Published in final edited form as:
Immunity. 2005 April ; 22(4): 519–531. doi:10.1016/j.immuni.2005.03.006.

A Role for Nuclear Factor Kappa B/Rel Transcription Factors in the Regulation of the Recombinase Activator Genes

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Summary

In developing B cells, expression of surface immunoglobulin is an important signal to terminate recombinase activator gene (*RAG*) expression and V(D)J recombination. However, autoreactive antigen receptors instead promote continued gene rearrangement and receptor editing. The regulation by B cell receptor (BCR) signaling of *RAG* expression and editing is poorly understood. We report that in editing-competent cells BCR ligand-induced *RAG* mRNA expression is regulated at the level of *RAG* transcription, rather than mRNA stability. In immature B cells carrying innocuous receptors, *RAG* expression appears to be under rapidly reversible negative regulation. Studies involving transduction of a superrepressive (sr) IκBα protein indicate that NF-κB/Rel proteins promote *RAG* transcription. Interestingly, NFκB1-deficient cells over-express *RAG* and undergo an exaggerated receptor editing response. Our data implicate NFκB transcription factors in the BCR-mediated regulation of *RAG* locus transcription. Rapidly activated NFκB pathways may facilitate prompt antigen receptor-regulated changes in *RAG* expression important for editing and haplotype exclusion.

Introduction

Recombinase activator gene products *RAG1* and *RAG2* are critical for the assembly of antigen receptor gene variable (V), diversity (D), and joining (J) elements by DNA recombination (Fugmann et al., 2000). *RAG* gene expression is highly restricted by cell type and maturational stage, with highest levels apparent in developing lymphocytes (Schlissel, 2003; Jankovic et al., 2004). *RAG1* and *RAG2* coding sequences are found on the chromosome near to one another with convergent transcriptional orientations, and these two genes are almost invariably expressed together (Fugmann et al., 2000). In addition, in T and B lymphocyte development *RAG* expression occurs in at least two separate waves, corresponding respectively to recombination of the two receptor chains: T cell receptor α and β or immunoglobulin (Ig) H and L (Grawunder et al., 1995; Wilson et al., 1994). These features indicate that *RAG* expression is under stringent and coordinate control.

B cells almost always express a single immunoglobulin (Ig) heavy (H) and light (L) chain on the cell surface, in part because in developing B cells, carrying surface Ig feedback signaling mechanisms can prevent recombination (for reviews see Storb, 1987; Karasuyama et al.,

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1996; Nemazee, 2000). However, B cell receptors that are ligated by self antigen fail to suppress V(D)J recombination and promote receptor editing, which can silence one receptor chain gene and replace it with another (Gay et al., 1993; Radic et al., 1993; Tiegs et al., 1993). Receptor editing in B cells is strongly correlated with elevated *RAG1* and *RAG2* mRNA levels (Hertz and Nemazee, 1997; Melamed and Nemazee, 1997; Melamed et al., 1998), but the basis of this regulation is unknown. Steady-state levels of message could be regulated by differential synthesis, differential degradation, or both. In B cell lines, both changes in the rates of *RAG* transcriptional initiation and degradation may be under the control of BCR or other signaling pathways (Neale et al., 1992; Ma et al., 1992; Verkoczy et al., 1995). However, there have been no studies to our knowledge that have examined the relative contributions of message stability and synthesis in the regulation of *RAG* genes in primary B cells. Nor is it known if the increased *RAG* levels in B cells undergoing receptor editing is regulated by increased transcription or decreased RNA degradation.

Key features of *RAG* transcriptional control have been elucidated: transcriptional start sites have been mapped and promoter regions characterized (Schlissel, 2003; Jankovic et al., 2004). *RAGs* are regulated both by promoter sequences and by more distant 5′ elements that are presumptive enhancers, locus control regions, or antisilencers (Kitagawa et al., 1996; Yu et al., 1999; Monroe et al., 1999; Yannoutsos et al., 2001; Wei et al., 2002; Hsu et al., 2003; Yannoutsos et al., 2004). Interestingly, the elements that regulate tissue-specific expression in B and T cells are different and these same elements appear to regulate both *RAG1* and *RAG2* expression (Yu et al., 1999; Monroe et al., 1999; Hsu et al., 2003). Transcription factors or cognate binding sites implicated in *RAG* gene expression include *NFY, Pax5, c-Myb, GATA-3, E2A, C/EBP, Runx, SP1*, and *Ikaros* (Fuller and Storb, 1997; Zarrin et al., 1997; Brown et al., 1997; Kee and Murre, 1998; Lauring and Schlissel, 1999; Fong et al., 2000; Kishi et al., 2000; Wang et al., 2000; Kishi et al., 2002; Miranda et al., 2002; Jin et al., 2002; Wei et al., 2002; Hsu et al., 2003; Yannoutsos et al., 2004; Quong et al., 2004). Despite this progress, much remains to be learned about the complex regulation of *RAG* gene expression.

The goals of the present study were to determine if the tolerance-induced increase in *RAG* expression of immature B cells is controlled at the level of RNA transcription or stability, and to determine how BCR signaling controls *RAG* mRNA levels. We find that the regulation *RAG* mRNA levels is virtually entirely at the level of transcriptional rate. In addition, we have uncovered an unexpected role for NFκB/Rel transcription factors in BCR-regulated transcription of the *RAG* genes, which may regulate both feedback suppression and tolerance-induced stimulation of *RAG* expression.

Results

RAG RNA Turnover and Transcription in Editing and Nonediting Primary B Cells

To assess the relative contributions of changes in RNA stability and transcriptional initiation to the regulation of *RAG* mRNA levels in immature B cells, we employed a culture system in which primary bone marrow (BM) cells from 3–83 antibody transgenic mice are stimulated with interleukin 7 (IL7). After 5 days of culture, >95% of nonadherent cells are $B220^+$ B lineage cells, which, upon removal of IL7, are competent to undergo receptor editing upon BCR ligation (Melamed and Nemazee, 1997). As shown in Figure 1A, treatment of cultured cells with anti-BCR antibody led to a marked increase in the levels of *RAG1* and *RAG2* mRNAs (compare lanes 1 and 2 to 11 and 12). Treatment of cells with the transcription inhibitor actinomycin D (ActD) allowed an assessment of RNA decay over time. We consistently found that the half-life of both *RAG1* and *RAG2* messages was ~30 min and that BCR ligation increased mRNA levels of *RAG1* and *RAG2* without significantly increasing mRNA half-life (Figure 1B).

BCR ligation increased steady state levels of *RAG* mRNA in the absence of significant changes in *RAG* mRNA stability, suggesting that transcription rates must be upregulated, a notion that was tested independently by transcriptional run-on assay (Figures 1C and 1D). Hybridization signals to immobilized *RAG1* and *RAG2* cDNAs were specifically increased several fold in the samples from BCR-stimulated cells, whereas hybridization to control β-actin cDNA was similar in treated and untreated samples (Figure 1C). To independently test the ability of BCR ligation to stimulate *RAG* transcription in primary cells, we studied cells from mice that carry both a GFP reporter/R/lG2 locus transgene (Yu et al., 1999) and the 3–83 Ig transgenes. As shown in Figure 1E, anti-BCR antibody stimulated a striking increase in the proportion of GFP^+ cells, suggesting enhanced activity of the *RAG2* promoter and associated *cis*-acting elements.

RAG Gene Expression Can Be Rapidly Upregulated by Treatment with Protein Synthesis Inhibitor

Experiments involving the addition of the protein synthesis inhibitor cycloheximide (CHX) indicated that *RAG* gene expression might be under continuous negative regulation. Within 1 hr of treatment, primary cells showed a striking increase in the mRNA levels of both *RAGs* (Figure 2A, compare lanes 1 versus 4 and 6 versus 9). Over time, this increase continued for *RAG1*, but not *RAG2* (lanes 2,3,7, and 8). Elevated levels of *RAG* mRNAs were seen in CHX-treated cells in either the presence or absence of anti-BCR treatment. Similar results were obtained in an editing-competent human B cell line and in the Jurkat T cell line (Figure 2B, lanes 1–4). RNA turnover and nuclear run-on analysis indicated that the rapid CHX-induced increase in *RAG* mRNA levels in primary B cells was the result of increased transcription rate (data not shown). Because new protein synthesis is blocked by CHX, these results suggested to us that *RAG* expression was under the negative regulation of a short-lived inhibitory protein. To test this idea, B cells were treated with CHX in combination with inhibitors that suppress proteasome-mediated degradation. ALLnL clearly blocked the CHX-mediated *RAG* mRNA induction as detected by Northern blot (Figure 2A, compare lanes 2 versus 5 and 7 versus 10), as did two other proteasome inhibitors, MG132 and lactacystin (not shown). Consistent with these results, these same proteasome inhibitors suppressed anti-BCR-induced GFP expression by primary *RAG2*/GFP Tg BM B cells (data not shown). These results indicated that *RAG* transcription was negatively regulated by a short-lived protein that is normally degraded in the proteasome.

