staudt and Lenardo 1991). The intron enhancer appears to regulate the developmental activation of the κ locus; it functions as a strongly inducible enhancer in transformed pre-B cell lines in which transcription of germ-line κ alleles is activated by bacterial lipopolysaccharide (LPS) stimulation (Atchison and Perry 1987; Lenardo et al. 1987). $E_{i\kappa}$ also appears to regulate rearrangement of the κ locus. Targeted deletion of $E_{i\kappa}$ in murine embryonic stem cells results in a complete block to rearrangement of the κ locus in B-lineage descendants of the mutant stem cells

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(Takeda et al. 1993). In contrast with the intron enhancer, the 3' enhancer is fully functional at later, mature B and plasma cell, stages of B-cell development (Meyer and Neuberger 1989; Pongubala and Atchison 1991).

Transcription factors of the NF- κ B/Rel family regulate the activity of the κ intron enhancer (for review, see Lenardo and Baltimore 1989; Blank et al. 1992). They bind to the κ B site in *E_H*, which is essential for the LPS-inducible activity of the enhancer in transformed pre-B cell lines (Atchison and Perry 1987; Lenardo et al. 1987). NF- κ B is a heterodimer comprised of two subunits, p50 and p65. The κ B site is additionally recognized by a diverse set of heterodimeric or homodimeric complexes including c-Rel, RelB, and p52 subunits. In unstimulated pre-B cell lines, NF- κ B/Rel complexes are sequestered in the cytoplasm in an inactive state by inhibitory I κ B subunits (Baeuerle and Baltimore 1988; for review, see Beg and Baldwin 1993). LPS stimulation results in highly accelerated degradation of I κ B α , thereby allowing nuclear translocation of NF- κ B/Rel complexes and their binding to the κ intron enhancer (Henkel et al. 1993; Rice and Ernst 1993). NF- κ B/Rel complexes are inducible in a wide variety of cell types by a diverse set of signals, including mitogens and inflammatory cytokines. However, the nature of the developmental signaling pathway that activates NF- κ B/Rel complexes in nontransformed pre-B cells is obscure.

Pre-B cell lines generated by in vitro transformation of fetal liver or adult bone marrow cells with the Abelson murine leukemia virus (Ab-MuLV) have been used extensively as models to analyze mechanisms regulating κ gene transcription and rearrangement (for review, see Baltimore et al. 1979). In almost every case, these cell lines have initiated or completed rearrangements at a transcriptionally active heavy-chain locus but contain germ-line κ alleles that are transcriptionally inactive (Alt et al. 1981; Nelson et al. 1985). A long-standing question has been whether transformation by the virus actively blocks transcription and/or rearrangement of the κ locus. We demonstrate that nontransformed pre-B cells expanded from the mouse bone marrow efficiently transcribe germ-line κ alleles. In addition, they contain activated complexes of the NF- κ B/Rel transcription factor family, in contrast with their Abelson-transformed counterparts. Using conditionally transformed pre-B cell lines, we demonstrate that the viral transforming protein, *v-abl*, blocks germ-line κ transcription and negatively regulates NF- κ B/Rel activity and the functioning of the κ intron enhancer. This analysis suggests a molecular pathway by which *v-abl* inhibits κ locus transcription and rearrangement. In an accompanying study (Chen et al., this issue) it is demonstrated that inactivation of *v-abl* in conditionally transformed pre-B cells results in efficient κ locus rearrangement. Because induction of rearrangement is also accompanied by increased expression of the recombinase genes, *Rag-1* and *Rag-2*, these results collectively suggest that *v-abl* inhibits κ rearrangement by two distinct mechanisms in pre-B cells.

Results

Nontransformed pre-B cells transcribe germ-line κ alleles and contain activated NF- κ B/Rel complexes

In experiments set up to explore the signaling pathways and mechanisms regulating transcription and rearrangement of the κ locus in nontransformed pre-B cells, we utilized IL-7-expanded pre-B cells from Whitlock–Witte bone marrow cultures (Whitlock and Witte 1982; Morrow et al. 1992). In these cultures, adherent stromal cells provide a feeder layer that specifically supports the growth and development of B-lineage cells. The addition of IL-7 to an established Whitlock–Witte culture permits the selective expansion of a well-defined population of B-lineage cells (Fig. 1) based on differential cell-surface expression of markers described by Hardy et al. (1991). These cells express the B-lineage-specific marker B220, and correspond to the late pro-B/early pre-B intermediate stage on the basis of the following criteria: (1) The cells express high levels of BP-1 and HSA (heat stable antigen), the latter being expressed at highest levels at the late pro-B and pre-B stages; (2) ~91% of the cells are S7 (leukosialin) positive, expression of which is down-regulated at the pre-B cell stage; and (3), as shown previously (Morrow et al. 1992), Southern blotting reveals that the vast majority of cells have undergone *J_H* rearrangements at both heavy-chain alleles but contain unrearranged κ alleles (data not shown). Although no heavy-chain (μ) protein is detectable on the cell surface, FACS analysis of

