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Chromatin opening is tightly linked to enhancer activation at the κ light chain locus

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Abstract

Enhancers play an important role in chromatin opening but the temporal relationship between enhancer activation and the generation of an accessible chromatin structure is poorly defined. Recombination enhancers are essential for chromatin opening and subsequent V(D)J recombination at immunoglobulin loci. In mice, the kappa light chain locus displays an open chromatin structure before the lambda locus yet the same proteins, PU.1/PIP, trigger full enhancer activation of both loci. Using primary B cells isolated from distinct developmental stages and an improved method to quantitatively determine hypersensitive site formation, we find the kappa and lambda recombination enhancers become fully hypersensitive soon after transition to large and small pre-B-II cells, respectively. This correlates strictly with the stages at which these loci are activated. Since these cells are short-lived, these data imply that there is a close temporal relationship between full enhancer hypersensitive site formation and locus chromatin opening.

Keywords: Enhancer; Chromatin; V(D)J recombination; Hypersensitive site; Immunoglobulin

Locus control regions (LCRs) and enhancers play a key role in chromatin opening but the precise mechanism by which they trigger an accessible chromatin structure many kilobases away is poorly understood. A number of models have been proposed including looping, where the enhancer physically touches other regulatory elements within the locus, and facilitated tracking, where the signal from the enhancer tracks directionally along the chromosome [1]. Chromatin conformation capture studies have provided strong support for the looping mechanism at the mouse β-globin [2], T(H)2 cytokine [3] and α-globin loci [4]. In the latter case, full formation of the upstream hypersensitive sites results in delivery of the preinitiation complex to the globin gene promoters. However, an open question is whether formation of the full enhancer hypersensitive site immediately triggers locus chromatin opening or whether the interaction between the enhancer and other regulatory elements in the locus is rate limiting.

To begin to address this, we used the immunoglobulin light chain loci as a model. Two light chain loci exist: kappa and lambda, that rearrange primarily in small pre-B-II cells [5]. In mice, the kappa locus rearranges ten-fold more frequently than lambda and is activated at an earlier developmental stage: Sterile transcription, which is elevated when loci are activated for V(D)J recombination, is upregulated at the kappa locus in large pre-B-II cells whereas at the lambda locus it is not increased until later in small pre-B-II cells [6]. Two enhancers of recombination have been characterised at the kappa locus: The intronic and 3′ enhancer; removal of either enhancer individually reduces recombination [7,8] whereas loss of both eliminates recombination [9]. At the lambda locus, the highly homologous $E\lambda_{2\text{-}4}$ and $E\lambda_{3\text{-}1}$ enhancers lie 3′ of each half of the duplicated locus (Fig. 2A) and activate expression of the rearranged lambda gene [10]. Using BAC constructs carrying the 3′ half of the locus in transgenic mice, we showed that $E\lambda_{3\text{-}1}$ is necessary and sufficient to activate recombination [11].

During development, hypersensitive sites associated with LCRs and enhancers become activated progressively.

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For example, the murine β-globin LCR is partially hypersensitive in multi-lineage progenitor cells; this is increased as cells commit to the erythroid lineage and lost if cells differentiate to non-erythroid types [12]. The kappa recombination enhancers are also partially hypersensitive in pro-B cells and become fully hypersensitive after the pro-B/pre-B transition. Complete hypersensitivity of the kappa 3′ enhancer (kappa 3′E) is correlated with increased binding of the PU.1/PIP complex [13–16]; importantly, this binding is also thought to trigger high level kappa locus recombination [17]. The temporal activation of the lambda recombination enhancer has not been determined but notably, the only lineage specific binding site at the lambda recombination enhancer is for the PU.1/PIP complex [18,19]. PU.1/PIP levels rise at the pro-B/pre-B transition [20] and it seems possible that this could trigger high level recombination of both light chain loci.

