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Immunoglobulin kappa enhancers are differentially regulated at the level of chromatin structure

Barbara S. Nikolajczyk^{1,*}, Sylvia H. Sardi¹, Joseph R. Tumang², and Lisa M. Ganley-Leal³

¹ Departments of Microbiology and Medicine, Boston University School of Medicine, Boston, MA 02118

² The Feinstein Institute for Medical Research, Manhasset NY

³ Division of Infectious Diseases, Boston Medical Center, Boston University School of Medicine, Evans Biomedical Research Center, Boston, MA

Abstract

The κ intronic (κ_i) and the $\kappa 3'$ enhancers synergize to regulate recombination and transcription of the Igk locus. Although these enhancers have overlapping functions, the κ_i enhancer appears to predominate during receptor editing, while the $\kappa 3'$ enhancer may be more important for initiating Igk germline transcription to target locus recombination and, later in development, somatic hypermutation. Changes in chromatin structure appear to regulate both enhancers, and previous reports suggest that both enhancers are packaged into an accessible chromatin structure only in B lineage cells. Why these enhancers cannot activate the demethylated, accessible, protein-associated Igk allele in pro-B cells is not known. Furthermore, how the enhancers function to reactivate the locus for receptor editing or to quantitatively promote hypermutation in B cells is vague. Quantitative analysis of Ig enhancer chromatin structure in murine pro-, pre- and splenic B cells demonstrated that the κ_i enhancer maintains highly accessible chromatin structure under a variety of conditions. This stable chromatin structure mirrored the highly accessible structure characterizing the Igu intronic enhancer, despite the fact that Igu is activated prior to Igk during B cell development. Surprisingly, parallel analysis of the $\kappa 3'$ enhancer demonstrated its accessible chromatin structure is markedly unstable, as characterized by sensitivity to changes in environmental conditions. These data unexpectedly suggest that κ locus regulation is compartmentalized along the gene in B lineage cells. Furthermore, these findings raise the possibility that environmentally dependent regulation of $\kappa 3'$ enhancer structure underlies changes in κ activation during B cell development.

Keywords

B cell; kappa enhancers; chromatin; molecular biology

1. Introduction

The κ intronic (κ_i) and the $\kappa 3'$ enhancers function in combination to regulate recombination and transcription of the immunoglobulin kappa gene (Igk) during B cell development. This functional interaction between the enhancers was first demonstrated in transient transcriptional activation assays (Fulton and Van Ness, 1993; Fulton and van Ness, 1994; Liu et al., 1999).

*Address correspondence to: Dr. Barbara Nikolajczyk, Boston University School of Medicine, Department of Microbiology, 715 Albany Street, L-516, Boston, MA 02118, Tel. 617 638-7019; Fax. 617 638-4286, E-Mail: bnikol@bu.edu.

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More recent analyses showed genetic deletion of both enhancers in combination resulted in an almost complete lack of κ -expressing B cells, probably due to the inability of the cells to demethylate the κ locus and activate the gene prior to recombination (Inlay et al., 2002). However, deletion of the either enhancer alone resulted in decreased (rather than absent) κ recombination, demonstrating neither enhancer is absolutely required for rearrangement (Chen et al., 1993; Gorman et al., 1996; Inlay et al., 2004; van der Stoep et al., 1998; Xu et al., 1996; Zou et al., 1993). More detailed analyses showed that the κ i enhancer, but not the κ 3' enhancer, may play a role in secondary κ rearrangements, some of which are the result of receptor editing in response to auto-antigen reactivity (Inlay et al., 2002). In contrast, the κ 3' enhancer is important in initiating κ germline transcription (Inlay et al., 2002). Taken together, these results suggest developmental changes in κ 3' (rather than κ i) activity may play a disproportionate role in early locus activation, but κ i enhancer activity predominates during events which define specificity of the surface Ig μ /Ig κ heterodimer. Additionally, recent work demonstrated that the κ 3' enhancer, but not κ i, plays a quantitative role in κ somatic hypermutation, suggesting the κ 3' enhancer may also influence surface Ig specificity by controlling κ i-independent processes (Inlay et al., 2006).