Figure 1. FACS analysis of IL-7-expanded pre-B cells. Shown are the levels of expression of BP-1, B220, S7, and HSA of a typical murine bone marrow-derived pre-B cell population. The IL-7-expanded cells are homogeneous for expression of BP-1, S7, and HSA but heterogeneous for expression of B220, a B-lineage specific marker, consisting of a B220 low and a B220 intermediate population. The B220 expression profile is similar to that observed in Whitlock–Witte cultures before the addition of IL-7 (Whitlock et al. 1985). This population is Thy 1.2[−], granulocyte[−], and IgM[−].

permeabilized cells shows that ~20% of the cells express μ intracellularly (data not shown). The presence of full-length μ protein has been confirmed by Western blotting. The IL-7-expanded pre-B cells seem to represent a developmentally stable transitional state preceding S7 down-regulation and the initiation of κ rearrangement. They proliferate rapidly in response to IL-7 for at least 7–10 days without undergoing significant κ rearrangement.

Although IL-7-expanded pre-B cells have yet to initiate κ rearrangement, they have been developmentally triggered to activate transcription of the κ locus (Fig. 2A). Ribonuclease protection analysis with a C_κ constant region probe showed that the nontransformed pre-B cells transcribe the κ locus at a high level. This level is comparable to that observed in Abelson-transformed pre-B cell lines after stimulation with LPS (Fig. 2A). The κ RNAs in nontransformed pre-B cells are derived from transcription of the unrearranged J-C region of the κ locus. Northern blot analysis of poly(A)⁺ RNA from IL-7-expanded cells with a C_κ probe detected four RNA species that are characteristic of germ-line κ locus transcription (Fig. 2B). The 8.4- and 4.7-kb transcripts are unprocessed forms of the mature 1.1- and 0.8-kb germ-line transcripts, respectively. These transcripts initiate from two germ-line promoters upstream of the J_κ gene

Figure 2. (A) RNase protection analysis of κ RNA in pre-B cells. RNA (10 μ g) from IL-7-expanded pre-B cells or from the Abelson transformant 38B9 was hybridized to C_κ and actin antisense riboprobes and processed as described in Materials and methods. 38B9 cells were cultured in the absence (–) or presence (+) of LPS (10 μ g/ml) for 24 hr. Lane M contains molecular weight markers. (B) Northern blot analysis of germ-line κ RNAs in pre-B cells. Poly(A)⁺ RNA (4 μ g) from the indicated cells was hybridized with a C_κ probe. MEL 201 and MPC 11 are murine erythroleukemia and myeloma cell lines, respectively. MPC 11 cells express a rearranged κ allele. 38B9 cells were cultured as indicated in the legend to Fig. 2A. Sizes of various κ RNAs are indicated at right.

Figure 3. Mobility shift analysis of NF- κ B/Rel complexes in pre-B cells. Nuclear extract protein (10 μ g) from the indicated cells was incubated with a κ B site probe derived from the κ intron enhancer. Protein-DNA complexes were resolved by polyacrylamide gel electrophoresis. Subunit composition of NF- κ B/Rel complexes in normal pre-B cells was analyzed using α -p65, α -p50, and α -c-Rel antipeptide antibodies. Nuclear extract protein was preincubated with the indicated antibody before the addition of κ B site probe. 38B9 cells were cultured as indicated in the legend to Fig. 2A for the isolation of nuclear extract protein. NF- κ B (includes p65:p50 and c-Rel:p50) and p50:p50 complexes are denoted at right.

segments and have been characterized previously in LPS-induced Abelson-transformed pre-B cells (Martin and Van Ness 1990). The high levels of germ-line κ transcription in nontransformed pre-B cells are not induced by exogenously added IL-7 in that pre-B cells isolated before the addition of IL-7 show similar levels of κ RNA (data not shown).

To determine the status of NF- κ B/Rel complexes in IL-7-expanded pre-B cells, nuclear extracts were prepared and assayed for DNA binding to a κ B probe using the mobility shift assay. Figure 3 shows that the nontransformed pre-B cells contain activated nuclear NF- κ B/Rel complexes at a level that is significantly higher than in an Abelson-transformed counterpart. Antipeptide antibodies directed against subunits of NF- κ B/Rel complexes detected comparable amounts of both p50:p65 and p50:c-Rel heterodimers in normal pre-B cells (Fig. 3). Both types of heterodimers are induced by LPS treatment of Abelson-transformed pre-B cell lines (data not shown). Therefore, high-level germ-line κ transcription in normal pre-B cells is correlated with activated nuclear NF- κ B/Rel complexes. In this regard, the nontransformed pre-B cells are reciprocally related to their Abelson-transformed counterparts. These observations led us to consider the hypothesis that transformation of pre-B cells by *v-abl* might result in a block of germ-line κ gene transcription as a consequence of negative regulation of NF- κ B/Rel activity.