We show here that the PU.1/PIP complex binds the kappa and lambda enhancers equivalently and thus, in principle, could trigger full hypersensitive site formation at both enhancers simultaneously. Since the kappa locus is activated earlier than lambda, this provides an ideal system to investigate the temporal relationship between chromatin opening and hypersensitive site formation. We find full formation of the hypersensitive site at kappa 3′E occurs shortly after the transition to large pre-B-II cells whereas that at $E\lambda_{3\text{-}1}$ does not occur until later in small pre-B-II cells. This therefore correlates strictly with the stage of development at which full locus activation is observed. Since pre-B-II cells are short-lived, this further suggests a tight temporal link between hypersensitive site formation and locus activation.

Materials and methods

Purification of pro- and pre-B cells. Bone marrow was flushed from femurs of 5- to 6-week-old mice into pro-B cell medium (McCoys with 15% foetal calf serum, 50 U/ml penicillin and 50 μg/ml streptomycin, essential and non-essential amino acids, 1 mM sodium pyruvate, vitamins, 50 μM β-mercaptoethanol and 5% IL-7-conditioned medium) and expanded for 1 week at 33 °C. Cells were washed in PBS and red blood cells lysed in 0.168 M ammonium chloride at room temperature for 10 min; white cells were then washed in PBS prior to staining with 5 μl Fluorescein isothiocyanate (FITC) labelled anti-CD19 antibody and 5 μl R-phycoerythrin (PE) labelled anti-CD43 antibody (Pharmingen) in 1 ml PBS/2%FCS/25 mM Hepes/1 mM EDTA for 7.5 min at room temperature. Double positive cells were purified by flow cytometry, centrifuged at 400*g* for 10 min, resuspended in 2 ml pro-B cell medium and incubated at 33 °C for 2 h prior to hypersensitive site analysis. Hardy fraction C cells were purified in the same way except staining was with FITC-BP1. Pre-B cells were prepared directly from bone marrow; staining was as above to isolate CD19 single positive cells. Large pre-B-II cells were purified by staining additionally with allophycocyanin (APC)-IgM; $CD19^{+}/CD43^{-}/sIgM^{-}$ cells with a high forward scatter were isolated.

Quantitative hypersensitive site analysis. Primary cells were centrifuged at 515*g* for 3 min and washed in PBS. Nuclei were prepared as described [21] except buffer A contained 40 mM KCl and centrifugation was at 800*g* for 3 min. Nuclei were resuspended in a 1× concentration of the relevant restriction enzyme buffer; incubation with or without 50 U of enzyme per 1×10^6 cells, was at 37 °C in a final volume of 250 μl for the times shown. Digestion was stopped by addition of EDTA to a final concentration of 10 mM.

DNA concentrations were measured using PicoGreen (Invitrogen) according to the manufacturer's instructions. The amount of digested and non-digested DNA for each sample was normalised and this was verified via real time PCR using Jκ5 RSS primers (that do not span a Sac I site). Real time PCR was quantified via incorporation of SYBR Green dye. Reactions were performed in triplicate using 2 ng of sample and the amount of product determined from a standard curve that used 0–6 ng of mouse genomic DNA. Accessibility of the hypersensitive sites was calculated from the equation: $X - Y/X$, where X and Y are the amounts of non-digested DNA and digested DNA, respectively.

To verify that nuclei remained intact during the digestion, control primers that span a Sac I site in a non-hypersensitive region were used. PCR primers are given in Supplementary Table 1. Southern blotting was performed as described in [22]. Hybridisation was at 65 °C using purified PCR products as probes.