Testing the Possibility that NFκB Proteins Play a Role in *RAG* Expression

The CHX superinducibility and proteasome sensitivity of *RAG* expression suggested that our putative short-lived inhibitor was an IκB family protein, which function by retaining NFκB in the cytoplasm (Ghosh and Karin, 2002). This hypothesis thus predicted that NFκB regulates *RAG* transcription. Accordingly, we tested the effects of NFκB inhibitors on *RAG* expression. The drug PDTC (Schreck et al., 1992) could readily suppress CHX-induced *RAG* expression (Figure 2C). Similarly, PDTC suppressed anti-BCR-mediated *RAG* expression in primary BM B cells (Figure 2E, compare lanes 1 and 4). As PDTC may have NFκB-independent effects, we studied two different, more specific peptide inhibitors of the NFκB pathway for their effect on *RAG* mRNA expression. First, we tested SN50 peptide (Lin et al., 1995) for its ability to affect anti-BCR-induced *RAG* expression or *GFP/RAG2* reporter expression by primary 3–83 BM B cells (Figures 2E and 2D). In both assays SN50, but not a control peptide, suppressed BCR- induced *RAG* expression. Next, we tested the ability of a transducible, superrepressive IκBα-TAT fusion protein (Kabouridis et al., 2002), to inhibit induced *RAG* gene mRNA expression in anti-BCR or CHX treated OCI-Ly8 cells. The srlκBα-TAT fusion protein lacks serine phosphorylation sites that normally target IκBα for ubiquitination and proteasomal degradation, and can efficiently enter cells owing to a linked peptide derived from the HIV TAT protein. As a consequence, treatment of cells with

srlκBα-TAT reduces NFκB/Rel nuclear activity (Kabouridis et al., 2002). The srlκBα-TAT fusion protein, but not control TAT fusion protein, was able to suppress significantly *RAG* mRNA expression induced by BCR ligation (Figure 3A, upper panel, compare lanes 7–9) or CHX treatment (lanes 4–6). In addition, srlκBα-TAT also suppressed the basal *RAG* expression seen in uninduced cells (lanes 1–3). Control immunoblot experiments showed that TAT-β-galactosidase fusion protein was transduced to a similar extent to srlκBα-TAT fusion protein (Figure 3A, lower panels). Consistent with the observed biological effect of srlκBα-TAT fusion protein, immunoprecipitation analysis confirmed that the trans duced protein was able to associate with cellular p65/RelA (Figure 3B). Because CHX treatment inhibits new protein synthesis, we conclude that the suppressive effect of srlκ Bα-TAT fusion protein on *RAG* expression (Figure 3A, lane 6, top) is mediated directly through its ability to sequester NF-κ B/Rel transcription factors away from the nucleus, and thus the *RAG* locus.

To test the ability of srlκ Bα-TAT fusion protein to modulate *RAG* expression and editing in more physiologically relevant cells, experiments were performed with ex vivo mouse BM B cells, tested in short term assays in the absence of exogenous IL7. As shown in Figure 3C, basal and BCR-induced *RAG2* expression was suppressed by srlκ Bα-TAT, but not control fusion protein, in a dose dependent manner (compare upper panels, lanes 10–15 versus 4–9), whereas the control HPRT gene expression was unchanged (lower panels). (Similar results were obtained using IL7 cultured cells, not shown). Cell recoveries were unaffected by srlκBα-TAT treatment, excluding selective cell loss as an explanation for the loss in *RAG2* expression. Similarly, PDTC specifically suppressed *RAG* expression (lanes 16–18). We conclude that, as in the human cell line, in primary BM B cells *RAG* expression is under the regulation of NFκB/Rel transcription factors.

NFκB Nuclear Activity in Editing Cells

To correlate the changes in nuclear NFκB binding activity with *RAG* expression, we analyzed nuclear extracts from induced or uninduced immature B cells by electrophoretic mobility shift assay (EMSA) and measured *RAG* mRNA levels in the same cell preparations. Nuclear NFκB binding activity was seen in uninduced cells that had low *RAG* mRNA levels (Figure 4B, lane 1), but significantly higher levels were consistently seen in cells stimulated with anti-lg (Figure 4B, lane 1 versus 4). Furthermore, other inducers of elevated *RAG* expression, such as CHX and wortmannin also stimulated an increase in overall EMSA activity (Figure 4B, lanes 2 and 3). As in the cases of CHX and anti-BCR induced *RAG* upregulation, the wortmannin-induced upregulation was sensitive to PDTC, SN50, and srlκBα (not shown). Interestingly, *RAG*-inducing stimuli appeared to preferentially increase the abundance of the slower migrating complexes. Supershift studies using antibodies against individual NFκB/Rel family members detected only three present in the nuclei of these cells, p65, c-Rel and p50 (Figure 4C). As expected, the irrelevant E2A antibody failed to cause a change in mobility in this assay (lanes 1 and 7). In addition, the p50 molecules appeared to be present in the faster migrating complexes, which were relatively deficient in p65. In anti-BCR treated cells, p65 activity appeared to increase most (Figure 4C, compare lanes 4 and 10), while p50 levels were relatively unchanged (lanes 2 and 8). These results indicated a correlation between *RAG* expression and the relative abundance of p50 compared to RelA and c-Rel in nuclear complexes.

Chromatin Immunoprecipitation, ChIP, Analysis

To assess the association of NFκB/Rel transcription factors with the *RAG* locus, we performed ChIP analysis using antibodies to p50, c-Rel and p65 to precipitate chromatin isolated from fibroblasts, mature B cells, or cultured immature B cells (Figure 5). Precipitated chromatin was analyzed by PCR for the presence of DNA derived from

different portions of the *RAG* locus or the Ig-κ intronic enhancer. Sites of interest (shown schematically in Figure 5A) included predicted NFκB sites shared between mouse and human *RAG* loci within the *RAG1* and *RAG2* introns, sites 25 kb and 32 kb upstream of *RAG2*, and two NFκB-like sites within known *RAG* enhancers, ERag (Hsu et al., 2003) and the D3 enhancer (Wei et al., 2002). Little to no NFκB/Rel association with the *RAG* or Ig-K loci was detected in TNF treated fibroblasts (Figure 5C), whereas analysis of BM B cells revealed clear associations of the Ig-κ enhancer and *RAG* locus κB sites with p50, p65 and c-Rel (Figure 5B). These associations were specific, as control CD8 locus DNA was not significantly precipitated (lower panel). Furthermore, in BM B cells stimulated with BCR antibody, the relative association of p50 to *RAG* loci was reduced while the p65 and c-Rel association was not lower; similar results were obtained in three independent experiments (not shown). In splenic B cells, all *RAG* sites except the *RAG2* in-tronic site were occupied solely by p50, while p65 and c-Rel associations were weak or not detectable (Figure 5D). These results show that NFκB/Rel transcription factors bind to the *RAG* locus, consistent with a regulatory function.

RAG Expression in p50-Deficient B Cells

To test genetically the notion that the NFκB/Rel factors regulate *RAG* transcription, we analyzed B cells of NFκB1/p50 deficient mice carrying the 3–83 antibody transgenes. In principle, p50 could either augment or suppress NFκB/Rel mediated transcription factors because, unlike p65 and c-Rel, p50 lacks a transactivation domain. In some contexts p50 dimers compete with NFκB binding and suppress transcription (Kang et al., 1992; Bohuslav et al., 1998; Zhong et al., 2002). As shown in Figure 6, IL7-expanded p50-deficient BM B cells had strikingly elevated *RAG* expression after cytokine withdrawal, compared to p50-sufficient cells, as assessed both in *GFP/RAG2* reporter and Northern blot assays (Figures 6A and 6B). GFP levels were elevated in p50-deficient cells that were challenged with control Ig alone (Figure 6A, upper right panel) and these levels were further elevated by anti-BCR treatment (Figure 6A, lower right panel). In both p50-deficient and sufficient cultures, GFP levels were extremely low prior to IL7 withdrawal. At 24 hr post-1L7, control Ig treated p50-deficient cells had elevated *RAG* mRNA levels compared to p50-sufficient cells (Figure 6B, compare lane 1 versus 2). Similarly, anti-lg treated, p50-deficient cells had a further elevation in *RAG* mRNA levels (Figure 6B, lanes 3 and 4). Quantitative changes in *RAG1* and *RAG2* mRNA levels were very similar, implying coordinate regulation (Figure 6C). Cultured p50-deficient cells had similar slg levels (not shown) and cell viability (Figure 6D) compared to p50-sufficient cells, ruling out the possibilities that changes in slg levels or selective apoptosis of cell subsets could explain alterations in *RAG* expression. The evidence that *RAG* expression was suppressed by inhibitors of NFκB, but augmented in the absence of p50, together indicate that other NFκB/Rel family members (probably p65 and/or c-Rel) play a positive role in *RAG* mRNA expression.