v-abl inhibits κ locus transcription in pre-B cells

To test the ability of *v-abl* to regulate the activity of the κ locus and NF- κ B/Rel complexes in pre-B cells, we utilized conditionally transformed cell lines derived by infection of bone marrow cells with an Abelson virus encoding a temperature-sensitive *v-abl* protein (Engelman and Rosenberg 1987, 1990). The tyrosine kinase activity of the mutant protein has been shown to be greatly reduced at the nonpermissive temperature (39.5°C) compared with the permissive temperature (34.5°C) (Engelman and Rosenberg 1990). As a result, the *v-abl^{ts}* cells arrest growth in the G₁ phase of the cell cycle soon after a shift to the nonpermissive temperature and undergo programmed cell death (Chen and Rosenberg 1992). To facilitate the analysis of differentiation events that might ensue upon the inactivation of *v-abl* kinase activity, both wild-type and temperature-sensitive transformed pre-B cell lines have been stably transfected with a *bcl-2* expression vector. Expression of the *bcl-2* gene prevents cell death when the *v-abl^{ts}* cells are shifted to the nonpermissive temperature. Both the *v-abl^{wt}* and the *v-abl^{ts}* cell lines have undergone rearrangement at the heavy-chain locus but do not express μ protein. In contrast, the κ loci are in a germ-line configuration. Both types of cell lines are B220 positive and express high levels of HSA and low levels of S7 (data not shown). Figure 4 shows the effect of inactivating the mutant *v-abl* protein on the expression of the κ locus. As expected, little κ RNA is detectable in the *v-abl^{wt}* pre-B cells at either the permissive or restrictive temperature.

Figure 4. RNase protection analysis of κ RNA in Abelson-transformed (*v-abl^{wt}* and *v-abl^{ts}* pre-B-cells). Wild-type (WT1/*bcl2-3*) and temperature-sensitive [103/*bcl2-4* and 103/*bcl2-13*] Abelson pre-B cell lines were cultured at 34.5°C and then shifted to 39.5°C. RNA was isolated at 0, 7, 24, and 48 hr after the temperature shift and analyzed as described in Materials and methods.

In contrast, κ RNA is highly induced (~50-fold) in the *v-abl^{ts}* pre-B cells within 24 hr after the shift to the restrictive temperature. It should be noted that the *v-abl^{ts}* cells express basal levels of κ RNA at the permissive temperature, which are approximately fivefold higher than those in *v-abl^{wt}* cells (discussed below). The induction of the κ locus upon inactivation of *v-abl* has also been confirmed in an independently derived temperature-sensitive pre-B cell line (data not shown). Inducible κ expression is not dependent on the expression of *bcl-2*. Similar levels of κ RNA are induced in the *bcl-2* nonexpressing parental line (data not shown).

v-abl negatively regulates NF- κ B/Rel complexes in pre-B cells

To determine whether the induction of the κ locus was accompanied by the activation of NF- κ B/Rel complexes, we also analyzed nuclear extracts prepared from the *v-abl^{wt}* and *v-abl^{ts}* cells during the course of the temperature shift (Fig. 5A). NF- κ B/Rel activity was induced approximately fivefold only in the *v-abl^{ts}* cells within 7 hr of the shift to the restrictive temperature. Maximal induction (approximately eightfold) was detected at 24 hr. Antibody supershift experiments revealed that the induced complexes contained p65, c-Rel, and p50 subunits and were similar in composition to NF- κ B/Rel complexes in nontransformed pre-B cells (data not shown). Two other transcription factors found in pre-B cells, Oct-1 and Oct-2 (Staudt et al. 1986), were unaffected by the temperature shift (Fig. 5A). The induction of NF- κ B/Rel complexes in the *v-abl^{ts}* cells closely paralleled the induction of κ RNA expression (see Fig. 4). As was the case for κ RNA levels, the *v-abl^{ts}* cells contained higher levels of activated NF- κ B/Rel complexes even at the permissive temperature when compared with the *v-abl^{wt}* cells. In this regard, the mutant *v-abl* protein has been shown to have lower kinase activity compared with its wild-type counterpart at the permissive temperature (Engelman and Rosenberg 1990). This may explain the higher levels of activated NF- κ B/Rel complexes as well as κ RNA in the *v-abl^{ts}* cells grown at the permissive temperature.