Electromobility shift assay. Nuclear extracts from the 103/BCL-2 pro-B cell line were prepared according to [23]. An oligonucleotide with a consensus PU.1/PIP binding site 5′-GTAACCAAGATGAGTTCACTTC CG-3' was end-labeled with [^{32}P] γ-ATP (3000 Ci/mmol) and T4 polynucleotide kinase. Ten fmol were incubated in binding buffer (5% ficoll, 50 mM KCl, 1 mM EDTA, 10 mM Hepes, pH 7.9) with 1 μg herring sperm DNA and 2–4 μg of extract. Double stranded competitor oligonucleotides were added in two-fold increments to give a 125- to 2000-fold excess over the labeled probe. Reactions were incubated on ice for 30 min. Samples were electrophoresed on 5% native polyacrylamide gels in 0.25× TBE at 180 V for 80 min. The oligonucleotides that span the PU.1/PIP sites in kappa 3′E and $E\lambda_{3\text{-}1}$ are: kappa 5′-CCCTTTGAGGAACTGAA AACAGA-3′ and lambda 5′-AAATAAAAGGAAGTGAAACCAAG-3′.

Results

The binding of the PU.1/PIP complex has been correlated with activation of kappa 3′E at the pro-B/pre-B transition and is the only B-cell-specific complex that binds $E\lambda_{3\text{-}1}$ [18,19]. Therefore, to determine if the earlier activation of the kappa locus could be explained by the preferential binding of the PU.1/PIP complex, we examined its affinity for the kappa and lambda recombination enhancers. Using extracts from 293T cells where PU.1 had been over-expressed, we find PU.1 binds both sequences with very similar affinity (Fig. 1A). Likewise, when oligonucleotides spanning the PU.1/PIP sites from the kappa and lambda enhancers are used as competitors, we find the PU.1/PIP complex binds the kappa and lambda enhancers equivalently (Fig. 1B).

Since the lambda locus is activated later than kappa but PU.1/PIP binds both enhancers similarly, this raised the possibility that enhancer activation and chromatin opening at the lambda locus might be uncoupled. We therefore investigated the hypersensitivity of the recombination enhancers at sequential stages of B cell development and chose restriction enzymes as a probe. Restriction enzymes have the advantage that they only cut their recognition site if it is accessible and, unlike DNase I, do not continue to cut chromatin non-specifically. Importantly, when combined with real-time PCR, this highly sensitive assay can accurately quantify chromatin accessibility using relatively few cells such as primary cells from early developmental stages [14].