To assess the possible effects of p50 deficiency on receptor editing in vivo, we compared p50-deficient and sufficient 3–83 Tg mice in the context of autoreactivity and measured *RAG* expression and L chain gene recombinations in BM $B220^+$ cells. The mice studied were bred to the B10.D2 ($H\text{-}2^d$) or B6 ($H\text{-}2^b$) genetic backgrounds. As the 3–83 antibody reacts with K^b molecules carried by $H\text{-}2^b$ cells but not to cells of the $H\text{-}2^d$ background, receptor editing normally occurs on the B6 but not the B10.D2 background. As predicted from the in vitro studies, p50-deficient mice had elevated *RAG1* and *RAG2* levels relative to wild type counterparts, and particularly elevated expression on the $H\text{-}2^b$ background (Figure 7A). This higher level *RAG* expression was correlated strongly with elevated endogenous L chain gene recombinations, including Vκ-to-Jκ1, RS-to-IRS, and λ1 excision product recombination (Figure 7B). These findings directly demonstrate that the NFκB1 gene

product negatively regulates *RAG* expression and L chain gene recombination in newly formed B cells in vivo.

Finally, because *RAG* expression was closely correlated with IgL recombinations, we assessed the effects of changing *RAG* expression levels on successful editing in vivo. This is conveniently measured by the appearance of IgD^+/λ^+ splenic B cells in 3–83/H-2^b mice, as roughly half of all edited cells in this model express λ chain (Lang et al., 1996; Tiegs et al., 1993). First, we assessed the possibility that *RAG* levels are limiting for receptor editing in vivo by comparing 3–83/H-2^b mice that were haploinsufficient for *RAG1* ($RAG1^{+/-}$) with *RAG 1* -sufficient controls. As shown in Figure 7C, $RAG1^{+/-}$ mice have severely reduced numbers of IgD^+/λ^+ splenic B cells, suggesting that *RAG1* is indeed limiting. Next, we compared the ability of p50-deficient or sufficient 3–83/H-2^b mice to generate IgD^+ splenic B cells. Curiously, despite the increased level of *RAG* expression and IgL gene recombination in 3–83/H-2^b/ p50-deficient BM B cells, peripheral IgD^+ B cell numbers were not enhanced (Figure 7D, compare second and fourth bars). The remaining cells had edited, as many expressed λ chain (not shown). In addition, p50-deficient 3–83/H-2^d mice (lacking cognate autoantigen) also had reduced peripheral B cell numbers compared to p50 sufficient controls (compare first and third bars). Apparently, the enhanced *RAG* gene expression and L chain gene recombination in autoreactive p50 deficient B cells did not increase the numbers of edited peripheral B cells, probably because of impaired survival of more mature B cells.

Discussion

Newly formed B cells are regulated to ensure both that they express a cell surface BCR and that this receptor is nonautoreactive; cells lacking an appropriate receptor continue *RAG1/2* expression and antibody gene recombination. Control must involve antigen receptor signaling, or the lack thereof. The present study makes several important findings regarding the BCR regulation of RAG gene expression in immature BM B cells. First, we conclude that BCR-induced receptor editing in primary B cells involves increased RAG gene transcription, relative to cells carrying an unligated receptor, with minimal effects on RAG transcript stability. Second, we find that RAG gene expression is suppressed in newly formed B cells carrying an innocuous, nonautoreactive receptor. This negative regulation can be rapidly reversed, for example, by treatment of cells with the protein synthesis inhibitor CHX or the PI3 kinase inhibitor wortmannin. Third, data presented here indicate that members of the NFκB/Rel transcription factor family regulate RAG gene transcription and that this transcription is limiting for L chain gene recombination. Overall, our results suggest that rapid changes in RAG gene transcription may regulate receptor editing.

NFκB/Rel transcription factors regulate innate immunity, cell survival, proliferation and other important processes, including BCR-mediated signals but have so far not been implicated in *RAG* expression (Karin and Ben Neriah, 2000). Analysis of genetic knockouts of NFκB/Rel genes has revealed surprisingly modest im pact on B cell development, with more striking effects on postimmune activation. B cell development in the BM is not intrinsically blocked in mice singly deficient in p50, p65, c-Rel, p52 or RelB (Sha et al., 1995; Cariappa et al., 2000; Doi et al., 1997; Kontgen et al., 1995; Liou et al., 1999; Franzoso et al., 1998; Weih et al., 1996). Similarly, variable numbers of surface IgM^+ B cells can be generated from precursors doubly deficient in both p50 and c-Rel, p50 and p65, p50 and p52, or c-Rel and p65 (Pohl et al., 2002; Horwitz et al., 1997; Cariappa et al., 2000; Franzoso et al., 1997; Grossmann et al., 1999; Grossmann et al., 2000), though not all of these studies examined BM B cell development at high resolution.

We provide several lines of evidence suggesting that NFκB/Rel family transcription factors can regulate *RAG* gene transcription in immature B cells. Introduction of inhibitors of NFκB (SN50, srlκB-TAT fusion protein, PDTC) block *RAG* expression in both primary BM B cells and in an editing competent B cell line. EMSA analysis indicates that *RAG* expression is correlated with nuclear NFκB/Rel binding activity, particularly the more slowly migrating species enriched in RelA and c-Rel. In editing-competent B cells, *RAG* expression is rapidly increased by treatment with CHX, a known inducer of NFκB activity (Sen and Baltimore, 1986), and this increase can be readily suppressed by protein transduction with srlκBα. Because in this context new protein synthesis is blocked, it is likely that NFκB/Rel factors directly regulate *RAG* transcription. ChIP analyses indicated that NFκB/Rel factors are bound to the *RAG* locus in immature B cells. The observations that the NFκB/Rel family proteins p50, RelA, and c-Rel are present in the nuclei of primary BM B cells, while p52 and RelB were not detected, along with the finding that *RAG* expression is increased in p50-deficient B cells, implicate RelA and/or c-Rel in promoting *RAG* transcription. These findings are consistent with other studies showing that p50, c-Rel, and p65 are the prominent NFκB/Rel factors present in the nuclei of immature B cells (Scherer et al., 1996; Shaffer et al., 1997; Kistler et al., 1998). Our results further suggest that p50 or its p105 precursor may play a negative regulatory role. Furthermore, because in this study *RAGs* 1 and 2 were essentially regulated in coordinate fashion under a variety of experimental conditions in which NFκB activity was modulated, the results suggest that nuclear NFκB/Rel transcription factor binding activity is involved in promoting transcription of both genes, probably through interaction with common enhancer (or antisilencer) elements.

Our results suggest that the relative amounts of p50 and c-Rel or p65 are important in regulating *RAG* transcription, but ChIP analysis indicated that higher *RAG* expression was correlated with reduced p50 association, whereas EMSA experiments revealed that nuclear p50 levels were similar, with enhanced p65 and c-Rel nuclear activity. The nature of this discrepancy is unclear but might be explained by differential affinities of different NF-κB/Rel family members for the binding sites involved in the assays: native sites associated with the *RAG* loci in the case of ChIP assays versus the consensus NFκB site used in the EMSA experiments.

Importantly, in immature B cells we find a very strong correlation between *RAG* mRNA expression and light chain gene recombination. Indeed, heterozygous *RAG1* gene mutant mice had significantly impaired receptor editing. Furthermore, enhanced *RAG* gene expression in p50 deficient cells was associated with elevated L chain rearrangements in the bone marrow. While p50 deficient immature B cells underwent more exuberant receptor editing than wild type cells, and manifested no obvious survival defects, in vivo their more mature peripheral splenic B cell decendants were less abundant. One interpretation of these results is that p50 deficiency results in reduced peripheral B cell lifespan independently of recombinations. NFκB/Rel transcription factors can enhance apoptosis (Abbadie et al., 1993; Jung et al., 1995; Grimm et al., 1996; Sheehy and Schlissel, 1999) but are more often associated with apoptosis protection (Sonenshein, 1997; Aggarwal, 2004). Alternatively, reduced peripheral B cell survival in 3–83 p50-deficient cells may be promoted by prolonged, excessive, or counterproductive V(D)J rearrangements. Furthermore, as Rel family members have been implicated in targeting Igκ for recombination (Scherer et al., 1996), possibly by regulating DNA methylation (Inlay and Xu, 2003), it is possible that NFκB activity could coordinately regulate *RAG* gene expression and IgL gene accessibility.