Differences in protein-binding motifs between the two enhancers have been detailed in multiple analyses. Despite the demonstration that the array of protein binding sites making up each enhancer overlaps substantially, it is not surprising that it is the organization of these sites that determines how each enhancer is regulated. It is furthermore clear that chromatin packaging also affects tissue-specific enhancer regulation. Previous analyses of tissue culture B cells demonstrated that the κ 3' enhancer is packaged into an accessible chromatin structure in pro-B cells, well before the κ locus was traditionally thought to become activated during B cell development (Ji et al., 2003; McDevit et al., 2005). Moderate levels of accessibility in pro-B cells have been attributed to the demonstration that the κ allele eventually "included" to produce protein for the surface immunoglobulin heterodimer takes on an accessible chromatin structure earlier in B cell development (Goldmit et al., 2002; Ji et al., 2003). The "excluded" allele becomes accessible at the κ 3' enhancer later in development at the early pre-B cell stage, probably prior to when cells are actively rearranging the κ locus. This allele is likely inactivated by sequestration into heterochromatin as κ rearrangement ensues as evidenced by association with Ikaros and HP-1 (Goldmit et al., 2005; Ji et al., 2003). Despite this inhibitory packaging of the κ 3' region on one allele of mature B cells, finer structural analysis indicates that marks of an accessible chromatin structure remain, including restriction endonuclease accessibility and histone tail hyperacetylation, although the latter point is controversial (Goldmit et al., 2005; Hodawadekar et al., 2006; McDevit et al., 2005). It is possible that changes in allele positioning into heterochromatin override these more local determinants of κ activity (Hodawadekar et al., 2006). Changes in chromatin structure of the κ i enhancer have not been documented in as much detail, although regulation of the enhancers at the level of local chromatin structure appears to be independently determined (Blasquez et al., 1992). This finding is consistent with earlier data suggesting that the two enhancers are activated at similar stages of B cell development but through fundamentally different mechanisms (Meyer et al., 1990).

In contrast to the Ig κ locus, the Ig μ locus is packaged into a constitutively accessible chromatin structure early in development, in the common lymphoid precursor stage (Borghesi et al., 2005; Rumfelt et al., 2006). Both alleles of the Ig μ locus remain highly accessible to restriction endonucleases and packaged into hyperacetylated histones throughout development, as assayed in both cell lines and primary B cells (McDevit et al., 2005). Like the excluded κ allele, the excluded μ allele remains packaged into acetylated histones and is endonuclease accessible despite its sequestration into perinuclear heterochromatin (Goldmit et al., 2005; Kosak et al., 2002; McDevit et al., 2005; Roldan et al., 2005).

Antigen specificity of the Ig μ / κ heterodimer is determined largely by receptor selection in the bone marrow. Following initial Ig μ recombination in pro-B cells and Ig κ recombination in pre-B cells, surface Ig+ cells can inappropriately react with self-antigen in the bone marrow, resulting in RAG-dependent receptor editing in an estimated 25% to 50% of B cells (Hertz and Nemazee, 1997; Sandel and Monroe, 1999; Tiegs et al., 1993). Receptor editing has been mainly characterized at the κ locus, but evidence suggests editing can occur at the μ heavy chain locus as well (Chen et al., 1995; Darlow and Stott, 2005). Receptor editing requires RAG activity (Xu et al., 1998; Yu et al., 1999), but how RAG activity is directed to replace autoreactive V κ gene segments is unknown. Finally, multiple pieces of evidence are consistent with a quantitative role for the κ 3' enhancer in targeting AID for κ somatic hypermutation during the germinal center reaction, although κ enhancers are not absolutely required for somatic hypermutation (Inlay et al., 2006; Yang et al., 2006). Details of how the enhancer affects/determines AID targeting remains ambiguous. Thus far, it has been assumed that accessibility of Ig loci is established developmentally and is determined by the stage of B cell development, rather than potential environmental cues. New analyses of both μ intronic and κ i enhancers confirms the assumption that these regions are stably accessible in B cells under a variety of conditions. In contrast, κ 3' enhancer accessibility is sensitive to environmental changes that are unique from the multiple signaling pathways characteristic of B cells. This unique feature of the κ 3' enhancer in primary B cells is muted in cell lines, suggesting analysis of κ chromatin structure in cell lines cannot accurately recapitulate the dynamic events in primary cells. Finally, our data highlight the likelihood that κ locus regulation is compartmentalized along the gene in B lineage cells and raise the possibility that environmentally dependent regulation of κ 3' enhancer structure may explain changes in κ activation during B cell development.