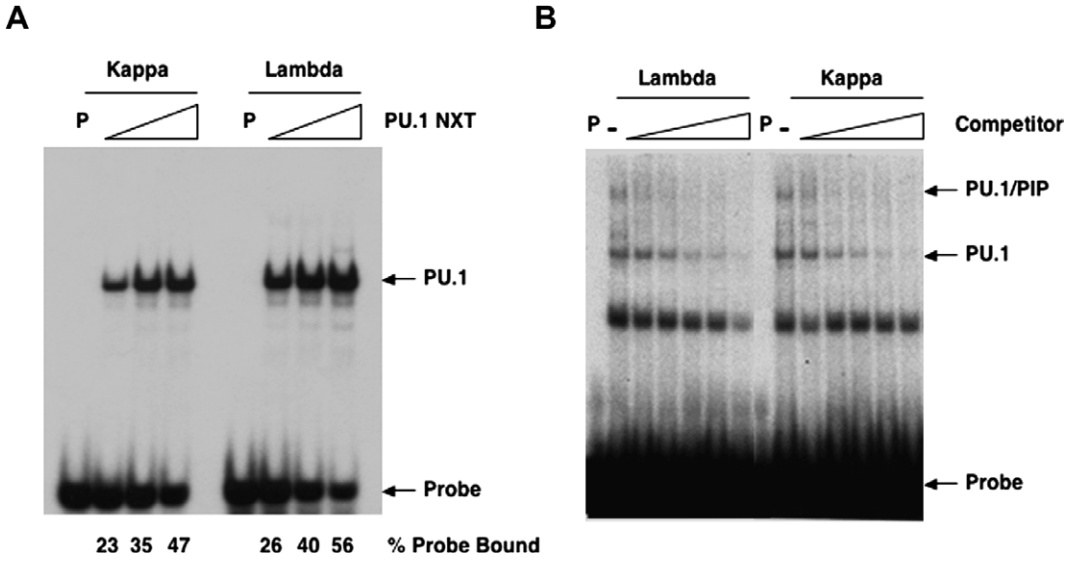

Fig. 1. PU.1/PIP binds kappa 3′E and Eλ$_{3\text{-}1}$ with similar affinity. (A) Increasing amounts of nuclear extract from 293T cells, transfected with a PU.1 expression vector (PU.1 NXT), were bound to oligonucleotide probes from Eλ$_{3\text{-}1}$ and kappa 3'E. P indicates probe alone. (B) Nuclear extract from the pro-B cell line 103/BCL-2 was bound to a consensus PU.1/PIP binding site. Cold competitor oligonucleotides spanning the kappa 3′E (Kappa) or Eλ$_{3\text{-}1}$ (Lambda) PU.1/PIP site were added in twofold increments from a 125- to 2000-fold excess. Control experiments confirmed that the bands indicated are bound by PU.1 and PU.1/PIP (Supplementary Figure 1 and data not shown).

The positions of the hypersensitive sites in the lambda locus are shown in Fig. 2A. A Sac I site lies 9 bp upstream of the 5′ E box within Eλ$_{3\text{-}1}$. The amount of Sac I required to achieve maximal digestion of the Eλ$_{3\text{-}1}$ hypersensitive site was determined by titration of the enzyme (Fig. 2B). Accessibility of Eλ$_{3\text{-}1}$ was then analysed in primary pro-B and pre-B cells: Nuclei were incubated in digestion buffer with or without Sac I for 1 h at 37 °C. To ensure that the same amount of digested and non-digested DNA was compared, the DNA concentration was measured using Pico-Green (Invitrogen) and we verified that the amounts of DNA were properly normalised by quantitative PCR of a control region that does not span a Sac I site. Next, we measured the amount of Sac I cutting at Eλ$_{3\text{-}1}$. However, using the standard protocol to determine hypersensitivity, the level of cutting was variable and no significant difference was observed between pro-B and pre-B cells (Fig. 2C).

Primary pro-B cells are prone to apoptosis and it seemed possible that endogenous nucleases might contribute to cutting when nuclei are incubated for 1 h at 37 °C in digestion buffer containing magnesium. To test this, nuclei were incubated in restriction buffer alone; an apparent cutting at the hypersensitive site of 33% was observed using two independent primer sets to different regions of the hypersensitive site (Fig. 2D and E). In contrast to the long digestion times typically used to probe accessibility with restriction enzymes, DNase I digestions are usually for 2–10 min; over this short time-scale, cutting by endogenous nucleases is minimised. Therefore, to develop a more accurate protocol to determine hypersensitivity with restriction enzymes, we asked if digestion conditions akin to those for DNase I could be used: A time course of Sac I digestion was performed and maximal digestion is reached within 20 min (Fig. 2F). Next, to accurately determine the level of hypersensitivity at Eλ$_{3\text{-}1}$, we repeated the experiment in Fig. 2C but digested nuclei for only 20 min. Now, there is a significant difference in hypersensitivity between pro- and pre-B cells (44% and 65%, respectively) as determined by both quantitative PCR (Fig. 3A) and semi-quantitative PCR followed by Southern blotting (Fig. 3B).

Using the improved protocol, we next assayed the level of accessibility at the kappa 3′ enhancer in primary pro- and pre-B cells. A NcoI site in kappa 3′E lies 10 bp upstream of a putative E box binding site (Fig. 3C) and thus is located similarly to the SacI site at Eλ$_{3\text{-}1}$. We find that accessibility increases from 48% in pro-B cells to 69% in pre-B cells (Fig. 3D); this is similar to the increased accessibility at Eλ$_{3\text{-}1}$ and to the level of accessibility of kappa 3′E reported previously in cell lines [14].