The experiments of Figures 4 and 5A are consistent with the hypothesis that transformation of a pre-B cell with *v-abl* negatively regulates the activity of NF- κ B/Rel factors, resulting in a block to κ gene transcription. To test whether *v-abl* can reversibly regulate NF- κ B/Rel activity, we analyzed *v-abl^{ts}* cells grown at the restrictive temperature for 24 hr to induce NF- κ B/Rel complexes and then shifted to the permissive temperature. Basal levels of NF- κ B/Rel complexes were restored after 24 hr at the permissive temperature (Fig. 5B). Thus, *v-abl* can reversibly regulate the level of activated NF- κ B/Rel complexes in a pre-B cell. In the above experiment, the level of κ RNA was also monitored (data not shown). A two-fold reduction in the induced κ RNA level was discernible at 48 hr after the shift to the permissive temperature. The slower decline in the level of κ RNA compared with the relatively rapid inactivation of NF- κ B/Rel com-

Figure 5. Mobility shift analysis of NF- κ B/Rel complexes in Abelson-transformed *v-abl*^{wt} and *v-abl*^{ts} pre-B-cells. (A) Nuclear extracts were prepared from the same cells described in the legend to Fig. 4. Mobility shift assays were carried out with 10 μ g of protein and either a κ B site probe or an octamer DNA probe. Positions of NF- κ B (includes p65:p50 and c-Rel:p50), Oct-1, and Oct-2 protein-DNA complexes are indicated. (B) Cells were cultured at 34.5°C, shifted to 39.5°C for 24 hr, and then shifted back to 34.5°C. Nuclear extracts were prepared at the indicated times (hours) and analyzed as described above.

plexes may be attributable to the greater stability of the RNA.

v-abl inhibits κ intron enhancer function in pre-B cells

If induction of NF- κ B/Rel complexes in the *v-abl*^{ts} cells promotes κ transcription, then the activity of the κ intron enhancer ($E_{i\kappa}$) should be induced upon inactivation of *v-abl*. This was tested by transiently transfecting chloramphenicol acetyl transferase (CAT) reporter constructs containing a *c-fos* promoter linked to either the wild-type $E_{i\kappa}$ or a mutant $E_{i\kappa}^*$ enhancer (Fig. 6). The mutant enhancer fragment contains clustered base substitutions in the κ B site that eliminate binding of NF- κ B/Rel factors (Lenardo et al. 1987). The CAT constructs were transfected into the *v-abl*^{ts} cells grown at the permissive temperature. After 24 hr, half of the cells were shifted to the restrictive temperature while the remaining half were maintained at the permissive temperature. Expression of the CAT gene was analyzed 48 hr after transfection. Compared with the parent *fos*-CAT reporter construct, the activity of the $E_{i\kappa}$ -*fos*-CAT construct was induced approximately sevenfold at the restrictive temperature (Fig. 6). This activity was dependent on the κ B site, as the mutant $E_{i\kappa}^*$ -*fos*-CAT showed sixfold reduced activity at the restrictive temperature. The activation of $E_{i\kappa}$ upon the temperature shift was specific to the *v-abl*^{ts} cells in that $E_{i\kappa}$ was inactive both at the permissive and restrictive temperatures in the *v-abl*^{wt} cells (data not shown). Therefore, *v-abl* negatively regulates $E_{i\kappa}$ function through a κ B-site-dependent

pathway in a pre-B cell. It should be noted that the mutant $E_{i\kappa}^*$ -*fos*-CAT construct has its activity compromised both at the restrictive as well as the permissive temperature. Its activity is reduced twofold compared with wild-type $E_{i\kappa}$ -*fos*-CAT at the permissive temperature. This is likely attributable to the basal levels of activated NF- κ B/Rel complexes in the *v-abl*^{ts} cells at the permissive temperature (see Fig. 5A). Additionally, the mutant $E_{i\kappa}$ -*fos*-CAT construct reproducibly shows a two- to threefold induction compared with the parent

Figure 6. Transient transfection analysis of the activities of κ locus enhancers in Abelson-transformed *v-abl*^{ts} cells. Each reporter construct (30 μ g) was cotransfected with a reference plasmid, Rous sarcoma virus (RSV) luciferase (8 μ g), into the *v-abl*^{ts} pre-B cell line (103/bcl2-4) cultured at 34.5°C using DEAE-dextran. The transfected cells were cultured for 24 hr at 34.5°C. Half of the cells were then shifted to 39.5°C while the remaining half were maintained at 34.5°C. Cells were harvested 24 hr after the temperature shift. CAT assays were performed with extracts from the remaining cells after normalizing to luciferase activity.

fos-CAT reporter. The basis of this residual inducibility remains to be explored. The κ locus contains a second enhancer, $E_{3,\kappa}$, positioned downstream of the C_{κ} exon. The activity of this enhancer (Fig. 6, $E_{3,\kappa}$ -*fos*-CAT) is not induced by inactivation of *v-abl* in Abelson-transformed pre-B cells.