2. Materials and Methods

2.1 Cells

Primary splenic Balb/c B cells prepared by "conventional methods" referred to in the text were purified from mature (6–8 wk-old) spleens by Thy-1.2-mediated complement lysis followed by a Lympholyte M gradient run at room temperature. Cells were kept on ice as much as possible throughout the preparation. Cells were routinely >90% pure as analyzed by flow cytometry. These cells were predominantly follicular B cells but include a small proportion of marginal zone B cells. All cells assayed after more than 12 hrs in culture (RPMI/10% heat inactivated fetal calf serum/10⁻⁵M β -mercaptoethanol/penicillin/streptomycin) were purified on a Lympholyte cushion to exclude dead cells. More than 90% of the cells were live at the time of nuclear preparation as indicated by trypan blue exclusion. Sort purified splenic B cells were selected as B220^{hi}/CD5⁻ splenocytes and were routinely less than 1.5% CD3⁺ or CD14⁺. Cells were always >98% pure upon re-analysis with additional lineage-specific markers. Pro B cells were selected from mouse bone marrow as the CD43⁻/CD23⁻/B220⁺ population. Pre B cells were similarly isolated as the CD43⁻/CD23⁻/B220⁺ population. This sort scheme allowed purification of a sufficient number of cells from one mouse for reproducible biochemical analyses (approx. 7000 pro B cells per experimental condition). Fresh splenocytes were prepared by gently squeezing cells from intact spleens, then pelleting cells for 5 mins at room temperature before resuspending in cytosolic lysis buffer as previously described (Boyes and Felsenfeld, 1996). Total elapsed time between mouse sacrifice and splenocyte lysis was 30–35 mins. Cells were kept at room temperature throughout this period. Percent B cells in the total splenocyte preparations was determined by flow cytometry on total splenocytes stained with biotin-labeled α -CD19 in 0.2% BSA/PBS followed by detection with streptavidin cy-chrome on a FACscan. Percent macrophages and T cells in total splenocyte preparations was similarly determined based on surface Mac-1 or CD4⁺/CD8⁺, respectively. Flow data was analyzed using WinMDI (Joseph Trotter, Scripps Institute). 1–2 cells are

RAG-1-null pro-B cells (kindly provided by Chris Roman, SUNY Downstate) and were grown in B cell media. Other cells and growth conditions have been described (McDevit et al., 2005).

2.2: Splenic B cell Stimulations

Splenic B cells were treated with 25µg/ml E. coli LPS for 18 hrs at 37°C, then live cells were purified through a Lympholyte M cushion before analysis. Alternatively, cells were incubated with human CD8-CD40 ligand chimeric protein for 5–10 mins before crosslinking the chimeric protein using anti-human CD8. Cells were incubated for an additional 18 hrs at 37°C before live cell purification and analysis as above.

2.3: Chromatin Accessibility by Real-Time PCR (CHART-PCR)

