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The Igκ3′ Enhancer Is Activated by Gradients of Chromatin Accessibility and Protein Association

Daniel C. McDevit,* Leslie Perkins,† Michael L. Atchison,† and Barbara S. Nikolajczyk2*‡

The Igκ locus is recombined following initiation of a signaling cascade during the early pre-B stage of B cell development. The Igκ3′ enhancer plays an important role in normal B cell development by regulating κ locus activation. Quantitative analyses of κ3′ enhancer chromatin structure by restriction endonuclease accessibility and protein association by chromatin immunoprecipitation in a developmental series of primary murine B cells and murine B cell lines demonstrate that the enhancer is activated progressively through multiple steps as cells mature. Moderate κ3′ chromatin accessibility and low levels of protein association in pro-B cells are increased substantially as the cells progress from pro- to pre-B, then eventually mature B cell stages. Chromatin immunoprecipitation assays suggest transcriptional regulators of the κ3′ enhancer, specifically PU.1 and IFN regulatory factor-4, exploit enhanced accessibility by increasing association as cells mature. Characterization of histone acetylation patterns at the κ3′ enhancer and experimental inhibition of histone deacetylation suggest changes therein may determine changes in enzyme and transcription factor accessibility. This analysis demonstrates κ activation is a multistep process initiated in early B cell precursors before Igκ recombination and finalized only after the pre-B cell stage. The Journal of Immunology, 2005, 174: 2834–2842.

R ecombinational activation of the Ig genes is cell stage specific, with Igμ generally activated before Igκ. The mechanisms controlling temporal differences in μ vs κ recombination are unknown. Regulated κ locus activation is controlled by two interacting regions, the κ intronic and κ3′ enhancers (1–4); however, in vivo footprint analyses clearly demonstrate that delayed Igκ activation as compared with μ is not due to an inability of proteins to simply access κ enhancer sequences (1–4). Analysis of a developmental series of B cell lines demonstrated similar levels of κ3′ enhancer chromatin accessibility in all B cells with at least partially recombined Igκ genes, suggesting κ activation is a temporally compact event beginning and ending at the pre-B cell stage (1, 5). Similarly, nonquantitative DNase I analyses showed little change in κ accessibility in a comparison between pro- and pre-B cell lines (6), additionally eroding the likelihood that changes in κ chromatin as the cells transition into pre-B cells is important for κ to become competent for recombination. In contrast, subsequent work in genetically modified primary B cells demonstrated that footprint patterns over the κ3′ enhancer, but not the κ intronic enhancer, changed during the pro- to pre-B transition, suggesting protein occupancy, rather than chromatin structure, changes to facilitate κ recombination (2). Specifically, hypersensitivity at the PU.1/IFN regulatory factor-4 (IRF-4)κ3′ element increased, suggesting that protein occupancy at the κ3′ enhancer, but not the κ intronic enhancer, changes as κ becomes active recombinationally. Mechanisms explaining these likely changes in protein occupancy have not been elucidated. However, these data indicate that assembleing a proper nucleoprotein complex, rather than changing chromatin structure per se, is key for regulating κ. Furthermore, these findings argue that the relevant target of the LPS-induced signaling cascade shown to activate the κ locus is not the κ intronic enhancer because NF-κB (a LPS target) or a related protein is already occupying the NF-κB site in the intronic enhancer before cellular stimulation (2). The latter finding is not unexpected given that the κ intronic enhancer does not impair stage or lineage-specific rearrangement of a κ transgene (7). On the basis of the implications of the above studies, we have focused our attention on more clearly understanding the relationship between an active κ3′ enhancer chromatin structure and the role protein/κ3′ association plays in activating the κ locus during B cell development.

Transcriptional activation of the κ3′ locus has been studied extensively outside of the constraints of chromatin structure, usually in the pre-B cell stage or later (8–10). Elements implicated by these in vitro and in vivo analyses include the cAMP response element (11) and a site occupied by a B cell lineage-specific activator protein (Pax5), an antagonist of PU.1 transactivation (12). However, the most thoroughly characterized κ3′ enhancer element is the PU.1/IRF-4 composite element. This element is activated due to PU.1 phosphorylation, which facilitates IRF-4 recruitment and subsequent transcriptional stimulation (9, 13). As noted above, the PU.1/IRF-4 composite element is also the element whose occupancy is most dramatically changed in vivo during the pro- to pre-B cell transition (2). Although PU.1 is unable to activate the κ3′ enhancer from a closed chromatin structure in nonlymphoid cells (14), the data are consistent with the model that PU.1, and by extension, IRF-4, takes advantage of an accessible κ3′ chromatin structure to activate the locus. However, this model has never been tested in the context of chromatin.

To elucidate the roles chromatin structural changes and transcription factor/κ3′ enhancer association play in appropriate temporal activation of the κ locus, we quantitatively examined κ3′
enhancer chromatin accessibility in a developmental series of B cell lines. Interestingly, pre-κ recombination B cell precursors package the κ3 enhancer into chromatin of intermediate accessibility as compared with non-B or κ-expressing B cells, which package the enhancer into inaccessible or highly accessible chromatin structures, respectively. Acetylation of histones packaging the κ3 enhancer is also increased in more mature cell types as compared with pre-κ recombination cells. Hence, κ3 activation is a prolonged multistep process initiated even before Igκ recombination begins. Examination of protein association with the κ3 enhancer in B cell lines and primary pro- and pre-B cells demonstrated that documented changes in κ3 footprint patterns are likely due to changes in PU.1 and IRF-4 association during κ locus activation independent of changes in protein expression levels. Overall, our data showing increases in PU.1/κ3 and IRF-4/κ3 association by chromatin immunoprecipitation (ChIP) concomitant with increased κ3 enhancer chromatin accessibility and histone acetylation support the model that PU.1 recruitment of IRF-4 to the κ3 locus is in part facilitated by changes in accessibility of the κ3 chomatin structure. κ locus activation is thereby a protracted multistep process ensuing before Igκ gene recombination.