v-abl inhibits NF- κ B/Rel activity via a post-translational mechanism

The activation of NF- κ B/Rel factors upon inactivation of *v-abl* could be indirect, representing an outcome of the resumption of the pre-B differentiation program when the conditionally transformed cells are shifted to the restrictive temperature. Alternatively, *v-abl* could post-translationally inhibit the activated state of NF- κ B/Rel factors, either directly or through a signaling pathway. To determine whether protein synthesis is required for the activation of NF- κ B/Rel factors upon *v-abl* inactivation, the *v-abl^{ts}* pre-B cells were cultured in the presence of cycloheximide (Fig. 7, top right). In the absence of cycloheximide, the temperature shift (6.5 hr) induced NF- κ B/Rel activity approximately fourfold (Fig. 7, top). A similar fourfold induction of NF- κ B/Rel activity was also observed by inhibiting protein synthesis at the permissive temperature. The activation of NF- κ B/Rel factors by the inhibition of protein synthesis has been demonstrated previously (Sen and Baltimore 1986; see below). Importantly, the combination of the temperature shift and protein synthesis inhibition resulted in an additive induction of NF- κ B/Rel activity (approximately eightfold). This additive induction was not observed in the *v-abl^{wt}* cells (Fig. 7, top left). Thus, *v-abl* inhibits the activated state of NF- κ B/Rel factors via a post-translational mechanism.

Recently, the activation of NF- κ B/Rel factors by stimulation of cells with extracellular agents such as tumor necrosis factor- α (TNF α), phorbol 12-myristate 13-acetate (PMA), and LPS has been shown to be accompanied by accelerated turnover of I κ B α (Brown et al. 1993; Henkel et al. 1993; Rice and Ernst 1993; Sun et al. 1993). Agents that rapidly activate NF- κ B/Rel (5–10 min), such as TNF α and PMA, cause highly accelerated turnover of I κ B α . This results in a rapid and dramatic decrease in total cellular I κ B α . Its level is gradually restored through new synthesis even in the presence of the stimulating agent (Brown et al. 1993; Sun et al. 1993). LPS is a somewhat slower acting agent and induces NF- κ B/Rel in 30–60 min. It has a less pronounced effect on I κ B α turnover, resulting in a smaller decrease in total I κ B α . To determine whether activation of NF- κ B/Rel factors upon inactivation of *v-abl* in pre-B cells is accompanied by increased turnover of I κ B α , its levels were monitored under conditions of protein synthesis inhibition. In the presence of cycloheximide, I κ B α levels were reduced two- to threefold more at the restrictive temperature than at the permissive temperature (Fig. 7, bottom right). The differential reduction in I κ B α levels was not observed upon temperature shift in the presence of cycloheximide in *v-abl^{wt}* cells (Fig. 7, bottom left). Direct measurement of

Figure 7. Mobility shift and Western blot analysis of NF- κ B/Rel and I κ B α in cycloheximide-treated, Abelson-transformed *v-abl^{wt}* and *v-abl^{ts}* pre-B-cells. (Top) Mobility shift analysis of NF- κ B/Rel complexes. Wild-type (WT1/*bcl*2-3) and temperature-sensitive (103/*bcl*2-13) Abelson-transformed pre-B cells were cultured in the absence or presence of cycloheximide (100 μ g/ml) at 34.5°C or 39.5°C. After 6.5 hr, aliquots were removed for preparation of nuclear extracts. Mobility shift assays were carried out with 10 μ g of protein and the κ B site probe. The position of NF- κ B (includes p65:p50 and c-Rel:p50) is indicated. NF- κ B/Rel activity was quantitated using a PhosphorImager after normalization to Oct-1 levels. (Bottom) Western blot analysis of I κ B α . Total cellular proteins from the cells described in the top panel were resolved on 10% SDS-polyacrylamide gels. The gels were blotted and probed with anti-I κ B α antiserum. The position of I κ B α is indicated. I κ B α levels were quantitated by densitometry after normalization to a cross-reactive band.

I κ B α stability by a pulse-chase protocol reveals a reduction in half-life from 30–40 min at the permissive temperature to 10–20 min at the restrictive temperature, specifically in *v-abl^{ts}* cells (data not shown). In the absence of cycloheximide, a slight decrease in I κ B α levels was observable 2–4 hr after the temperature shift but not at 6.5 hr (Fig. 7, bottom; data not shown). The failure to observe a sustained decrease in the steady-state levels of I κ B α upon a temperature shift in the absence of cycloheximide is likely attributable to increased transcription of the I κ B α gene caused by activated NF- κ B/Rel complexes. NF- κ B/Rel complexes have been shown recently to positively regulate expression of the I κ B α gene (Sun et al. 1993). These results indicate that inactivation of *v-abl* results in increased turnover of I κ B α . In contrast, neither the steady-state levels of the p50, p65, and c-Rel subunits nor the half-lives of p50 and p65 were affected

by inactivation of *v-abl* (data not shown). Thus, *v-abl* appears to regulate NF- κ B/Rel activity via a post-translational mechanism that alters the stability of I κ B α .