Relevant restriction endonuclease sites used for accessibility assays of the κ i, κ 3' and μ enhancers are shown in Figs. 1A and 4A. CHART-PCR was completed as previously detailed (Rao et al., 2001). Briefly, nuclei from $0.1-2 \times 10^6$ cells were isolated (Boyes and Felsenfeld, 1996), then treated for 1 hr at 37°C with restriction endonucleases recognizing accessible target sequences in the κ 3' (Bsu 36i or Nco I, 5–10 or 10–50 units, respectively), κ i (Scr FI or Sau 3AI: 0.5–10 units for both) or μ (Pvu II or Pst I: 10 or 20 units, respectively) enhancers proximal to core activation sequences for each enhancer. Partially digested DNA was purified using the Qiagen Blood Purification kit then quantitated with Pico Green (Invitrogen). Equivalent amounts (1–5ng) of genomic DNA were quantitatively amplified using primers that flank the endonuclease recognition palindrome (see sequences below). Standard curves generated by amplification of 0.5–10ng uncut genomic DNA allowed quantitation of DNA in each enzyme treated sample. DNA accessibility was calculated as follows: $(X-Y)/X \times 100$, where X= initial DNA quantity according to Pico Green quantitation and Y= apparent DNA quantity according to the Ct value and the standard curve. Some experiments analyzing the κ locus normalized κ amplification to amplification of a region of Igu refractory to the κ accessibility enzymes. Results for this alternative method of normalization were indistinguishable from normalization to amount of DNA using Pico Green. All cell types gave overlapping standard curves upon amplification of genomic DNA with a given primer set.

2.4: Primer Sequences

μ Pvu II or Pst I:	Forward: 5'-TGGCCGATCAGAACCAGAA-3' Reverse: 5'-CCAAATAGCCTTGCCACATGA-3'
κ 3'/Bsu36i:	Forward: 5'-TAGCTACCGTCACACTGCTTTGATC-3' Reverse: 5'-GCTGTGGAGGAGGATGGGAG-3'
κ 3'/Nco I:	Forward: 5'-ACTGGCCTGAGAAGATTAATAAGTAATG-3' Reverse: 5'-CAGTGTGACGGTAGCTATGACAGTTG-3'
κ i/Scr FI and κ i/Sau 3AI:	Forward: 5'-GGACTTCCGAGAGCCATCTG-3' Reverse: 5'-TGTAATCTGGGCCACCTGC-3'

3. Results

3.1. The κ 3' enhancer is variably accessible in B cells

Although previous reports showed that the κ 3' enhancer is endonuclease hyper-sensitive in pro- pre- and mature murine B cell lines (McDevit et al., 2005), our preliminary analyses in primary murine splenic B cells surprisingly demonstrated that the κ 3' enhancer is resistant to restriction endonuclease cleavage. These cells were prepared by conventional methods, kept on ice as much as possible, and assayed for κ 3' accessibility at the Bsu 36i site 1/2–1 hr after purification. Furthermore, the κ 3' enhancer chromatin structure in cells assayed shortly following purification by fluorescence-activated cell sorting was indistinguishable from the inaccessible structure in RAW 264.7 macrophages, as measured at the Bsu 36i site (not shown).

These preliminary experiments suggested that our published cell line analyses revealed an incomplete picture of the dynamic κ chromatin structure in primary normal splenic B cells. However, B cells incubated for 16–18 hrs at 37°C packaged the $\kappa 3'$ enhancer into a relatively accessible chromatin structure (Fig. 1B, warm B2, open bar). In contrast, the $\kappa 3'$ enhancer remained relatively inaccessible in B cells incubated on ice for 16–18 hrs (Fig. 1B, cold B2, dark stippled bars), recapitulating preliminary results on freshly purified cells. This treatment protocol was based on the storage methods used in many investigations to limit cell death yet allow cells to rest between purification and further experimental manipulations. These results suggest that chromatin structure of the $\kappa 3'$ enhancer is sensitive to environmental changes introduced during preparation of primary cells.

Because accessibility at a single restriction site could inaccurately reflect more global events, we analyzed splenic B cells incubated either on ice or at 37°C for accessibility at the Nco I site of the $\kappa 3'$ enhancer. The $\kappa 3'$ Nco I site was moderately accessible (an average of 41% of alleles cleaved) in B cells stored on ice (Fig. 1C, cold B2, dark stippled bar), as compared to relatively robust accessibility (an average of 59% of alleles cleaved) in cells stored under more physiological conditions (Fig. 1C, warm B2, open bar). Although the change in $\kappa 3'$ enhancer accessibility is less dramatic at the Nco I site, the data are consistent with Bsu 36i accessibility data showing that the cellular environment can determine $\kappa 3'$ enhancer chromatin structure.