Although these observations suggest that the kappa and lambda recombination enhancers become hypersensitive to the same extent and at the same stages of development, to better determine when the hypersensitive sites become fully open, we next examined hypersensitive site formation in cells undergoing the pro-B/pre-B transition. Firstly, Hardy Fraction C cells were purified by flow cytometry ($CD43^+$/$BP1^+$); these cells are a mixture of pro-B and large pre-B-I and are immediate precursors to large pre-B-II cells. As can be seen in Fig. 4A, there is no difference in the level of accessibility of kappa and lambda recombination enhancers which remains very similar to that in the total pro-B cell population. Next, we purified large pre-B-II cells ($CD43^-$/$CD19^+$/$sIgM^-$; Hardy Fraction C′ and D). Now, hypersensitivity at kappa 3′E is increased and importantly, this is as accessible as the full hypersensitive site in the total pre-B cell population (Fig. 4B and C). In contrast, no

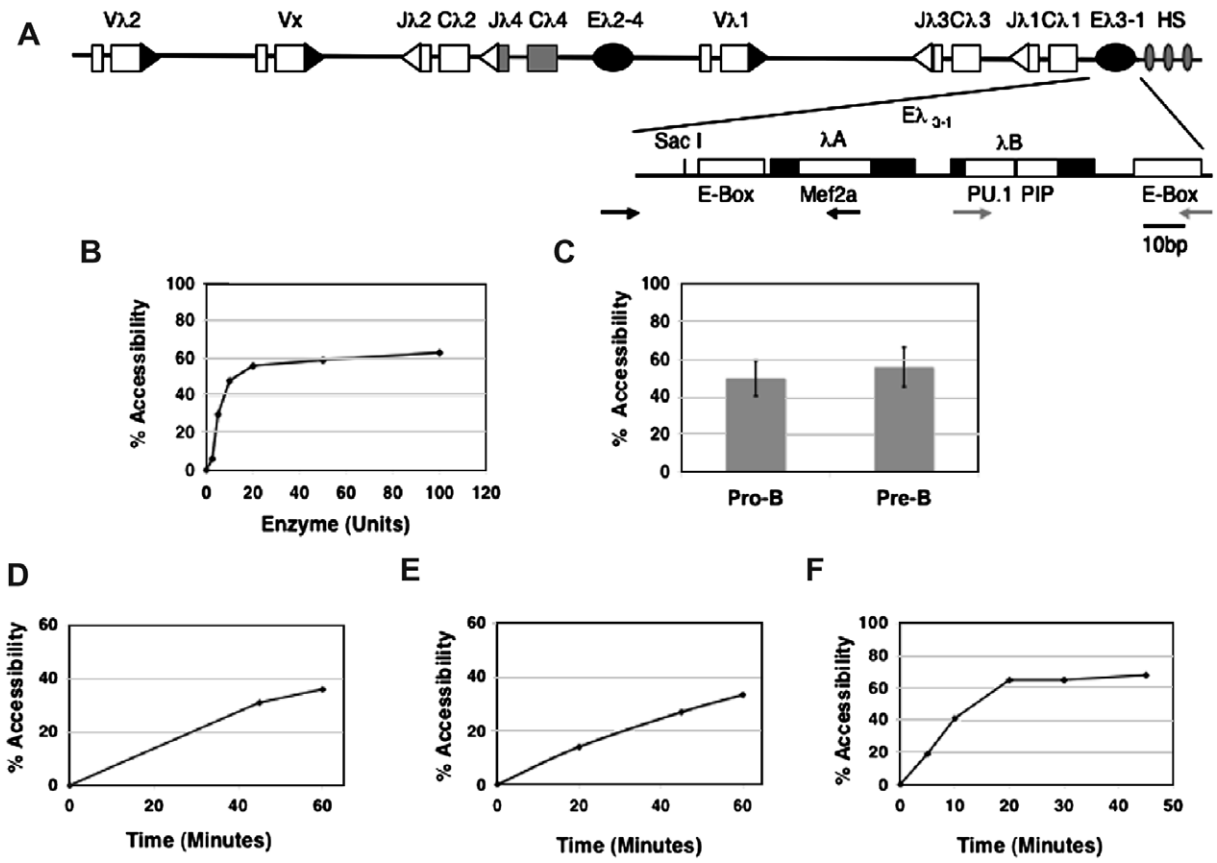

Fig. 2. Accessibility at $E\lambda_{3\text{-}1}$. (A) Location of the hypersensitive sites in the lambda locus (upper) and fine map of the transcription factor binding sites in $E\lambda_{3\text{-}1}$ (lower). Filled ovals show enhancers and triangles indicate recombination signal sequences. Arrows show the position of PCR primers. (B) Titration of SacI in primary pro-B nuclei. Nuclei were incubated with the amounts of enzyme indicated for 1 h at 37 °C. The level of digestion at $E\lambda_{3\text{-}1}$ was determined by quantitative PCR. In this experiment and 2D-F, PIP was over-expressed, resulting in greater hypersensitivity of $E\lambda_{3\text{-}1}$ than in other pro-B cells. (C) Accessibility of $E\lambda_{3\text{-}1}$ in pro- and pre-B nuclei. Primary pro-B and pre-B cells were purified by flow cytometry and nuclei digested with SacI. Error bars show standard deviation. (D and E) Incubation of pro-B cell nuclei in digestion buffer alone induces cutting by endogenous nucleases. The apparent amount of cutting at $E\lambda_{3\text{-}1}$ was determined by comparison to DNA from nuclei that had not been incubated. Primers that span the SacI site and control primers that do not (shown in grey in 2A) were used. The latter also assayed accessibility at 20 min. (F) Time course of digestion. Pro-B cell nuclei were digested with SacI for the times indicated; accessibility at $E\lambda_{3\text{-}1}$ was analysed via real time PCR.