Discussion

Our observation that nontransformed pre-B cells expanded from the mouse bone marrow efficiently transcribe germ-line κ alleles and contain activated NF- κ B/Rel complexes, in contrast with their Abelson-transformed counterparts (Figs. 2 and 3), led us to consider one specific mechanism by which *v-abl* might actively inhibit transcription of the κ locus. Using a temperature-sensitive *v-abl* mutant protein, we have demonstrated that the transforming protein blocks germ-line κ gene transcription and negatively regulates NF- κ B/Rel activity and the functioning of the κ intron enhancer (Figs. 4–6). Significantly, the inactivation of *v-abl* does not lead to the induction of the second κ enhancer, E₃ κ (Meyer and Neuberger 1989; Pongubala and Atchison 1991). This enhancer is not dependent on NF- κ B/Rel factors for its activity. Thus, it is likely that transcription of the germ-line κ alleles in the nontransformed pre-B cells is primarily controlled by the NF- κ B/Rel-dependent κ intron enhancer, which is susceptible to inhibition by *v-abl*.

In an accompanying study (Chen et al., this issue), it has been shown that the *v-abl*^{ts} pre-B cells also efficiently activate rearrangement of light-chain loci, both κ and λ , when shifted to the restrictive temperature. Induction of recombination is accompanied by up-regulation of *Rag-1* and *Rag-2* RNAs, as well as *V(D)J* recombinase activity. Therefore, *v-abl* can impose a block to κ light-chain gene rearrangement at a minimum of two levels in pre-B cells. It inhibits the functioning of the κ intron enhancer (Fig. 6), which has been genetically demonstrated to be required for rearrangement (Takeda et al. 1993), and it also down-regulates expression of the *Rag-1* and *Rag-2* genes. The relative contributions of these two negative regulatory mechanisms to the inhibition of κ rearrangement remain to be determined. In this regard, it is noteworthy that Abelson-transformed pre-B cell lines more frequently rearrange heavy-chain alleles than κ light-chain alleles in culture (Alt et al. 1981). In these cells, the heavy-chain locus is actively transcribed in contrast with the κ light-chain locus (for review, see Blackwell and Alt 1989). Although *v-abl* likely antagonizes rearrangement of both heavy- and light-chain loci by down-regulating recombinase activity, rearrangement of the κ locus is inhibited much more severely because of inactivation of the κ intron enhancer. We propose that this latter mechanism accounts, in part, for the striking stage specificity of the developmental block imposed by *v-abl* transformation (Alt et al. 1981). It remains to be determined whether *v-abl* inhibits λ light-chain rearrangement at both the level of recombinase and λ enhancer activity. If so, this would involve a distinct mechanism, as the λ enhancers do not appear to require NF- κ B/Rel factors for their function (Hagman et al. 1990).

In these experiments we have demonstrated that the

v-abl tyrosine kinase can reversibly inhibit the activated state of NF- κ B/Rel complexes in a pre-B cell. Although *src*-like tyrosine kinases have been implicated previously in the activation of NF- κ B/Rel factors (Jamieson et al. 1991; Devary et al. 1993), this study represents the first demonstration of inhibition of NF- κ B/Rel activity by a *src* family member. It will be of interest to determine whether this inhibition is restricted to pre-B cells and the *v-abl* tyrosine kinase, as NF- κ B/Rel factors can be activated in many different cell types by diverse signaling pathways (Lenardo and Baltimore 1989). *v-abl* inhibits the activated state of NF- κ B/Rel factors in pre-B cells via a post-translational mechanism that is associated with increased stability of I κ B α (Fig. 7). Recently the LPS, PMA, and TNF α -mediated activation of NF- κ B/Rel factors has been shown to be accompanied by accelerated turnover of I κ B α (for review, see Beg and Baldwin 1993). The activation of NF- κ B/Rel factors upon inactivation of *v-abl* in pre-B cells is also associated with increased turnover of I κ B α , but the effect is less pronounced as compared with the extracellular signaling modes of activation. Significantly, neither the steady-state levels of the p50, p65, and c-Rel subunits nor the half-lives of p50 and p65 are affected by inactivation of *v-abl*. Although I κ B α -independent mechanisms of activation cannot be ruled out, it seems likely that the increased turnover of I κ B α is the basis for the activation of NF- κ B/Rel factors in the conditionally transformed pre-B cells. From a theoretical standpoint, one may assume that the levels of I κ B α are limiting, in vivo, such that the system is poised to respond to a stimulus. Then, at the permissive temperature, the rate of degradation of I κ B α and NF- κ B/Rel release in the cytoplasm is balanced by reassociation of newly synthesized I κ B α . Thus, the majority of NF- κ B/Rel complexes remain cytoplasmic. Upon shifting to the restrictive temperature, a twofold decrease in I κ B α half-life would enable twice as many NF- κ B/Rel complexes to be released during a given time period. Only half of these complexes would be inhibited by the available I κ B α , as I κ B α levels are limiting during the initial period of activation. The remaining half of the newly released NF- κ B/Rel complexes could escape inhibition and transit to the nucleus. Thus, a small change in I κ B α half-life could result in a disproportionately increased flux of NF- κ B/Rel factors to the nucleus.