Because only a maximum of about 65% of Ig κ alleles could be cleaved regardless of storage conditions or enzyme amount (Fig. 1 B&C and data not shown), while up to 95% of Ig μ alleles were accessible in parallel experiments (Fig. 3 and McDevit et al., 2005), we questioned whether activating immunoglobulin production would result in recruitment of a higher proportion of κ alleles into the accessible pool. We treated cells with CD40 ligand or LPS and measured $\kappa 3'$ enhancer accessibility at the Bsu 36i site (Fig. 1D). Note that cells were incubated at 37°C in these assays; incubation on ice would prevent activation of signal transduction pathways normally stimulated by these treatments. Neither stimulation protocol resulted in significant changes in $\kappa 3'$ chromatin structure (white stippled and striped bars show stimulation through CD40 or TLR4, respectively). This same maximum $\kappa 3'$ enhancer accessibility was also measured in B cell lines, represented by the RAG-1-null line 1–2 (Fig. 1E). This result contrasts with findings in a second RAG-1-null line, AH-7, which packages the $\kappa 3'$ enhancer into a relatively inaccessible chromatin structure (McDevit and Nikolajczyk, 2006), discounting the possibility that RAG-1 expression and $\kappa 3'$ accessibility are somehow related. Preliminary results suggested that cell lines representing pro-, pre- or mature B cells (38B9, Wehi 231 or BAL-17, respectively) package the $\kappa 3'$ enhancer into an equivalently accessible structure when incubated on ice or at 37°C for 16–18 hours before chromatin structural analysis (not shown). This finding indicates that, despite similar maximal levels of $\kappa 3'$ enhancer accessibility between B cell lines and purified primary cells, the cell lines do not accurately recapitulate the dynamic nature of the $\kappa 3'$ structure documented in primary splenic B cells. Overall, the data demonstrate that the accessible structure of the $\kappa 3'$ enhancer is unstable in primary splenic B cells.

To test whether the dynamic nature of the $\kappa 3'$ enhancer is typical of only mature B cells, or is a general characteristic of all B lineage cells, we measured enhancer accessibility in pro- and pre B cells isolated from mouse bone marrow by FACS. Purified pro- and pre B cells were incubated on ice or at 37°C for 3 hours prior to analysis by CHART-PCR with Bsu 36i. Pro B cells incubated on ice package the $\kappa 3'$ enhancer into a relative inaccessible chromatin structure (Fig. 1F, dark stippled bars), but incubation at 37°C resulted in an accessible structure (open bars). Identical analysis using pre B cells demonstrated a similar trend (i.e. cells incubated on ice packaged the $\kappa 3'$ enhancer into a less accessible structure) but differences were not statistically significant (white stippled bars). These data demonstrate that responsiveness of

$\kappa 3'$ enhancer chromatin structure to environmental cues is characteristic of multiple members of the B lineage: pro B cells and mature B cells.

3.2. The κ intronic enhancer, κi , is reliably accessible in B lineage cells

Non-quantitative analyses of J_{κ} accessibility have suggested the κi region is accessible only in B cells (Maes et al., 2001). Quantitative analyses testing the accessibility of κi to the restriction endonuclease Scr FI (position indicated in Fig. 1A) specifically demonstrated κi is packaged into a highly accessible chromatin structure in the pro B cell line 1–2 and is inaccessible in RAW 264.7 macrophages (Fig. 2A). However, because analysis of B cells lines versus primary B cells showed that lines do not accurately reflect the dynamic nature of the $\kappa 3'$ region, we tested κi accessibility to Scr FI in splenic B cells under conditions described for our $\kappa 3'$ enhancer assays. In contrast to the relative inaccessibility of the κi enhancer in macrophages (Fig. 2B, black bars), the κi enhancer is accessible to Scr FI in splenic B cells incubated at both 0° and 37°C (dark stippled or open bars, respectively). Preliminary analyses further indicated Scr FI cleavage was not changed by treating cells with LPS or CD40 cross-linking, that in combination with similar findings at the $\kappa 3'$ enhancer, suggested the overall chromatin structure of the κ locus was not changed upon cellular stimulation (data not shown). In contrast, κi accessibility to Sau 3AI was similar between macrophages and B cells, regardless of whether the B cells were incubated on ice or at 37°C (Fig. 2C, dark stippled or open bars, respectively).