**Materials and Methods**

**Cells and Abs**

Characteristics of murine cell lines used in this study have been described previously (1). Lines are defined by recombination status at the Igκ and κ loci. Briefly, AH-7 or 63-12 pre-B cells are RAG 1−/− or RAG 2−/−, respectively; hence, these lines have no opportunity to initiate Igκ or Igλ recombination and represent early pro-B cells. 358B9 pre-B cells are re-centered in D3-1 at the Igκ locus. These cells complete Igκ recombination and initiate Igκ sterile transcription and κ recombination upon LPS stimulation (15). 3-1 are pre-B cells that have completed Igκ recombination (VDJκ, VDJκ) and initiate Igκ transcription in response to LPS (16). Wehi 231 cells have both Igκ and κ recombined; hence, they are considered immature B cells. BAL-17 B cells express surface Igκ and model mature splenic B cells in signaling assays. S194 are nonsecreting plasmacytoma cells that have both Igκ and κ recombined; hence, they are considered immature B cells. BAL-17 B cells express surface Igκ and model mature splenic B cells in signaling assays. S194 are nonsecreting plasmacytoma cells obtained from American Type Culture Collection (TIB19). 2017 cell lines were grown in RPMI 1640 supplemented with 5% heat-inactivated FCS, 10% 2-ME, penicillin, and streptomycin. The exception was S194 cells, which were grown in DMEM plus 10% horse serum, NIH 3T3 fibroblasts or RAW 264.7 macrophages were grown in DMEM supplemented with 5% heat-inactivated FCS, 10% 2-ME, penicillin, and streptomycin. The exception was S194 cells, which were grown in DMEM plus 10% horse serum, NIH 3T3 fibroblasts or RAW 264.7 macrophages were grown in DMEM supplemented with 5% heat-inactivated FCS, respectively, penicillin, and streptomycin. Primary splenic (B2) B cells were prepared from mature BALB/c mice by Th y-1.2-mediated complement lysis, followed by a lymphocyte gradient. Cells are routinely >90% pure as analyzed by FACS. These cells are largely follicular B cells but include a small proportion of marginal zone B cells. Primary pro-B cells were isolated from the ells of 15 BALB/c mice by fluorescence sorting for the B220+CD43+CD32+ population (see Fig. 3. A and B). Dead cells were excluded based on forward vs side scatter. Pre-B cells were isolated similarly as the B220+CD43−CD32− bone marrow population. Although CD32 status is a relatively simplistic approach to identifying pro- vs pre-B cells, purifying sufficient numbers of cells for analysis is a practical approach. Primary pro-B cells were formaldehyde fixed for ChIP immediately following purification. For some experiments, B cells were treated with LPS or trichostatin A (TSA) at a concentration of 10 μg/ml or 33 nM, respectively, for 18–24 h.

The Ab to the C-terminal peptide of PU.1 was purchased from Santa Cruz Biotechnology (T-21) and used in Western blot analyses (1/500 dilution) by standard methods. Ten microliters of this Ab were used for ChIP assays in at least two independent experiments. PU.1 protein used for ChIP assays was made by immunizing rabbits with recombinant PU.1, then purifying α-PU.1 Ig on a PU.1 affinity column. A total of 1.5 μl of this Ab was used per ChIP sample. Similarly, Ab to the full-length IRF-4 protein was raised in rabbits (21) and was used without additional purification from serum. A total of 2.5 μl of α-IRF-4 was optimal for ChIP assays. ChIP assays for histone tail modifications used α-acetylated H3 (06-599) or H4 (06-598) Abs from Upstate Biotechnology (24 and 6 μl, respectively). Control precipitations were performed with preimmune rabbit serum or rabbit α-histidine tag Ab as noted in each figure.

**ChIP**

ChIP was completed on 1–10 × 106 cells as we have described in detail previously (22). Analysis was quantitated by real-time PCR using SYBR green incorporation. Melt peaks indicated formation of a single product in all template-containing reactions. Identity of all PCR products was verified on native–8% acrylamide gels. Two methods were used to quantify ChIP products: the ∆(ΔCt) method (23) or the 2−(ΔΔCt) quantitative PCR method (24), as designated in the figure legends. The former method was used to compare results between primary cell subpopulations and the latter to judge binding in absolute terms compared with the amount of PCR target present in the sample input before enrichment by immunoprecipitation. Primer sequences used in quantitative PCR are listed below. Alternatively, ChIP products in Figs. 7 and 8 were quantified by dot blot after 25 cycles of PCR amplification. Five, 10, or 20 ng of template were amplified in independent reactions to ensure analysis was in the linear range of amplification. Primers for the amplification were 5′-GACAGAGATTACACCCCATACCTC-3′ and 5′-CTGGAAAGGTTGGAGTGCACCA-3′. Amplified samples loaded onto Hybond-N (Amer sham Biosciences) were detected with a probe made from the 1.1-kb EcoRI–SacI fragment of the κ3 enhancer. Signals were quantified by phosphorimager analysis to determine the percentage of DNA bound as a fraction of total input DNA. Note that absolute values from the PCR- vs blot-based ChIP quantitation methods are not comparable mathematically, although relative differences can be compared accurately.