The biochemical mechanism by which *v-abl* increases the stability of I κ B α and inactivates NF- κ B/Rel factors remains to be elucidated. Previously, serine/threonine protein kinases (e.g., protein kinase C and protein kinase A) have been shown to activate NF- κ B in vitro by phosphorylating I κ B α and promoting its dissociation (Ghosh and Baltimore 1990). *v-abl* may inactivate NF- κ B/Rel factors by stimulating the activity of a protein phosphatase or inhibiting the activity of a kinase. This would result in reduced phosphorylation of I κ B α , thereby promoting its association and stabilization as a complex with NF- κ B/Rel. It should be noted that although *v-abl* inhibits the activated state of NF- κ B/Rel factors in pre-B cells, this block is alleviated by LPS or PMA stimulation (Sen and Baltimore 1986; data not shown).

In this study we have demonstrated activated NF- κ B/Rel complexes correlating with germ-line κ transcription in two types of nontransformed pre-B cells. The first type represent pre-B cells expanded from the mouse bone marrow in the presence of stromal cells and IL-7. The second type represent conditionally transformed pre-B cells that have been reverted to their nontransformed state. Importantly, activation of NF- κ B/Rel factors in the latter cells is observed in the absence of stromal cells and IL-7. This suggests that the activation of NF- κ B/Rel factors in pre-B cells is an intrinsic feature of their differentiation state. Therefore, during B-cell development, NF- κ B/Rel factors may be activated as a secondary response (dependent upon protein synthesis) to an extracellular differentiation signal derived from the stroma. For example, stromal cell-dependent development of pre-B cells could involve increased expression of an I κ B α kinase gene. This would promote increased dissociation and turnover of I κ B α , thereby activating NF- κ B/Rel factors.

Our results suggest that the NF- κ B/Rel system may have evolved two distinct modes of regulation for executing different functions during cellular activation and differentiation. In the former situation, NF- κ B/Rel factors are activated as a primary response (independent of protein synthesis) to an extracellular signal such as TNF α . Signaling results in highly accelerated turnover of I κ B α , which promotes a rapid and short-lived activated state. In the latter situation, NF- κ B/Rel factors are activated as a secondary response to a differentiation signal. Differentiation results in a modest increase in I κ B α turnover. This promotes the generation of a slower but extended activated state. It is interesting to speculate that the inhibition of NF- κ B/Rel factors by *v-abl* may be required for transformation of pre-B cells. In this regard, it has been shown recently that high levels of c-Rel expression induce apoptosis of bone marrow cells in culture (Abbadie et al. 1993). The conditionally transformed *v-abl^{ts}* pre-B cells also undergo apoptosis upon inactivation of *v-abl* (Chen and Rosenberg 1992), which is associated with the activation of NF- κ B/Rel factors.

Our results with bone marrow-derived pre-B cell cultures provide evidence for a normal stage of B-cell development in which activation of germ-line κ transcription is uncoupled from κ light-chain rearrangement. This is based on the observation that transcription of the germ-line κ locus is not accompanied by rearrangement during the course of the IL-7 expansion (Fig. 2). This strongly suggests that the two events are regulated independently and therefore activated by distinct developmental signals. Cell-surface expression of the pre-B cell receptor appears not to be required for activation of germ-line κ transcription, as the IL-7-expanded pre-B cells are surface μ negative. Furthermore, expression of a membrane form of the μ protein is not necessary for inducing germ-line κ transcription. IL-7-expanded pre-B cells from bone marrow cultures established from μ MT mice (Kitamura et al. 1991), which do not express the membrane form of the μ protein, transcribe the κ locus at high levels (S. Gerety and H. Singh, unpubl.). The IL-7-expanded pre-B

cells appear to represent a novel intermediate in development, as κ transcription but not rearrangement has been activated. It will be of significant interest to determine what signals can induce κ rearrangement in these normal pre-B cells, particularly in light of the observation that proficient rearrangement is activated simply by inactivating *v-abl* in conditionally transformed pre-B cells (Chen et al., this issue). It should be noted that the IL-7-expanded pre-B cells express *Rag-1* and *Rag-2* RNAs at detectable levels (Morrow et al. 1992; data not shown). However, these RNA levels as well as recombinase activity have yet to be directly compared with the *v-abl^{ts}* pre-B cells. Both the IL-7-expanded and the conditionally transformed *v-abl^{ts}* pre-B cells represent new systems to explore the signaling pathways and mechanisms by which κ rearrangement is induced during B-lymphocyte development.