change in hypersensitivity is observed at the lambda recombination enhancer that remains at the level in pro-B cells. Together, these findings suggest that full formation of the enhancer hypersensitive site correlates tightly with the stage of development at which these loci are activated.

Discussion

During development, enhancer and LCR hypersensitive sites are formed progressively, firstly generating a primed state that is ultimately converted to the full hypersensitive site. Here, we have examined whether formation of the full hypersensitive site automatically triggers locus activation. By examining purified B cells at sequential stages of development, we provide the first evidence that full formation of the kappa 3′ enhancer hypersensitive site occurs rapidly upon transition to large pre-B-II cells. Likewise, the lambda recombination enhancer becomes fully hypersensitive in small pre-B-II cells. Since this correlates strictly with the stage in which the respective loci become activated and since these pre-B-II cells are short-lived, these data indicate a tight temporal coupling between full hypersensitive site formation and locus chromatin opening.

Our analyses used an improved protocol to assay chromatin accessibility via restriction endonucleases. Previous studies typically examined accessibility in cell lines and digested nuclei for 1 h. Using primary pro- and pre-B cells that are prone to apoptosis, we find digestion due to endogenous nucleases is considerable. The regions that are most affected by this cutting, due to their high accessibility, are hypersensitive sites. Consequently, by lowering the digestion times and using high amounts of restriction enzyme, endogenous cutting is minimised (Fig. 2E) and a more accurate measurement of hypersensitivity is obtained.

Notably, we demonstrate that the kappa 3′ enhancer is as hypersensitive in large pre-B-II cells as in the total pre-B population. This suggests that the hypersensitive site is fully formed in nearly all cells in the population and thus is likely to have been formed soon after the transition to large pre-B-II cells. Not only this, but the level of kappa sterile transcription in large pre-B-II cells is higher than in small pre-B-II cells [6]; this suggests that the locus