**Chromatin accessibility by real-time PCR (CHART-PCR)**

CHART-PCR was completed as described previously (25). Briefly, nuclei of cells from 1–2 × 106 cells were isolated (26), then treated for 1 h at 37°C with restriction endonucleases recognizing accessible target sequences in the κ3 (Bus 36 i or NcoI, 10 or 30 μl, respectively) or υ (PvuII or PstI, 10 or 20 μl, respectively) enhancer chromatin structure in a developmental series of B cell lines (Fig. 1A, B). NcoI, 10 or 30 μl, respectively) or υ (PvuII or PstI, 10 or 20 μl, respectively) enhancer proximal to the relevant PU.1-binding element. Partially digested DNA was purified using the Qiagen Blood Purification kit (Qiagen) then quantitated with PicoGreen (22). Equivalent amounts (25 ng) of genomic DNA were quantitatively amplified using primers that flank the endonuclease recognition palindromes (see sequences below). Standard curves generated by amplification of 1–50 ng of uncut genomic DNA allowed quantitation of DNA in each enzyme treated sample. DNA accessibility was calculated as follows: (25 − X)/25 × 100, where X = initial DNA quantity according to the standard curve. All cell types, except BAL-17, gave overlapping standard curves upon amplification with a given primer set. For this reason, only BAL-17 samples were quantified based on a BAL-17 genomic DNA standard curve.

**Primer sequences**

ChIP: μ forward, 5′-AGACAGTGCGAACGAGCAG-3′, and reverse, 5′-TCAAAACCACTTCTAACAACACAG-3′; υ forward, 5′-TACATCCAG TCACACTGTTTGATC-3′; and reverse, 5′-GCGTTGAGGAGAT GGAG-3′; β-globin forward, 5′-GGCTTTGCCTGTCTCGTC-3′, and reverse, 5′-CAGACATTAAAACTGTTTTTATAGCC-3′; and reverse, 5′-GGGTCAGATGCGCCAG-3′. BAL-17 CHART-PCR: μ forward, 5′-PvuII or PstI, 10 or 20 μl, respectively) or υ (PvuII or PstI, 10 or 20 μl, respectively) enhancer proximal to the relevant PU.1-binding element. Partially digested DNA was purified using the Qiagen Blood Purification kit (Qiagen) then quantitated with PicoGreen (22). Equivalent amounts (25 ng) of genomic DNA were quantitatively amplified using primers that flank the endonuclease recognition palindromes (see sequences below). Standard curves generated by amplification of 1–50 ng of uncut genomic DNA allowed quantitation of DNA in each enzyme treated sample. DNA accessibility was calculated as follows: (25 − X)/25 × 100, where X = initial DNA quantity according to the standard curve. All cell types, except BAL-17, gave overlapping standard curves upon amplification with a given primer set. For this reason, only BAL-17 samples were quantified based on a BAL-17 genomic DNA standard curve.

**Results**

κ3 enhancer chromatin is accessible in pro-B cells

Although both characterized κ enhancers associate with proteins before κ recombination (1, 2), the protein footprinting pattern over only the 3′ enhancer changes upon comparison between pro- and pre-B cells (2). Genetic deletion of the κ3 enhancer additionally demonstrates that this enhancer plays an important role in κ rearrangement and expression in vivo (27). To further understand the changes occurring at the κ3 enhancer as the locus is activated during B cell development, we measured accessibility of the enhancer to endonucleases as an indication of chromatin structure. CHART-PCR (25) was used to measure quantitatively κ3 enhancer chromatin structure in a developmental series of B cell lines using non-B cells as controls. For these assays, two restriction endonucleases that recognize sequences 82 bp apart in the κ3 enhancer, Bus 36 i and NcoI (Fig. 1A), were used to probe chromatin accessibility. Analysis with two enzymes eliminates the possibility of drawing conclusions from a single chromatin region
Packaged into a nonregulated (or inaccessible) structure. Such a region might not provide an accurate representation of \(\kappa^3\) chromatin structure. Chromatin accessibility profiles for the \(\kappa^3\) enhancer demonstrated three discernible levels of accessibility on a population basis. As expected, nonlymphoid cells such as NIH 3T3 fibroblasts or RAW 264.7 macrophages were virtually inaccessible to Bsu 36I (Fig. 1B, []). At the opposite end of the spectrum, immature (Wehi 231) and mature B cells (BAL-17) in []). Averages and errors were calculated based on nanograms of DNA amplified by comparison to a standard curve generated from amplification of known amounts of genomic DNA, then displayed as percent total input DNA (25 ng).