This site can therefore be cleaved by restriction endonucleases in a cell type that never activates the κi enhancer, indicating this region is not regulated by a tightly closed/inaccessible chromatin structure. This finding contrasts with the $\kappa 3'$ enhancer, which is completely refractory to endonuclease cleavage in macrophages and 3T3 cells (Fig. 1).

To further test the conclusion that the κi enhancer maintains an accessible structure in B lineage cells, we measured κi accessibility in primary pre B cells. Pre B cells packaged the enhancer into a highly accessible structure regardless of incubation conditions prior to analysis (Fig. 2D). Preliminary analyses of pro B cells also showed the κi enhancer is highly accessible (50–70%) in cells incubated on ice or at 37°C for 3 hrs prior to analysis (data not shown), further supporting the idea that the κi enhancer is packaged into an accessible chromatin structure in all B lineage cells. Taken together, characterization of κ enhancers uncovered differences between the κi and $\kappa 3'$ enhancers by showing that 1. an accessible κi chromatin structure may not be uniquely B lineage-specific; and 2. in contrast to the $\kappa 3'$ enhancer, the κi enhancer is stably packaged into a chromatin structure that is unaffected by changes in environmental conditions.

3.3. The μ intronic enhancer is constitutively accessible in B cells

Given that stability of the chromatin structure of the two κ enhancers differs, we questioned whether either enhancer mirrored the accessible chromatin structure characterizing the μ intronic enhancer (E_{μ} ; Fig. 3A) in B cell lines and primary splenic B cells prepared by conventional methods. E_{μ} was highly cleaved in mature B cells, indicative of accessibility at both alleles, under all conditions tested (Figs. 3B&C). Specifically, cells incubated at either 0° or 37°C for 16–18 hrs packaged E_{μ} into a >75% restriction endonuclease accessible structure as measured by CHART-PCR at recognition sites for Pvu II (Fig. 3B) or Pst I (Fig. 3C). Control cells packaged E_{μ} into a relatively inaccessible structure (black bars), consistent with previous findings (Chowdhury and Sen, 2001; McDevit et al., 2005). Therefore, like the κi enhancer, the chromatin structure of E_{μ} is not subject to regulation by environmental conditions. The finding that chromatin structure within different regions of the κ locus, κi vs. $\kappa 3'$, may vary more than the differences between κi and E_{μ} was unexpected, and suggests that different regions of κ may be regulated largely independently.

3.4. Igk enhancers are highly accessible in fresh splenocytes

Our data show that E μ and κ i are packaged into highly accessible chromatin in primary B cells regardless of environmental conditions. In contrast, pro- and mature B cells package the κ 3' enhancer into a relatively pliable chromatin structure. We questioned how the κ 3' enhancer is packaged in unstimulated splenic B cells prior to manipulations that could inadvertently alter chromatin structure. We measured κ 3' enhancer accessibility in total splenocytes at approximately 30 mins post-mortem, the first time point at which we could reliably prepare single cell suspensions suitable for chromatin structural analysis *in vitro*. Because the analysis was completed on a mixed cell population, we performed flow cytometry on the single cell suspension to measure % B cells. 50–55% of total splenocytes were CD19⁺ B cells in 3 mice analyzed (Fig. 4A shows results from a representative spleen). Accessibility measurement of the splenocytes analyzed in Fig. 4A is shown in Fig 4B; Fig. 4C shows an average of multiple accessibility measurements at both κ enhancers. Both the κ 3' enhancer (as analyzed with Bsu 36i and Nco I) and the κ i enhancer (as indicated by Scr FI and Sau 3AI cleavage) were highly accessible in the mixed splenocyte population (Figs. 4B&C). Although Sau 3AI accessibility in non-B cells prevents us from predicting the contribution of B cells to this measurement, the approximate 50% accessibility at the other endonuclease recognition elements, coupled with the demonstration that only about 50% of the cell population is B cells (Fig. 4A) suggests that both enhancers are highly accessible in splenic B cells, with measurements approaching 100% accessibility. Technical considerations prevent a more accurate measure of the likelihood that maximum κ 3' enhancer accessibility in purified B cells underestimates actual accessibility *in vivo*. On a technical note, the data demonstrate that murine B cells used for *in vitro* analyses, especially analyses of κ 3' enhancer-dependent events, may or may not accurately recapitulate the physiological state of B cells *in vivo*, depending on culture conditions.