Experimental Procedures

Mice and Cell Lines

3–83 transgenic mice (Russell et al., 1991) and 3–83/HYG Rag2-GFP transgenics (Yu et al., 1999) were bred on a B10.D2 genetic background.

Bone Marrow Cultures, Antibodies, Peptides, and Chemicals

Immature B cells were generated from BM using IL7 cultures as described (Melamed et al., 1997). Hybridoma S23 (lgG2b anti-3–83 idiotype) was generated in a fusion of sp2/0 myeloma cells with spleen cells from a 3–83-immunized Igκ-deficient mouse. Y3 (lgG2b anti-H-$2K^b$) was obtained from ATCC. These antibodies were isolated by protein G-agarose affinity chromatography. All other antibodies used in flow cytometry were purchased from Pharmingen. For anti-BCR ligation of OCI LY8 C3P, a polyclonal goat anti-human mu-F(ab)′2 fragment (Biosource) was used. Actinomycin D and CHX were purchased (Sigma). ALLnL, MG-132, lactacystin, PDTC, Wortmannin, and the Sn50 and Sn50c peptides were obtained from Calbiochem.

Nuclear Extract Preparation and EMSA

Extracts were prepared as described (Dyer and Herzog, 1995). EMSA probe was a preannealed oligonucleotide consisting of 5-TCGAGTTGAG**GGGACTTTCCC**AGGC-3′ and 5-TCGAGCCT**GGGAAA**GTCCCCTCAAC-3′, which carry a consensus NFκB binding site indicated in bold. Binding reactions were in 10mM Tris pH 8.0, 50mM NaCI, 1mM MgCI, 0.5mM EDTA, 0.5mM dithiothreitol, 4% glycerol, 50μg/ml poly(dl/dC), 500μg/ml BSA, approximately 50 pg ^{32}P-end labeled probe and nuclear extract (5 μg protein). Reactions were incubated for 20 min at room temperature and electrophoresed at 7 V/cm through 5% native acrylamide gels in 0.5X TBE buffer. For supershift assay, nuclear extracts were preincubated with 1 μg of indicated antibody (Santa Cruz Biotechnology) for 30 min on ice prior to EMSA binding reaction.

Expression and Purification of TAT Fusion Proteins

Plasmids encoding TAT-srlκBα (Kabouridis et al., 2002) or TAT-β-gal (Schwarze et al., 1999) fusion proteins were kindly provided by Drs. Panos Kabouridis and Steven Dowdy, respectively. Recombinant proteins were expressed in BL21 (DE3)pLysS bacteria as described (Kabouridis et al., 2002).

Northern Blot Analysis, Nuclear run-on Analysis, and Quantification

Northern blots and nuclear run-on assay were performed as described (Verkoczy et al., 1995) using *RAG* cDNA probes (plasmids kindly provided by Dr. David Schatz), or a mouse 18S RNA gene probe (Ambion). Hybridization signals were quantified using Molecular Dynamics phosphorimager and Image Quant 1.2 software.

Chromatin Immunoprecipitation

Cells were fixed in 1 % formaldehyde, the nuclei isolated, and lysed in 1% SDS/10 mM/ EDTA/50 mM TrisHCI, pH8 for 10 min on ice. Chromatin was sheared by sonication to an average size of ~500– 1000 bp, suspended in ChIP immunoprecipitation buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris pH 8, 167 mM NaCI), and immunoprecipitations were carried out overnight at 4°C. Immune complexes were collected on protein G agarose, extensively washed, eluted in 1% SDS and 0.1 M $NaHCO_3$, and DNA cross-links were reverted by heating at 65°C overnight prior to DNA isolation. Isolated DNA from precipitations and input chromatin (diluted a further 1/20) were amplified by PCR for 34 cycles (94°C 1 min; 60°C 1 min.; 72°C 1 min, with a 7 min. final extension at

72°C). Oligonucleotide primers used were as follows: intronic R1, 5-GCC TTGGGTGATTGTGCTAA-3′ and 5-TCCTGAGGGCTGGTTCTGA-3′; intronic R2, 5′-ATGCTGGACCTGGATTTCTT-3′ and 5-TATGGGCTG GAATCGATTTT; 25 kb 5R2, 5-TATAAAGGCATCCACTGAAGA-3′ and 5-AGGGAACCGACTTACACAAA-3′; 32 kb 5R2 5-GTGTGG TCAAGAGCAATATAAA-3′ and 5′-GCTTATCCCCTGTTCTCTTT-3′; ERag 5-CCTCGGGTTCTGTCTAATACTC-3′ and 5-GGAGACAG CACATAAAACAACA-3′; D3 enhancer 5-CTCTGGCTATCACTGTC ACTT-3′ and 5-TGACCGTTGGCTTTATTACTTA-3′; mouse kEnh, 5-GGGAAAGGCTGCTCATAATT-3′ and 5-CCCTGGTCTAATGGT TTGTAA-3′; CD8, 5-TCTCTAAGTTAACAAGCATCTACTG-3′ and 5′-GTCGCGCAGAAGTAGAAGTCAC-3′.

Rearrangement and RT-PCR Assays

Rearrangement products present in genomic DNA were detected using the probes and PCR conditions/primers described for the following rearrangements: Vλ1-Jλ1 excision product (Tiegs et al., 1993), RS-IRS (Retter and Nemazee, 1998), or Vκ-Jκ1 (Hertz and Nemazee, 1997). cDNA synthesis, RT-PCR, and detection of PCR products by hybridization for mouse *RAG2* was as described (Tiegs et al., 1993). PCR primers for mouse HPRT were 5-GCTGGT GAAAAGGACCTCTC-3′ and 5-CACAGGACTAGAACACCTGC-3′. PCR cycle conditions used were: 25 cycles (94°C 1 min; 62°C 1 min.; 72°C 1 min with a 7 min. final extension at 72°C). PCR products were resolved on 1.5% TBE agarose gels, transferred to Zetaprobe membranes and probed with appropriate [^{32}P]-labeled probes.

Acknowledgments

The authors thank D. Schatz for *RAG* cDNA probes, S. Dowdy and P. Kabouridis for TAT-fusion protein constructs and advice, M. Nussenzweig for GFP/RAG2 bac transgenic mice, H. Kishimoto for SN50 peptide and C. Espinoza for advice on ChIP. The authors thank N. Klinman, M. Diaz, K. Murre, R. Rickert, G. Bokoch, A. Feeney and J. Kaye for their advice on the manuscript. We thank the National Institutes of Health (RO1AI33608) and The National Cancer Institute of Canada (postdoctoral award to LKV). DAA was supported by NIH training grant T32HL07195.