To verify that \(\kappa\) activation correlates with a more accessible \(\kappa^3\) region, we took advantage of the demonstration that LPS treatment of 38B9 pro-B cells (which are normally D-J recombined at the \(\mu\) locus and germline at \(\kappa\)) activates the \(\kappa\) locus. \(\kappa\) locus activation by LPS in these cells can be measured by production of \(\kappa_0\) sterile transcripts (15), which we have independently verified (14). Therefore, stimulated cells represent the pre-B stage of development. Interestingly, LPS-treated 38B9 cells also package the \(\kappa^3\) enhancer in a highly accessible chromatin structure when probed with Bsu 36I (Fig. 1B, []), approximating the 60% \(\kappa\) accessibility measured for more mature B lineage members. In contrast, \(\kappa\) is only moderately accessible (approximating 40%) to NcoI (Fig. 1C). Because neither enzyme appears to cleave DNA at an unrepresentative structure in five other cell types as discussed above, we discount this possible explanation. Instead, different accessibility to the two enzymes may represent a transient structure formed during the demonstrated prolonged opening of \(\kappa^3\) enhancer chromatin. Overall, the \(\kappa\) locus is packaged into at least three distinct, stable chromatin structures as measured by endonuclease accessibility in CHART-PCR: closed, moderately accessible, and highly accessible. This analysis is consistent with DNase I hypersensitivity site analyses showing hypersensitive sites in the \(\kappa^3\) enhancer of both 38B9 pro-B and BALB mature B cells (1). However, the 20–30% differences in chromatin accessibility indicated by the CHART-PCR analysis would be difficult to detect on Southern blots, thus the moderately accessible chromatin structure packaging the \(\kappa^3\) enhancer has not been described previously. More importantly, these data show that the first steps of \(\kappa\) locus activation occur well before the developmental transition from pro- to pre-B cell. Differences in protein occupancy shown by the in vivo footprint assays (1, 2) thereby correlate with notable changes in \(\kappa\) chromatin structure, eventually forming a highly accessible nucleoprotein structure concomitant with \(\kappa\) locus activation.

The \(\mu\) enhancer (\(\mu\)EN) is packaged into an accessible chromatin structure at all stages of B cell development

To determine whether Ig enhancer accessibility and protein association generally increases during B cell development, we analyzed the Ig\(\mu\) intronic enhancer (\(\mu\)EN). \(\mu\)EN is activated early in B cell development, as evidenced by production of sterile Ig\(\mu\) transcripts in the earliest identified B cell precursor cell (28). The \(\mu\)EN is also packaged by hyperacetylated histone H3 in pro- and pre-B cells (29). Chromatin structure of \(\mu\)EN during maintenance of Ig\(\mu\) transcription in mature B cells has not been directly analyzed. We used CHART-PCR to document putative changes in \(\mu\) chromatin structure during B cell development, taking advantage of PvuII or PstI recognition elements within the core enhancer (30) to probe accessibility in the same cells used for \(\kappa^3\) analysis in Fig. 1. \(\mu\)EN was in a highly accessible chromatin structure in all B cells tested, as indicated by cleavage of \(\sim80\%\) of enhancers in the cellular population by PvuII (Fig. 2A) or PstI (Fig. 2B). Primary splenic B2 cells also package \(\mu\)EN into a highly accessible chromatin structure as demonstrated by analysis with both enzymes (Fig. 2, A and B, []), indicating our cell line analyses reflect structure in primary cells. As expected, the B cell-specific \(\mu\)EN was not accessible in Ig\(\mu\)-negative NIH 3T3 fibroblasts or RAW 264.7 macrophages (Fig. 2, A and B, []). These data demonstrate that Ig\(\mu\) enhancer accessibility does not generally change as B cells transition from pro-B to surface Ig\(\mu\)-positive effector cells. Hence, the intermediate accessibility demonstrated for the \(\kappa^3\) enhancer is a novel characteristic of the \(\kappa\) locus.

\(\kappa^3\) enhancer/transcription factor association increases during B cell development

\(\mu\)1 is an important transcriptional activator for both the \(\kappa^3\) enhancer and \(\mu\)EN (9, 31). \(\mu\)1 acts as a chromatin accessibility determinant for \(\mu\) but not \(\kappa\), as indicated by the ability of ectopically
expressed PU.1 to increase μ (but not κ) chromatin accessibility (14, 18). We questioned whether PU.1 association with the κ3′ enhancer, suggested by changes in footprinting patterns over the κ3′ PU.1 site in primary B cells, changes with increased chromatin accessibility of an activating κ locus. We used PU.1-specific Abs in ChIP assays to demonstrate PU.1/κ3′ enhancer association in primary pro- and pre-B cells sorted from murine bone marrow-based CD23, B220, and CD43 surface expression (Fig. 3, A and B). PU.1 specifically associated with the κ3′ enhancer in both pro- and pre-B cells (Fig. 3C, □ or ■, respectively), and apparent association is 3.2-fold more robust in pre-B cells as compared with pro-B cells (65.6- or 20.3-fold over 2017 levels, respectively). PU.1/κ3′ association is additionally enriched to 93.7-fold over 2017 levels in primary splenic B2 cells as compared with pre-B cells (Fig. 3C, □). Specificity of PU.1/κ3′ association is indicated by the inability of an unrelated α-histidine tag-specific Ab to precipitate the κ3′ enhancer (Fig. 3C, right bars). α-PU.1 Ab specificity is demonstrated by the lack of PU.1 association with the inactive β-globin promoter in all B cell subpopulations (Fig. 3D). Overall, this result is consistent with the interpretation that PU.1 exploits increased chromatin accessibility (Fig. 1) at the κ3′ enhancer as the cells activate the κ locus at the pre-B stage. The ChIP results from primary cells mirror data from B cell line analyses using the same set of cells used for κ accessibility analyses (D. McDevit and B. Nikolajczyk, unpublished observation).