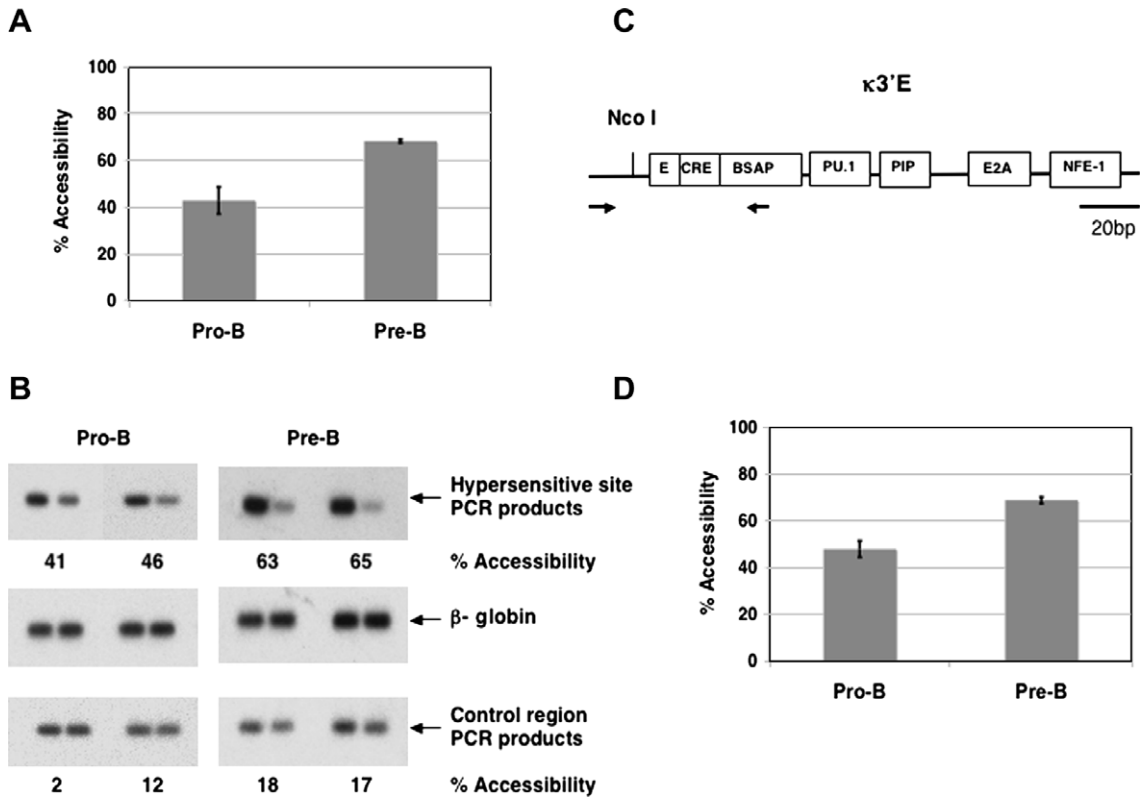

Fig. 3. Accessibility of E$\lambda_{3\text{-}1}$ and kappa 3′E in primary pro- and pre-B cells. Nuclei were digested for 20 min at 37 °C. The level of hypersensitivity of E$\lambda_{3\text{-}1}$ was determined (A) by real time PCR and (B) by semi-quantitative PCR followed by Southern blotting. Duplicate experiments are shown in each panel. The lowest panel shows cutting of SacI at a control (non-hypersensitive) region of the genome. (C) Diagram of kappa 3′E, showing transcription factor binding sites and NcoI site. Arrows show the position of PCR primers. A time course showed maximum NcoI cutting is reached by 20 min (data not shown). (D) Accessibility of kappa 3′E. Primary pro- and pre-B cells were purified by flow cytometry and their nuclei incubated with NcoI for 20 min. Accessibility was quantified by real time PCR. Error bars show standard deviation.