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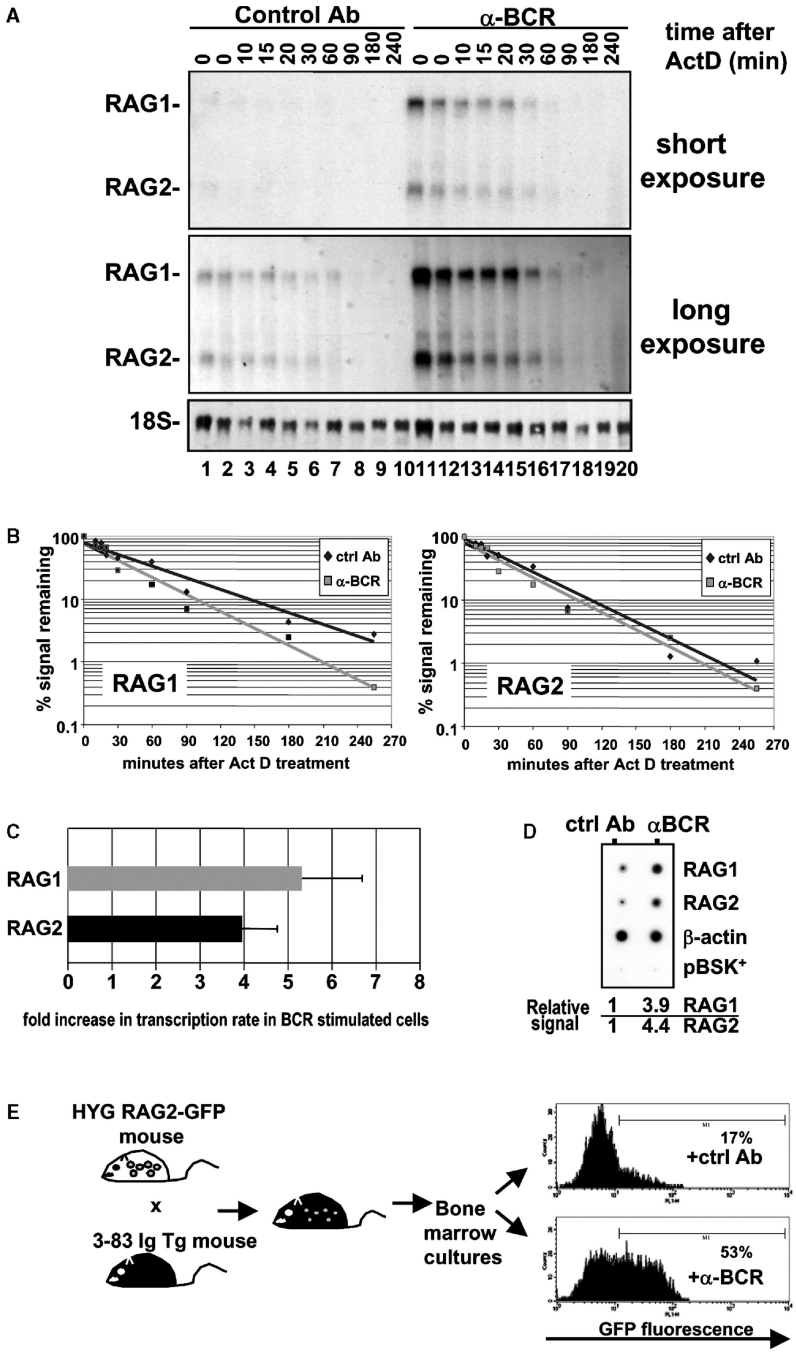

Figure 1. Assessment of *RAG1* and *RAG2* mRNA Stability and Transcription in Immature slg$^+$ B Cells Carrying an Unligated or Anti-BCR Ligated Antigen Receptor

Primary bone marrow cells were cultured for 5 days in IL7-containing medium, and then cells were challenged in the absence of IL7 with control or anti-BCR antibody.

(A and B) Assessment of decay of *RAG1* and*RAG2* mRNA levels upon Actinomycin D treatment.

(A) Northern blot RNA analysis of 3–83 transgenic BM B cells cultured in the presence of control antibody (lanes 1–10) or anti-BCR antibody (lanes 11–20) for 24 hr post IL7 withdrawal, followed by addition of the transcriptional inhibitor Actinomycin D and incubation for the indicated times.

(B) Graphical representation of the data shown in (A), showing *RAG1* and *RAG2* mRNA levels relative to 18S RNA signal. Initial levels were set to 100% and the percent signal remaining was plotted as a function of time.
(C and D) Nuclear run-on analysis of *RAG1* and *RAG2* transcription at 24 hr post IL7. Data shown indicate the fold increases in BCR-stimulated compared to control Ig-treated cells.
(E) GFP reporter analysis of *RAG2* transcription in 3–83 Ig / HYG *RAG2*-GFP double transgenic BM cells. Panels on right show flow cytometry analysis of cells cultured post IL7 withdrawal for 48 hr with control or anti-BCR antibody.

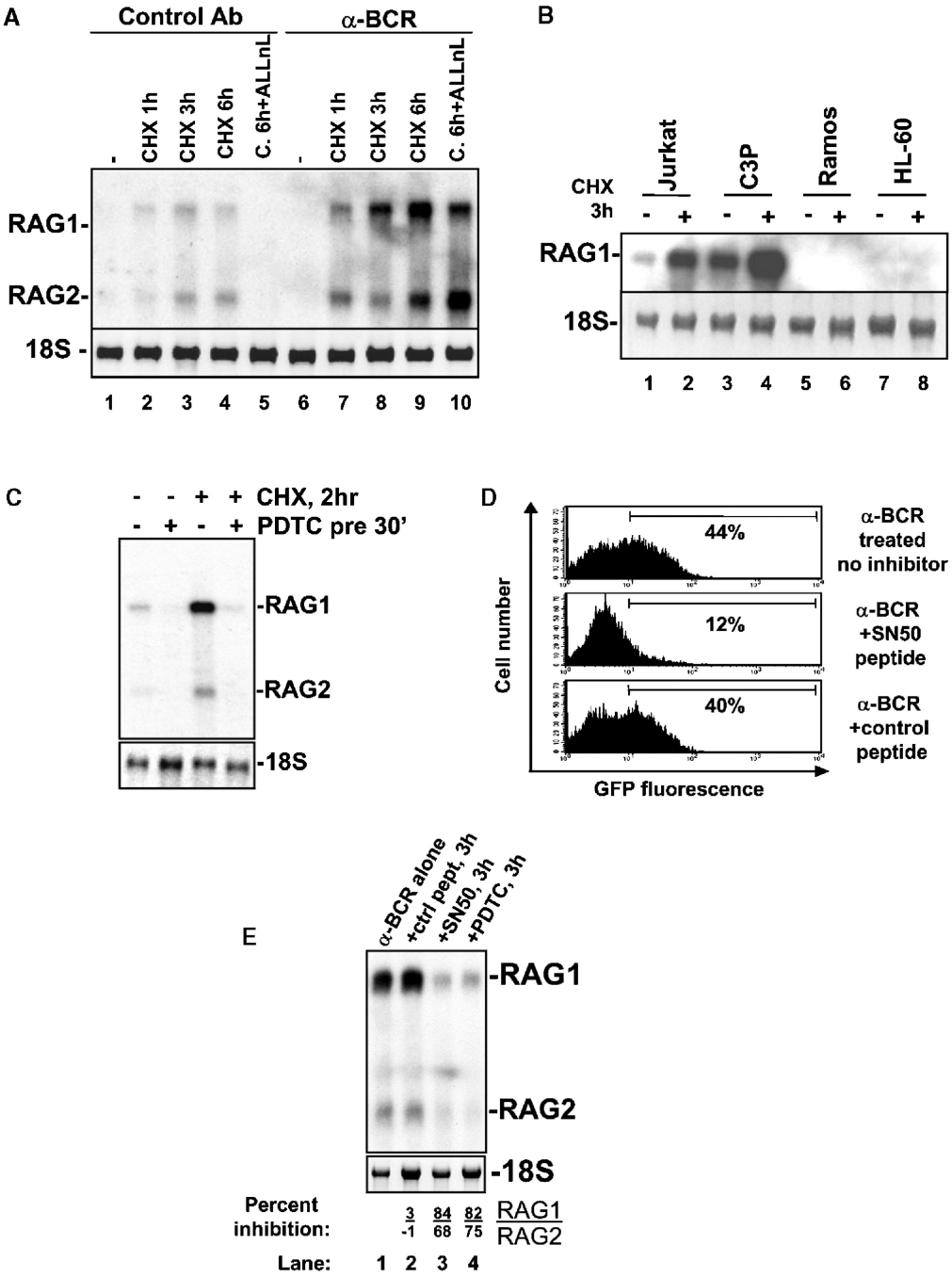

Figure 2. Effects of Cycloheximide, Proteasome, and NFκB Inhibitors on BCR-lnduced and -Uninduced *RAG* Gene Transcription in Primary B Cells and Cell Lines

(A and B) Stimulation of *RAG1* and *RAG2* gene expression by CHX treatment and its reversal by treatment with a proteasome inhibitor.

(A) Northern blot analysis of treated primary BM B cells. 3–83 Tg BM cells were cultured for 5 days in IL7-containing medium, then cells were challenged for 24 hr in the absence of IL7 with control antibody (lanes 1–5) or anti-BCR antibody (lanes 6–10). Cycloheximide (10 μg/ml) was added at the indicated times prior to harvest. ALLnL was added 30 min prior to the indicated CHX addition (lanes 5 and 10).