Additionally, to investigate the relationship between κ3′ recombinational activation at the pre-B stage and PU.1 association with the κ3′ enhancer, we compared the ability of an epitope-specific α-PU.1 Ab to precipitate PU.1 in unstimulated and LPS-stimulated 3B9 cells. This Ab associates with the C-terminal PU.1 peptide. PU.1-specific ChIP demonstrated increased PU.1 association with the κ3′ enhancer poststimulation (Fig. 3E). Specifically, PU.1 associated ∼5.1-fold over input genomic DNA in LPS-stimulated cells (Fig. 3E, □) compared with 9.1-fold over input DNA in unstimulated cells (Fig. 3E, ■) for a 78% increase. Control assays show negligible α-his/κ3′ association by ChIP (Fig. 3E, right bars) or α-PU.1/β-globin promoter association (data not shown). This data corroborates data from primary bone marrow B cells showing that PU.1/κ association is increased in pre- vs pro-B cells.

To test whether PU.1/DNA association also changes during development at another PU.1-regulated locus characterized by uniform chromatin accessibility, we measured PU.1/Eμ association by ChIP. Consistent with the 3.2-fold increase in apparent PU.1/κ3′ enhancer association at the pro- to pre-B transition, PU.1/Eμ association is 3.2-fold more robust in pre-B cells as compared with pro-B cells (Fig. 3F, □ or ■, respectively). Specificity of PU.1/Eμ association is demonstrated by the lack of PU.1 association with the inactive β-globin promoter in all B cell subpopulations (Fig. 3G). Overall, this result is consistent with the interpretation that PU.1 exploits increased chromatin accessibility (Fig. 1) at the κ3′ enhancer as the cells activate the κ locus at the pre-B stage. The ChIP results from primary cells mirror data from B cell line analyses using the same set of cells used for κ accessibility analyses (D. McDevit and B. Nikolajczyk, unpublished observation).

**FIGURE 2.** Eμ accessibility to restriction endonucleases as measured by CHART-PCR. A, Eμ accessibility to PvuII. Nuclei of indicated cells were treated with 10 U of PvuII before analysis by quantitative PCR. B, Eμ accessibility to 20 U of PstI and analyzed as in A. Cell stage is color coded as outlined in Fig. 1. Primary B2 cells, most similar to mature BAL-17 B cells based on surface Ig expression and signaling pathways upon BCR-mediated activation, are represented by ◼. Averages and error bars were calculated from two to four analyses as outlined in Fig. 1.
association was 4.2-fold stronger in bone marrow pre-B cells (8.4-fold over input; Fig. 4, A) as compared with pro-B cells (2.0-fold over input; Fig. 4, B). Although PU.1/Ea association was somewhat less robust in mature splenic B2 cells (6.8-fold over input; Fig. 4, C) as compared with pre-B cells, it is unclear whether this reproducible 20% decrease in association is biologically meaningful. Specificity in this analysis is indicated by lack of α-histidine association with the Eα (Fig. 4, right bars) and lack of PU.1/β-globin association (Fig. 3D). Note PU.1/Ea association (Fig. 4) and PU.1/κ3′ association (Fig. 3C) were measured from the same pool of precipitated B cell DNA in parallel PCR reactions.

PU.1 recruits a member of the IRF transcription factor family, IRF-4, to activate the κ3′ enhancer in an extrachromosomal context (13). If PU.1/κ3′ enhancer association is limited by an immediately accessible κ3′ enhancer chromatin structure in pro-B cells, we would expect IRF-4/κ3′ enhancer association to also be limited before κ locus recombinational activation. To test this prediction, we completed ChIP assays using an α-IRF-4 Ab on B cell lines with demonstrated intermediate or high accessibility. These experiments showed IRF-4-associated with the κ3′ enhancer in all B cells tested, but a significantly higher proportion of κ3′ enhancers were associated with IRF-4 in Wehi 231 immature B cells as compared with 38B9 pro-B cells, i.e., IRF-4 association increased after κ locus activation (Fig. 5A). Specifically, IRF-4 enriched κ3′ DNA 1.4-, 3.7-, or 5.7-fold over input DNA in pro-, late pro-, or immature B cells, respectively. Preliminary analysis shows IRF-4/κ association in primary splenic B2 B cells quantitatively approximates Wehi 231 association, predicting IRF-4 association is maximum on the recombined κ locus. The α-IRF-4-specific Ab did not specifically precipitate the inactive β-globin promoter (Fig. 5B). Because our previous analyses ruled out the possibility that the PU.1/IRF-4 combination establishes an accessible κ locus (at least in 3T3 fibroblasts and pro-T cells; Ref. 14), the ChIP data are more likely explained by increased κ chromatin accessibility in activated pro-B cells. Specifically, the data support the model that increased PU.1/κ3′ association (Fig. 3C) results in increased IRF-4 recruitment to the κ3′ enhancer and hence increased IRF-4/κ3′ association by ChIP in pro- vs pro-B cells.