4. Discussion

We have demonstrated that the κ 3' enhancer, but not the κ i or μ enhancers, is packaged into a chromatin structure that responds to environmental cues. Although it is highly unlikely that B cells would be exposed to widely varying temperatures under normal mammalian conditions, our data raise the possibility that changes in chromatin structure at the κ 3' enhancer play a role in initial κ locus activation or somatic hypermutation. In contrast, the static character of the κ i enhancer discounts the likelihood that changes in κ i chromatin structure plays a role beyond initial activation of this region as soon as the pro-B cell stage (Maes et al., 2001; Shaffer et al., 1997). This interpretation is consistent with the *in vivo* footprint studies in primary B cell precursors, which show protein association patterns at the κ i enhancer do not change in the pro-to pre-B transition, while protein association with the κ 3' enhancer differs between pro- and pre-B cells (Shaffer et al., 1997). We have now documented a conceptually similar trend in pro- and mature primary B cells responding to changes in environmental conditions. Due to the functional synergy between the enhancers, our new data raise the possibility that changes in κ 3', but not κ i chromatin structure affect overall κ activation.

Multiple mechanisms may regulate the dynamic nature of the κ 3' enhancer. One possibility is that ATP-dependent chromatin remodelers may require continuous ATP hydrolysis (severely abrogated at low temperatures) to maintain an accessible structure at the κ 3', but not κ i, enhancer. Because previous findings suggest that PU.1 and IRF-4 association with the κ 3' enhancer appears to take advantage of a developmentally determined accessible chromatin structure (McDevit et al., 2005), the new data suggest that changes in the cellular environment could have multiple effects on the enhancer nucleoprotein complex. Chromatin immunoprecipitation (ChIP) showed that the ATP-dependent chromatin remodeler Brg-1 was broadly distributed over the μ enhancer (Morshead et al., 2003). However, our findings that in the absence of ATP hydrolysis (i.e., in cells incubated on ice) the μ locus remains highly

accessible strongly suggests that Brg-1 association with Ig genes in mature B cells may be a vestige of Brg-1 activity at an earlier stage of development. Non-essential Brg-1 association has been documented at promoters of multiple genes, as demonstrated by the lack of an effect of Brg depletion on promoter activation, despite promoter association with Brg-1 in ChIP assays (Ramirez-Carrozzi et al., 2006). This work strongly questions the physiological significance of remodeler association with genes packaged in an active chromatin structure.

Although accessibility at the κ i enhancer was not subject to environmental changes, the region proximal to the Scr FI recognition site was accessible in B cells but not macrophages. In contrast, a nearby element, the Sau 3AI recognition site, was equivalently accessible in both cell types. These findings may be explained by the location of each element relative to protein binding sites in κ i: the Sau 3AI element lies at the 3' end of the NF- κ B element, while the Scr FI site is in the 5' region of the κ E2 site. Perhaps constitutive NF- κ B element occupancy (Shaffer et al., 1997) allows Sau 3AI cleavage in both cell types, while potential κ E2 occupancy only in B cells allows Scr FI access in B cells but not macrophages. However, the NF- κ B element is occupied in primary pro- and pre-B cells, but not in T cells, while the κ E2 site may not bind protein in B cells as assayed by *in vivo* footprinting (Shaffer et al., 1997). An alternative explanation for differences in Sau 3AI versus Scr FI accessibility in macrophages may trace to the closer proximity of the Sau 3AI site to the κ i enhancer-adjacent matrix attachment region (Yi et al., 1999). It is also possible that the natural structure of the enhancer renders it constitutively accessible (i.e. unconstrained by chromatin packaging) at one site but not the other, based on primary DNA sequence. Finally, the κ i enhancer may be stably accessible due to its role as an RNA polymerase II template. In other words, lack of pol II association with the κ 3' enhancer may enable a more flexible structure sensitive to regulatory pressures. Regardless, these findings, coupled with the tissue-specific accessibility of the κ 3' enhancer, suggest that chromatin structure plays an ongoing role in tissue-specific κ 3' regulation, but a substantially less important role in κ i control. Based on our work, integrating the new findings of a dynamic κ 3' enhancer structure into understanding how this enhancer regulates κ germline transcription and somatic hypermutation becomes an important direction for future analyses.