(B) Northern analysis of cycloheximide-treated hematopoietic cell lines. Jurkat (T cell), OCI LY8-C3P (editing-competent B cell), Ramos (mature B cell), and HL-60 (myeloid cell) were treated for 3 hr in the presence or absence of CHX (10 μg/ml).
(C-E) Effect of two NF-κB inhibitors on *RAG1* and *RAG2* expression in post IL7-cultured primary mouse BM B cells.
(C) Northern analysis of cycloheximide-induced *RAG* expression. CHX (10 μg/ml) was added to the indicated cultures 2 hr prior to harvest (22 hr post IL7). PDTC was added to the indicated cultures 2.5 hr prior to harvest. No antibodies were added to these cultures.
(D) Effect of SN50 on anti-BCR-induced *RAG2* reporter expression. Cells were treated at time of IL7 withdrawal with anti-BCR antibody alone (top panel), anti-BCR with the NF-κB inhibitor SN50 (middle panel), or anti-BCR with a control peptide, SN50c (lower panel). Cells were analyzed for GFP expression at 36 hr.
(E) Northern of *RAG1* and *RAG2* expression in cells treated for 24 hr post IL7 with anti-BCR antibody. Lanes 2–4, cells were treated 3 hr prior to harvest with lane 2, SN50, 50 μM; lane 2, control peptide, 50 μM; lane 4, PDTC, 5 μM. Percent inhibition of *RAG1* and *RAG2* RNA signal (normalized to 18S RNA content) is shown under each lane.

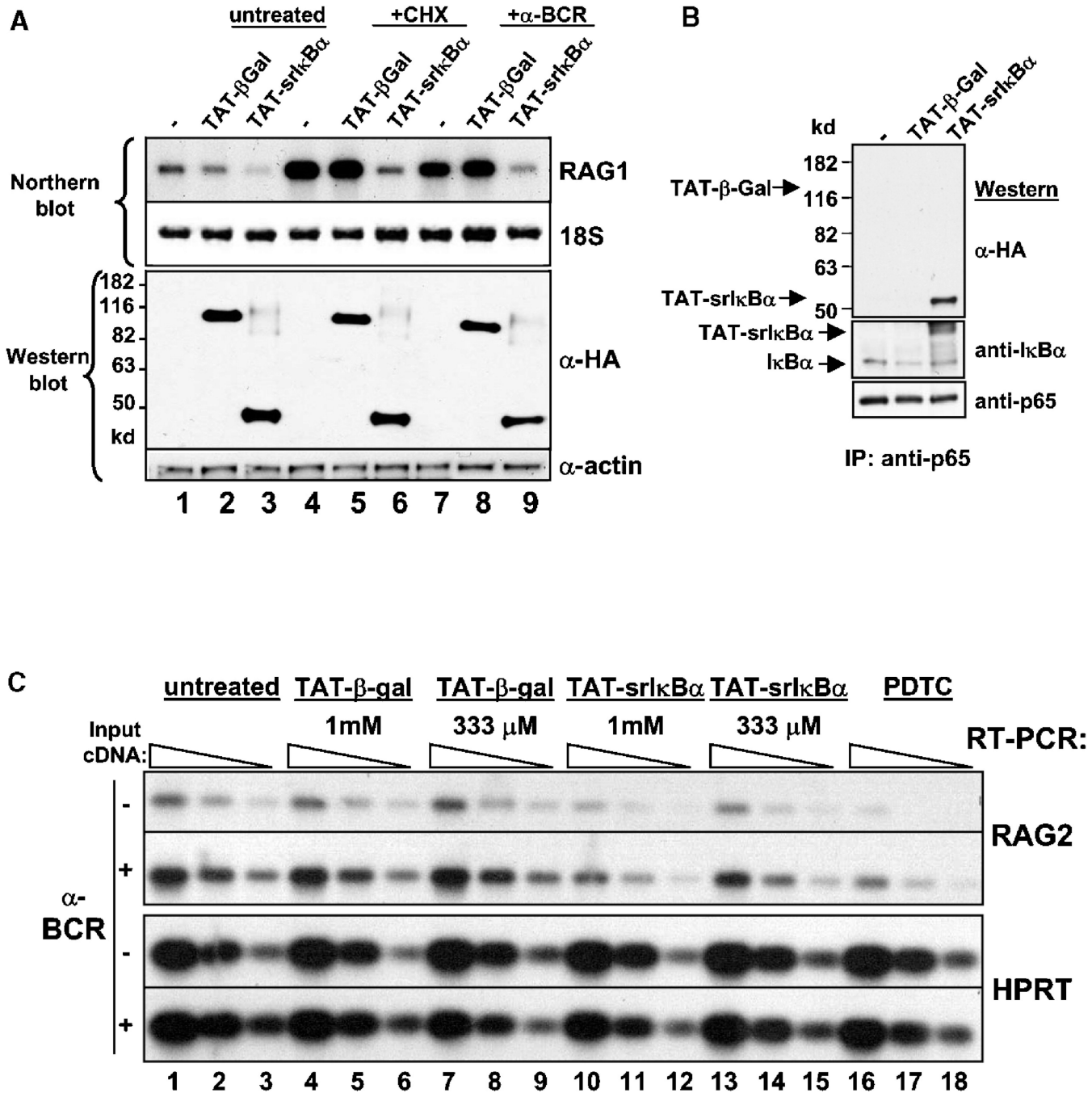

Figure 3. The Ability of Protein Transduction with a Dominant Negative IκBα Protein to Suppress *RAG* Expression in Editing Competent Primary Mouse BM B Cells or Transformed Human B Cells

(A and B) Rapid suppression of anti-BCR- and cycloheximide-induced *RAG1* expression in a human editing-competent B cell line by TAT- srκ Bα.

(A) Upper panels: Northern analysis of *RAG1* expression and 18S RNA loading control in treated OCI LY8-C3P cells. Cells were cultured for 3.5 hr with control fusion protein TAT-β-Gal (lanes 2, 5, and 8), the NF-κB inhibitor TAT-srIκBα (lanes 3, 6, and 9), or without TAT fusion protein (lanes 1, 4, and 7). To induce *RAG1* expression, CHX (lanes 4–6) or anti-BCR antibody (lanes 7–9) was added 30 min after the start of culture. Lower panels:

Immunoblot analysis of the uptake of TAT fusion proteins using antibodies to hemaglutinin epitope tags carried by the fusion proteins.

(B) Analysis of interaction between transduced proteins and p65/RelA. Cells treated as in (A) were washed, lysed, and the p65 subunit of NFκB was immunoprecipitated. Immune complexes were resolved by SDS-PAGE, transferred onto a PVDF membrane, and probed with α-HA, α-lκBα, or α-p65 Abs. Arrows next to middle panel indicate positions of fusion proteins; arrows in lower panels indicate the positions of the endogenous IκBα and the transduced TAT-srlκ Bα.

(C) RT-PCR analysis of the effects of TAT-srlκ Ba on BCR-stimulated *RAG* expression in freshly isolated BM B cells. Total 3–83 Tg BM cells were stimulated for 24 hr with anti-BCR antibodies in vitro in the absence of exogenous cytokines as described (Hertz and Nemazee, 1997). TAT fusion proteins or PDTC were added 4 hr prior to harvest. Wedges indicate serial 4-fold dilution of input cDNA.