Transcription factor expression level does not correlate with κ3′ enhancer association

Our preliminary work demonstrated that PU.1 expression levels may, at least in part, explain increased PU.1/κ3′ enhancer association detected in more mature members of the B lineage (D. McDevit and B. Nikolajczyk, unpublished observation). We analyzed PU.1 levels in additional members of the B lineage to additionally define the mechanisms by which PU.1 activates the κ3′ enhancer. Western blot analyses using an α-PU.1-specific Ab demonstrated PU.1 levels increase substantially as cells begin recombining the Igκ locus at the pro-B stage (Fig. 6, top panel, lane 3). Approximately equivalent protein loading is indicated by actin levels detected in each sample (Fig. 6, lower panel). These data show that PU.1 levels in the immature Wehi 231 B cells are low relative to 38B9 (compare Fig. 6, lanes 3 and 4), despite demonstrating that the κ3′ enhancer is in a highly accessible structure in Wehi 231 but not 38B9 cells (Fig. 1). Clearly PU.1 levels do not strictly correlate with κ3′ enhancer accessibility or PU.1/κ3′ association by ChIP.
Acetylation of κ3′ enhancer-packaging histone H3 increases upon κ recombination

The interpretation that association of PU.1 and IRF-4 with the κ3′ enhancer follows rather than initiates the demonstrated increases in chromatin accessibility does not explain how PU.1 and IRF-4/κ3′ binding is more robust in mature B cells as compared with pre-B cells (Figs. 3C and 5A). Analysis of a third set of staged B cells, 3-1 pre-B and S194 plasmacytoma cells, confirmed that IRF-4 binding to the κ3′ enhancer was more robust in later cell stages (Fig. 7, A and B, lanes 5 and 6 or lanes 3 and 4, respectively). Because increased histone tail acetylation is widely associated with gene activation (32), we questioned whether this alternate measure of chromatin accessibility would correlate with changes in chromatin structure in later B cells not apparent in restriction endonuclease assays (Fig. 1). We addressed this possibility using ChIP assays to precipitate κ3′ enhancer DNA associated with α-acetylated histone H3 or α-acetylated histone H4 in 3-1 vs S194 cells. Acetylated H3 was more commonly associated with the κ3′ enhancer in S194 plasma cells as compared with 3-1 pre-B cells (Fig. 7A, lanes 7 and 8). Expressing the data relative to levels of DNA precipitated in 3-1 cells (Fig. 7B, lanes 5 and 6) showed a 4.1-fold increase in acetylated H3 associated with κ3′ in the more mature S194 cell. Similarly, both methods of analyses demonstrated that acetylation of histone H4 packaging the κ3′ enhancer increased ~2.3-fold in S194 cells as compared with 3-1 pre-B cells (Fig. 7B, lanes 7 and 8). This finding extends previous H4 acetylation analyses in 63-12 pro-B and HC8 pre-B cells, which demonstrated that H4 packaging the Jκ region is probably more highly acetylated in the more mature cells (6). H3 acetylation analyses were not included in these related studies. Overall, our data suggest that increased histone acetylation, especially H3 acetylation, correlates with increased transcription factor binding at the κ3′ enhancer and is consistent with the possibility that the transcription factors are taking advantage of increased chromatin accessibility even at later stages of B cell development.

Differential acetylation of histones packaging the κ3′ enhancer may indicate that such changes hold biological relevance to κ locus activation during B cell development. To test this possibility, we took advantage of the demonstration that the κ locus can be activated by LPS in 3-1 cells, as measured by κ3′ transcript production (16). ChIP specific for acetylated H3/κ3′ association demonstrated that LPS treatment increases association between acetylated H3 and the enhancer ~4.3-fold (Fig. 8, lanes 4 and 5). This increase is comparable to the 4.1-fold difference in acetylated H3/κ3′ association measured in unstimulated 3.1 pre-B cells vs S194 plasma cells (Fig. 7B, lanes 5 and 6). Similarly, association between acetylated histone H4 and the κ3′ enhancer increases 2.8-fold upon LPS stimulation (Fig. 8, lanes 7 and 8), replicating the 2.3-fold enhancement of acetylated histone H4 association in S194 vs 3-1 cells (Fig. 7B). PU.1/κ3′ association does not change significantly upon LPS treatment (1.1-fold increase post-LPS; Fig. 8, lanes 1 and 2), demonstrating specificity of increased acetylated histone/enhancer association upon experimental κ activation in pre-B cells.

We speculated that the change in acetylation status of the κ3′ enhancer during B cell development may be explained either in
selective expression of a histone deacetylase (HDAC) in pre-B cells or the selective expression of a histone acetylase in more mature B lineage cells. To differentiate between these possibilities, we treated 3-1 pre-B cells with TSA, a HDAC inhibitor, then measured association of acetylated histones with the κ3′ enhancer by ChIP. Acetylated H3 precipitates the κ3′ enhancer ~20-fold over control (untreated) 3-1 cells (Fig. 8, lanes 4 and 6). In contrast, association of acetylated histone H4 with the enhancer increases only marginally (2-fold) upon TSA treatment (Fig. 8, lanes 7 and 9). Specificity of these increases is demonstrated by the finding that PU.1/κ3′ enhancer association changes insignificantly upon TSA treatment (0.6-fold; Fig. 8, lanes 1 and 3). Overall, these data suggest that pre-B cells express an inhibitable HDAC that prevents acetylation of κ3′-associated histone H3 (and less likely, H4) but does not affect PU.1/κ3′ enhancer binding. Inhibition of this putative HDAC has a particularly pronounced effect on acetylation of histone H3 at the 3′ enhancer.