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Abbreviations

CHART-PCR

chromatin accessibility assayed by real-time PCR

Ig

immunoglobulin

Figure 1.

Chromatin accessibility of the $\kappa 3'$ enhancer under various environmental conditions for 16–18 hrs. (A) Schematic of the κ enhancers showing location of restriction endonuclease cleavage sites relative to identified protein association sequences. (B) Accessibility of the $\kappa 3'$ enhancer in nuclei treated with 10 units Bsu 36i as measured by CHART-PCR. (C) Accessibility of the $\kappa 3'$ enhancer in nuclei treated with 30 units Nco I as measured by CHART-PCR. (D) Accessibility of the $\kappa 3'$ enhancer in nuclei from cells treated as indicated before treatment with 5 units Bsu 36i. (E) Chromatin accessibility of the $\kappa 3'$ enhancer to different amounts of Nco I as indicated in RAW 264.7 macrophages (black bars) or 1–2 pro-B cells (white bars). In panels B–E, bars show averages from three independent determinations; error bars show standard deviation. Results were similar for all accessibility assays when cells were incubated at 37°C (or on ice) for 3 hrs (data not shown). (F) Accessibility of the $\kappa 3'$ enhancer to the indicated amount of Bsu 36i in multiple primary cell types as shown. For this assay cells were incubated at 37°C (or on ice) for 3 hrs. Bars show the average and error bars show range of 2–3 independent experiments.

Figure 2.

Chromatin accessibility of the ki enhancer at two unique restriction endonuclease recognition sequences: (A) Accessibility to Scr FI (amounts as indicated) in RAW or 1–2 proB cells. (B) Chromatin accessibility in primary splenic B cells (incubated for 16–18 hrs as shown) measured with ScrFI or (C) Sau 3 AI. Nuclei were treated with units of enzymes indicated below each data set. (D) Accessibility of pre B cells incubated for 3 hrs as shown and assayed with 5 units Scr FI (left bars) or Sau 3AI (right bars). Shown is average of at least three independent determinations in each assay. Error bars indicate standard deviation. Low levels of negative accessibility in panel B are likely due mathematical amplification of slight error in DNA measurements and are not biologically significant.

Figure 3. Chromatin accessibility of the μ enhancer. (A) Schematic of the enhancer showing relative position of the restriction endonuclease sites with respect to protein binding sites. Accessibility measurements are shown at the Pvu II (A) or Pst I (B) recognition elements. Data show average and range of 2–3 independent determinations.

Figure 4.

Chromatin accessibility of the $\kappa 3'$ enhancer or the κi enhancer in fresh splenocytes using the amount of enzyme indicated. (A) flow analysis of a representative spleen. 51.6% of splenic cells from this particular animal were B cells as indicated by surface staining for CD 19. % B cells in the total splenocytes preparation was 50–55% for each of three spleens analyzed by flow. (B) Accessibility of the $\kappa 3'$ or κi enhancers in splenocytes shown in (A). Bsu 36i and Nco I measure accessibility of the $\kappa 3'$ enhancer; Scr FI and Sau 3AI measure κi enhancer accessibility. Because the data are from a single digestion of cells for each condition, no error bars are shown. Error of Ct values from triplicate PCR reactions was less than 0.5 cycles. (C) Average accessibility from 2 (Bsu 36i) or 4 (all other enzymes) independent experiments as in (B). Error bars show standard deviation (Nco I, Scr FI or Sau 3AI) or range (Bsu 36i).