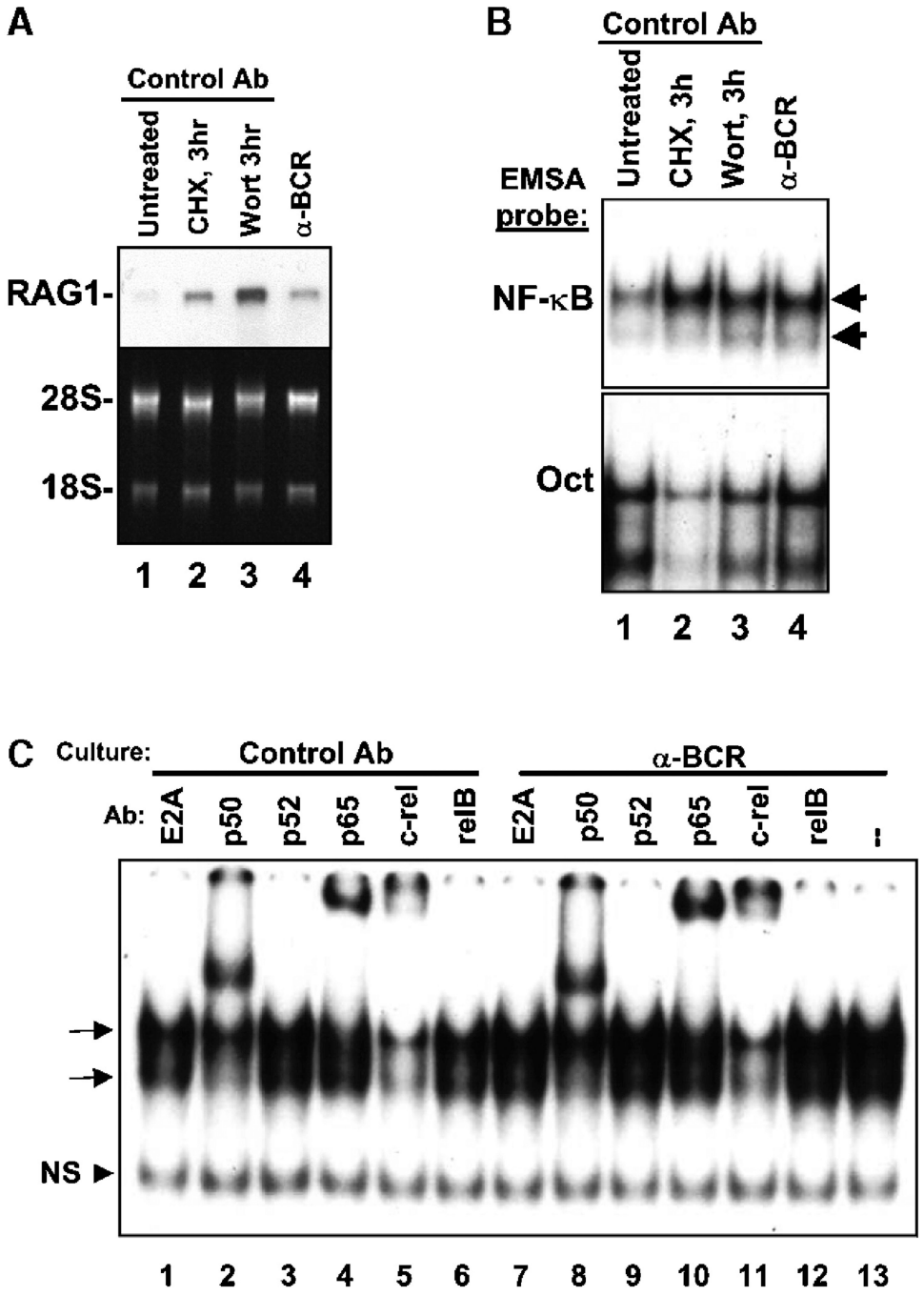

Figure 4. Correlation between Induced *RAG* Expression and Nuclear NF-κB DNA Binding Activity in BM B Cells

(A) Northern analysis of *RAG1* expression in cells treated for the indicated time periods prior to harvest at 24 hr post IL7 withdrawal. Abbreviations are as follows: Cyclo, CHX; wort, wortmannin.

(B) Electrophoretic mobility shift analysis (EMSA) of nuclear extracts using a consensus NF-κB probe. Lanes 1–4 show nuclear binding activity of consensus NF-κB probe (top panel) or Oct-1 (lower panel) using extracts from cells treated as indicated in (A).

(C) Analysis of NF-κB containing EMSA complexes by antibody supershift. Nuclear extracts from control (lanes 1–6) or anti-BCR treated (lanes 7–13) cells were bound to

consensus NF-κB probe in the presence of the indicated antibodies. E2A antibodies served as a negative control (lanes 1 and 7).

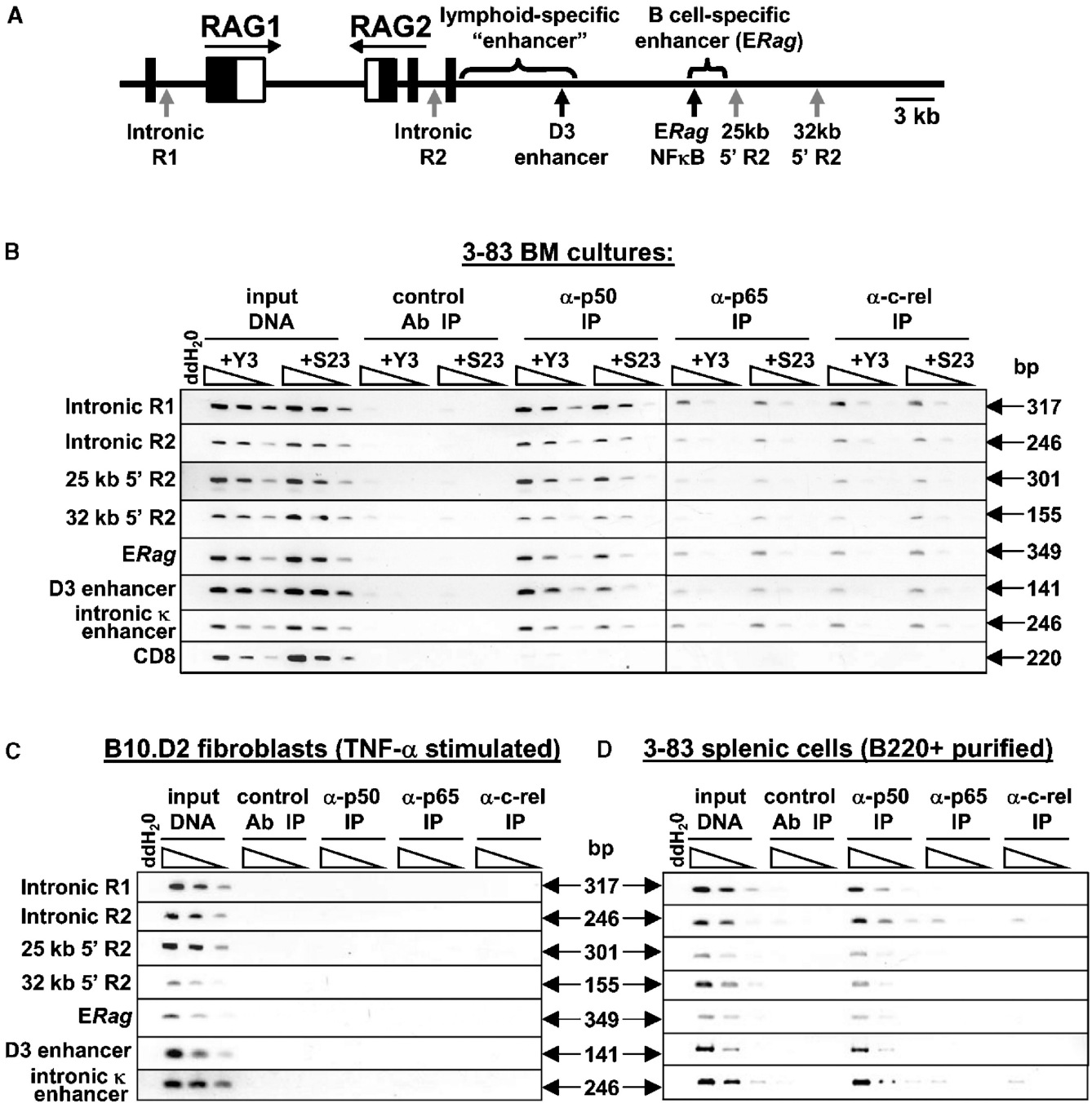

Figure 5. ChIP Analysis of NFκB/Rel Association with the *RAG* Locus

(A) Schematic representation of the *RAG* locus with the approximate locations of the sites studied. Putative NFκB sites, indicated with vertical arrows, were identified using default settings of the TESS program (http://www.cbil.upenn.edu/tess/), and were conserved between mouse and human *RAG* loci; also shown are possible sites within known D3 and Erag enhancer elements.

(B–D) Immunoprecipitation assays were carried out with chromatin from the indicated cell types using antibodies to the indicated NFκB/Rel proteins. Bound DNA was detected and quantitated using semiquantitative PCR assays. (B) Shows results from cultured 3–83 BM B cells stimulated for 24 hr with control antibody (Y3) or anti-idiotypic antibody (S23).