Discussion

Previous analyses of the κ3′ enhancer have largely focused on demonstrating activating protein associations and chromatin structures during the early pre-B cell stage during which, by definition, κ recombination ensues. Although analyses on the Jκ and Vκ regions have been published (6), we analyzed the region most likely involved in initiating κ locus activation by selective protein/DNA interaction, the κ3′ enhancer. A second potential κ regulatory region, the intronic enhancer, does not impart stage- or lineage-specific κ expression, at least in some experimental scenarios (7). Furthermore, because intronic enhancer occupancy does not change at the pro- to pre-B transition (2), we did not include it in our studies. The κ3′ chromatin structure and protein association analyses herein clearly demonstrate κ activation begins in early pre-B cells before the initiation of Igμ recombination. Specifically, κ3′ chromatin is moderately accessible in pre-B cells, and intermediate levels of the transcriptional activators PU.1 and IRF-4 associate with this incompletely accessible structure. We propose these events are priming the κ locus for the more dramatic and functionally important event, Vκ-Jκ recombination. Taken together with the demonstration that RAGs cannot cleave the κ locus in primary or 63-12 pre-B cells (33, 34), locus accessibility is necessary but not sufficient for κ recombination, even in the presence of RAG protein in 38B9 cells. Hence, κ accessibility is probably mechanistically distinct from κ recombination. Overall, our work demonstrates that κ locus activation is a relatively lengthy, multistep process, regulated at several points, as opposed to a process initiated only after a signal is generated by surface pre-BCR. Clearly the data are consistent with the demonstration that κ can recombine in the absence of μ recombination during normal B cell development (35).

Because our analyses measure accessibility to small molecules rather than large complexes such as the transcription or recombinase machinery, the data suggest that the κ locus is activated by multiple regulated changes in chromatin accessibility. This model can be incorporated into either proposed explanation of κ activation by large RAG- or polymerase-containing complexes. Both of these models were developed initially to explain κ allelic exclusion, but due to the nature of the questions asked, these models incorporate explanations of how an accessible κ chromatin structure is initially established. The first model is based on the demonstration that a single κ allele is preferentially replicated (36), demethylated (37–39), and recombined during B cell development. Because this allele is highly likely to be allelically included in mature B cells, it follows that early biochemical changes in that κ allele predispose the DNA to biologically relevant activation (i.e., recombination). The κ accessibility data herein are consistent with this model because it is likely that the partial κ accessibility to endonucleases in Fig. 1 is measuring relatively high accessibility at one allele and low accessibility at the second (excluded) allele. Toward this end, we speculate that the demonstrated 40% accessibility of the enhancer is indicating a high degree of accessibility at one allele in each individual pro-B cell and that 60–80% accessibility (to Bsu 36i or NcoI, respectively) is indicating a relatively uniform activation of the second and more likely excluded allele upon transition to the pre-B cell stage. This interpretation is consistent with the demonstration that the κ3′ (but not κ intronic) enhancer plays an important role in allelic exclusion (40) and hence must likely be activated on both alleles in pre-B cells when exclusion is important. The demonstration that κ sterile transcripts are produced from both alleles in μ/κ-RAG-null (pre-B) and mature B cells (41) is consistent with the nearly complete κ access measured from both alleles of pre-B and mature B cells. Taken together, the data argue that establishing κ access occurs before successful μ recombination and that κ chromatin accessibility is mechanistically distinct from κ recombination. The second model describes κ activation as a rare stochastic event occurring at the pre-B cell stage but not in pre-B cells (42); our data likely detects less dramatic but more prevalent changes in κ chromatin that are putative harbingers for subsequent accessibility to large complexes such as the polymerase complex measured Liang et al. (42). Although our analyses do not support one model of κ allelic activation over the other, the data are consistent with either model based on the likely structural limitations put on access to small endonucleases vs large multisubunit complexes.

The new data are consistent with our previous observation that PU.1 is unlikely to serve as a chromatin accessibility factor for the Igκ locus (14) and the likelihood that other factors such as Pax-5 establish κ3′ enhancer accessibility (43), at least to the intermediate level shown in Fig. 1. Although our data do not directly address the suggestion that Pax-5 is the critical κ accessibility factor (43) responsible for opening a closed κ structure at the appropriate point in development, the interpretation that PU.1 and IRF-4 take advantage of pre-established accessibility is consistent with this possibility. κ3′ structure in stimulated 38B9 cells shows that κ activation results in a structure moderately accessible to NcoI yet highly accessible to Bsu 36i. Because both enzymes yield similar accessibility profiles in all other cell types, it is unlikely that we are assaying nonrepresentative chromatin regions. Because the enzymes cleave κ DNA at 82 bp or approximately half a nucleosome’s worth of DNA apart, it is instead possible that we have
captured incomplete establishment of a mature κ3’ chromatin structure. The possibility of an intermediate chromatin structure is consistent with the demonstration that association of hyperacetylated histones with the κ3’ enhancer increases upon stimulation of pre-B cells (Fig. 8). Therefore, we interpret these results as highlighting the continuum of κ locus opening en route to the highly accessible locus in immature/mature B cells.