Materials and methods

Isolation and analysis of nontransformed pre-B cells

Pre-B cells were expanded from Whitlock–Witte cultures using the method of Morrow et al. (1992). Whitlock–Witte cultures were established by plating bone marrow cells from 4- to 8-week-old BALB/c female mice at a density of 1×10^6 cells/ml in RPMI 1640 supplemented with 5% fetal calf serum (FCS), 5×10^{-5} M 2-mercaptoethanol and 2 mM L-glutamine. Colonies of B-lineage cells were readily observable after 28 days of culture. At this point, pre-B cells were selectively expanded by the addition of IL-7 (Genzyme) to a concentration of 300 U/ml. Seven days following IL-7 addition, the nonadherent B-lineage cells were isolated for analysis. IL-7 stimulation resulted in expansion of the B-lineage cells from an initial density of $\sim 1 \times 10^5$ cells/ml to a final density of 2×10^6 to 3×10^6 cells/ml. Aliquots of cells were stained with the following reagents: α -Thy 1.2 (PharMingen, clone 53-2.1); α -granulocyte (PharMingen, clone RB6-8CS); α -IgM (Southern Biotechnology, G α M-FITC); α - κ (Becton-Dickinson); α -BP-1 (PharMingen, clone 6C3); α -B220 (PharMingen, clone RA3-6B2); α -leukosialin (PharMingen, α -CD43, clone S7); and α -HSA (PharMingen, clone MI/69). Data were acquired using a propidium iodide (1 mg/ml) viability gate on a dual laser cell sorter (FACScan, Becton-Dickinson).

Abelson-transformed pre-B cell lines

38B9 is an Abelson-transformed pre-B cell line derived from BALB/c fetal liver (Alt et al. 1981). The derivation of Abelson transformants WT1/*bcl2-3*, 103/*bcl2-4*, and 103/*bcl2-13* is described in Chen et al. (this issue). Briefly, WT1 and 103 were derived using the wild-type Ab-MLV-P120 and temperature-sensitive Ab-MLV-P120/G&H strains, respectively (Engelman and Rosenberg 1990). Transfectants expressing *bcl2* were obtained by electroporating and selecting for the construct, spleen focus-forming virus (SFFV)-*bcl2-nl*. Temperature-sensitive pre-B lines, 103/*bcl2-4* and 103/*bcl2-13*, represent two different *bcl2*-expressing derivatives of the temperature-sensitive transformant designated 103. All cell lines were grown in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 50 μ M 2-mercaptoethanol, and 0.01% penicillin-streptomycin. The WT1 and 103 cell lines were routinely grown at 34.5°C (permissive temperature). The nonpermissive temperature used in all experiments was 39.5°C.

RNA isolation and analysis

Cellular RNA was isolated according to Feldhaus et al. (1992). Poly(A)⁺ RNA was isolated using the FastTrack kit (Invitrogen). Northern blot and RNase protection analyses were performed according to Miller et al. (1991). RNase protection assays were quantitated using an Ambis radioanalytic imager.

Mobility shift assays

Nuclear extracts from 3×10^7 to 5×10^7 cells were prepared and assayed as described previously (Feldhaus et al. 1992). Protein-DNA complexes were resolved by polyacrylamide gel electrophoresis (Singh et al. 1988). The κ B site probe is a cloned oligomer

CTCTCGGAAAGTCCCCTCTGTTGGTAC
CATGGAGAGCCTTTCAGGGGAGACAAC

derived from the κ intron enhancer. The octamer site DNA probe is described in Staudt et al. 1986. For antibody supershift assays, nuclear extract protein was preincubated with the antibody for 20 min at room temperature before the addition of κ B site probe. Anti-p65 antipeptide antibodies were raised against the carboxyl terminus of p65, residues 537–550 (Rice et al. 1992). Anti-p50 and α -Rel antibodies were raised against residues 2–15 of mouse p50 and residues 573–586 of mouse c-Rel, respectively (Rice and Ernst 1993). NF- κ B/Rel complexes were quantitated using a PhosphorImager (Molecular Dynamics). Fold induction of NF- κ B/Rel complexes was calculated after normalization to Oct-1 levels.

Transient transfection analysis

The parent reporter construct (fosCAT) contains a minimal c-fos promoter (Δ 71) fused to the CAT gene (Lenardo et al. 1987). $E_{i\kappa}$ and $E_{i\kappa}^*$ contain the wild-type and mutant κ B site κ intron enhancers, respectively (Lenardo et al. 1987). $E_{3'\kappa}$ contains a wild-type κ 3' enhancer fragment (Meyer and Neuberger 1989) cloned into the *SalI* site of fos-CAT. All enhancer fragments are positioned upstream of the fos promoter. Pre-B cell lines were transiently transfected using DEAE-dextran according to Feldhaus et al. (1992). CAT assays were carried out as described in Gorman et al. (1982). Luciferase assays were performed using the Promega luciferase assay system. Each reporter construct was transfected in duplicate at least three independent times. CAT assays were quantitated using an Ambis radioanalytic imager.

Western blot analysis

Total cellular proteins were resolved on 10% SDS-polyacrylamide gels and transferred to Immobilon P membrane (Millipore). The blots were incubated with a 1:500 dilution of anti-I κ B α antiserum, raised against an amino-terminal I κ B α peptide (Rice and Ernst 1993), or a 1:1000 dilution of α -p50, α -p65 or α -c-Rel antisera (described above). Immunoreactive proteins were detected using the ECL reagent system (Amersham). Quantitation of immunoreactive bands was performed by scanning densitometry.

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