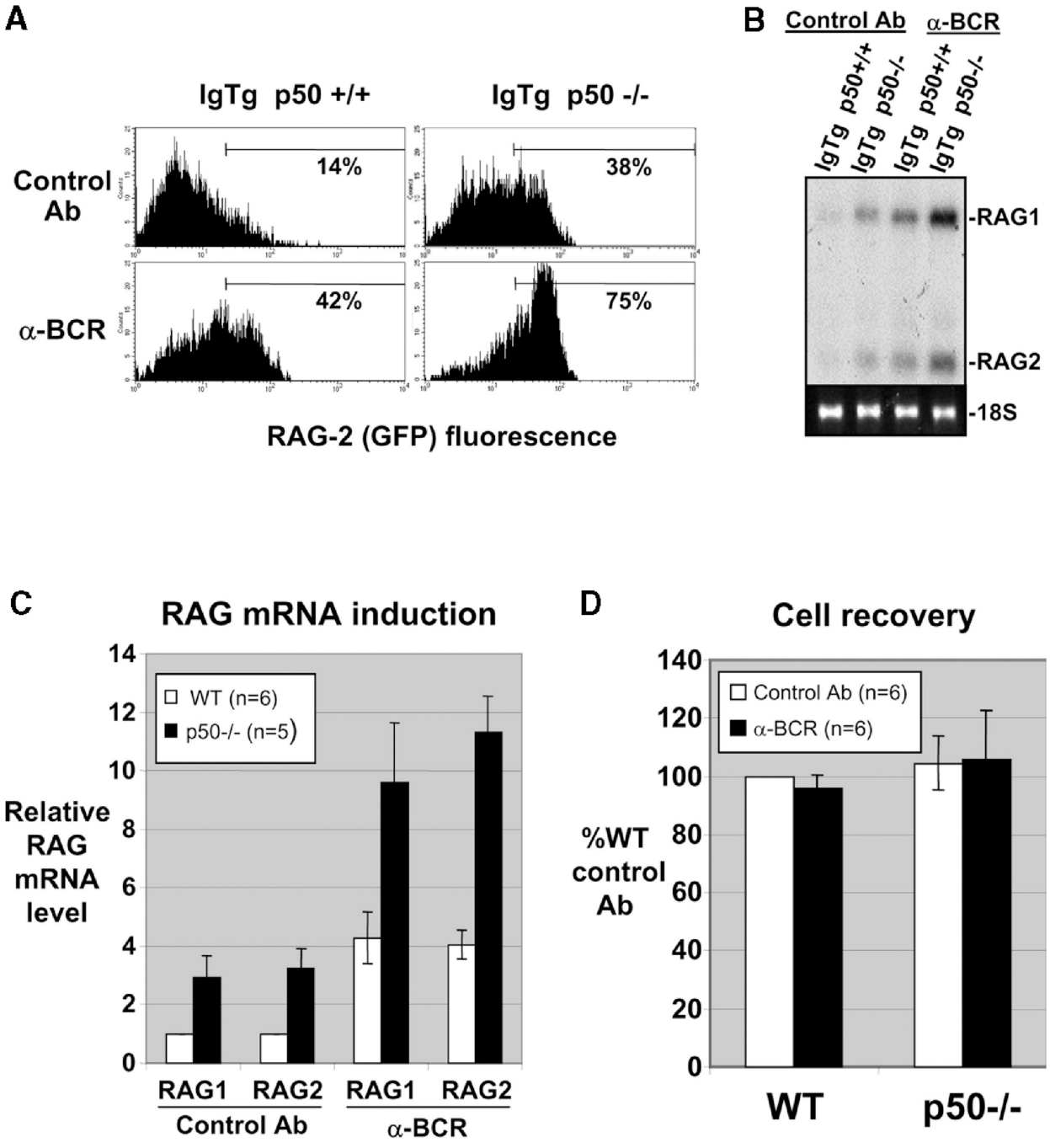

Figure 6. Effect of p50 Deficiency on *RAG2* GFP Reporter Expression, *RAG* mRNA Levels, and Cell Survival

IL7 expanded p50-deficient or p50-sufficient 3–83 Ig/ HYG *RAG2-GFP* double transgenic BM cells were recultured without IL7 in the presence of anti-BCR or control antibody and assessed for GFP expression or *RAG* mRNA levels.

(A) GFP expression at 48 hr of culture of the indicated BM cells.

(B and C) Northern blot analysis of *RAG* mRNA expression at 24 hr. Lower panel of (B) shows 18S RNA levels. The relative message levels of *RAG1* and *RAG2* mRNAs were calculated as ratios of the RNA signal/18S relative to control Ig-stimulated p50-sufficient cells.

(D) Nonadherent cell recovery assessed at 24 hr.

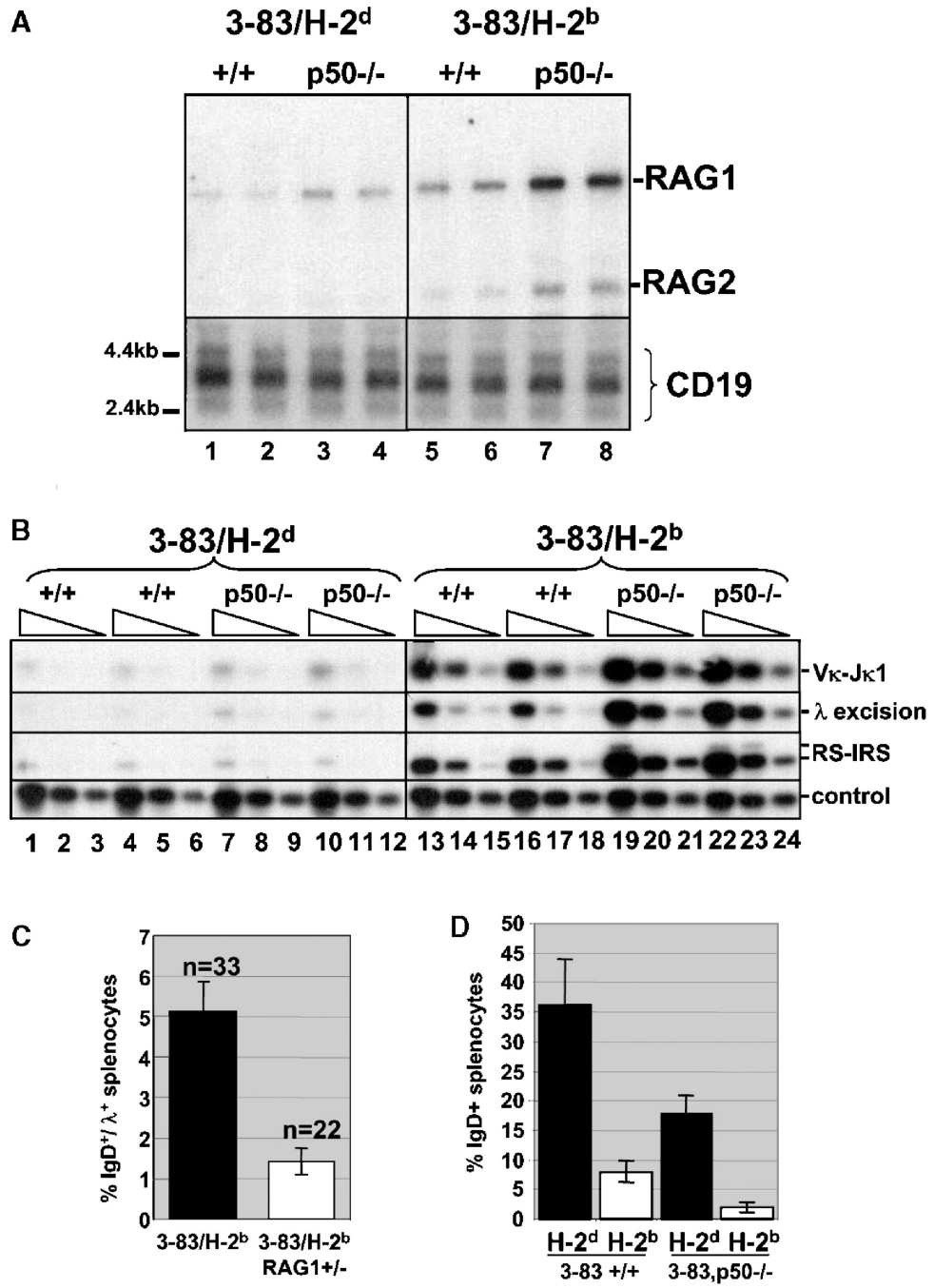

Figure 7. Effect of p50 Mutation on In Vivo *RAG* mRNA Expression and Light Chain Locus Recombination in B Cells

Mice expressing (3–83/H-2^b) or lacking (3–83/ H-2^d) BCR reactive antigen were bred to be p50-deficient or sufficient.

(A) $B220^+$ cells purified from BM were assessed for *RAG* and CD19 gene expression by Northern blot. Samples from two independent mice of identical genotype were assessed.

(B) Analysis of L chain locus DNA recombinations by PCR. DNA from $B220^+$ BM B cells of the indicated genotypes was tested for the following recombinations: endogenous VκS-to-Jκl (top panel), Vλ1-to-Jλ1 excision product (middle), and RS-to-IRS (lower). Samples from two independent mice/genotype were assessed.

(C) Reduction in the frequency of $lgD^+\lambda^+$ splenocytes in centrally tolerant 3–83/H-2^b/ *RAG1*$^{+/-}$ mice compared to nonmutant 3–83/ H-2^b mice. N = number of individual mice analyzed.
(D) Flow cytometry analysis of lgD^+ B cell frequency in spleens of mice of the indicated genotype. Statistics are based on four mice per group.