Previous analyses on the effect of TSA on κ activation agree with our suggestion that a pre-B cell HDAC limits the extent of acetylation at histones packaging the κ3’ enhancer. In these studies, treating p815 mast cells with TSA led to enhanced bulk histone acetylation and specifically increased acetylation of histone H4 packaging the Jk region (6). However, TSA-mediated histone hyperacetylation in mast cells did not successfully establish the B cell-specific micrococcal nuclease pattern over κ locus, suggesting that changes in histone acetylation alone cannot establish a B cell-specific κ chromatin structure. Our new data complement these findings by emphasizing the likelihood that changes in histone acetylation status following establishment of an accessible κ locus (or at least κ3’ region) regulates activation of the locus in pre-B cells, introducing the concept that histone acetylation may act as a regulatory rheostat for κ activation even after initial events establish chromatin accessibility.

Data herein demonstrate that activation of signaling cascades by LPS leads to a new step in the continuum toward full κ chromatin accessibility and maximal PU.1/κ3’ and IRF-4/κ3’ enhancer association. An alternative interpretation, that the transcription factors are becoming better targets for immunoprecipitation, is unlikely, at least for PU.1, due to our demonstration that the α-PU.1 antisera detects a largely available epitope (or epitopes) in DNA-associated protein (D. McDevit and B. Nikolajczyk, unpublished observation). In vivo footprinting (1, 2) and our new data focus on the likelihood that the κ3’ enhancer rather than the κ intrinsic enhancer mediates LPS-induced κ activation. One attractive hypothesis stems from the demonstration that LPS treatment activates the cellular kinase CK2, which phosphorylates PU.1, thereby altering PU.1 function as a transcriptional activator (13, 44). Although both LPS (and TSA) have multiple ill-defined effects on cells, the data presented interesting, testable hypotheses on mechanisms regulating κ3’ activation. Whether PU.1 phosphorylation alone could lead to increased PU.1/κ3’ association or increased chromatin accessibility remains to be tested. Preliminary data showing no change in PU.1/κ3’ association in EMSA following in vitro PU.1 phosphorylation by CK2 may or may not be relevant to effects on the endogenous chromatinized locus. Of critical importance will be development of a phospho-PU.1-specific ChIP competent Ab for ascertaining phospho-PU.1/κ3’ association in vivo.

It is important to consider that the data shown, as with the published footprint data (1, 2), are averages of a population of cells and that each κ3’ allele is either cut (or not) in the CHART-PCR endonuclease step and bound (or not) by protein detected in ChIP assays. Hence, our data show the proportion of cells within the population with accessible chromatin and/or protein-associated increases as the cells display maturity characteristics, such as inducible κ, transcription in 38B9 cells. Analysis of a population of cells using a gain-of-signal assay such as ChIP may also explain how our PU.1/IRF-4/κ3’ association data corroborate the in vivo footprinting data of Shaffer et al. (2). In the latter analyses, footprint patterns over the κ3’ enhancer are indistinguishable in naked vs pro-B cell DNA, consistent with meager PU.1/IRF-4/κ3’ association measured by ChIP. Similarly, the substantial changes in footprint pattern detected at the PU.1/IRF composite element in pre- vs pro-B κ3’ enhancer likely reflect the substantial increases in PU.1 and IRF-4 association with the enhancer in pre- vs pro-B cells detected by the more quantitative ChIP method. Hence, our data provide a more mechanistic and perhaps more quantitative explanation of changes previously demonstrated from the standpoint of DNA protection/hypersensitivity to dimethyl sulfate modification.

The Western blot data for IRF-4 (Fig. 6), although in agreement with relatively invariable IRF-4 mRNA levels before terminal B cell differentiation (45), do not completely agree with IRF-4 protein analysis in a separate developmental series of B cells, wherein IRF-4 levels increase at the pre-B to immature stage of B cell development (46). Our demonstration that IRF-4 levels can vary dramatically between representatives of the same B cell stage (i.e., RAG-null 63-12 and AH-7 pro-B cells) suggests that additional analysis on primary cells will be needed to determine how IRF-4 levels change during B cell development. Regardless of the results of this analysis, it is difficult to argue that IRF-4 - protein levels dictate IRF-4/κ3’ association when multiple pieces of evidence, including our ChIP data, are more consistent with this association being determined by PU.1-dependent IRF-4 recruitment to κ3’ DNA (13, 45). In fact, IRF-4/DNA association in the absence of PU.1 allows IRF-4 to suppress, rather than activate, gene expression in some cases (46). Hence, our interpretation that IRF-4 levels do not dictate increased IRF-4/κ3’ association demonstrated in pre-B vs pro-B cells follows mechanistic analyses from independent investigations.

PU.1 and IRF-4 association can, in the simplest interpretation, serve as markers for highlighting changes in chromatin structure that precede Igκ recombination. However, the likelihood that these κ3’ activators play more important roles in establishing a functional κ locus is significant based on the rich literature demonstrating the role PU.1 and IRF-4 play in activating the κ3’ enhancer in a relatively accessible (i.e., transfected plasmid) context. Although these proteins do not appear to dictate κ chromatin accessibility, they are clearly able to take advantage of access mediated by other (perhaps Pax-5 dominated) mechanisms. Hence, PU.1 and IRF-4 potentially play important roles in the demonstrated progressive changes in κ at the pro-B cell stage and beyond.

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References