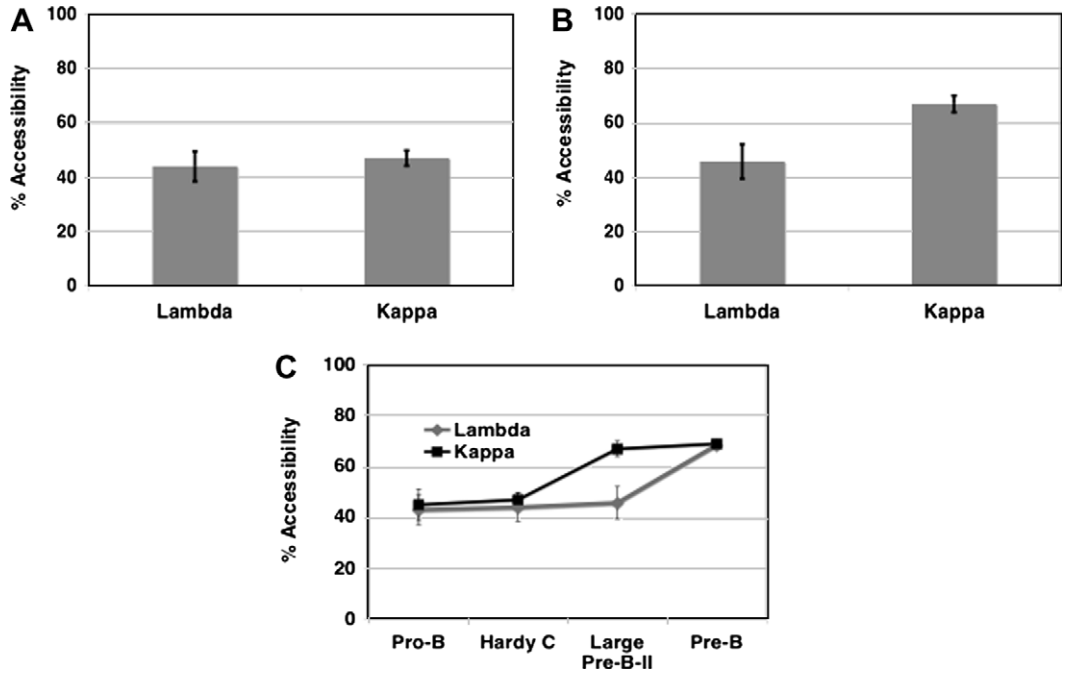

Fig. 4. Accessibility of E$\lambda_{3\text{-}1}$ and kappa 3′E at the pro-B/pre-B transition. (A) Hardy fraction C cells ($CD43^+/BP1^+$) were purified by flow cytometry and accessibility at kappa 3′E and E$\lambda_{3\text{-}1}$ quantified by real time PCR. (B) Accessibility was determined in purified large pre-B-II nuclei ($CD43^-/CD19^+/sIgM^-$) by real time PCR. (C) Summary of the accessibility of E$\lambda_{3\text{-}1}$ (Lambda) and Kappa 3′E (kappa) during B cell development.

becomes fully open within a very short time of enhancer hypersensitive site formation. Our finding that the kappa recombination enhancer becomes hypersensitive earlier than that at lambda also helps to explain the observation that the kappa locus recombines 10-fold more frequently than lambda [6]. However, more transcription factors bind to kappa 3′E than $E\lambda_{3\text{-}1}$ [15,18,19] and it seems possible that increased enhancer interaction with regulatory elements in the kappa locus might also help to trigger more frequent kappa recombination.

Although PU.1/PIP binds to the kappa and lambda recombination enhancers equivalently, and this complex triggers full hypersensitive site formation, activation of the kappa and lambda enhancers is temporally distinct. This suggests that events beyond the simple affinity of an activator for its binding site regulate hypersensitive site formation. The most obvious possibility is that other factors either prevent PU.1/PIP binding (at $E\lambda_{3\text{-}1}$) or facilitate binding (at kappa 3′E). Direct analysis of this by chromatin immunoprecipitation in large pre-B-II cells, however, is not feasible due to the low numbers of these cells obtainable (1×10^5/mouse). Preferential kappa hypersensitivity might also be explained by the relocation of the kappa locus from the heterochromatic nuclear periphery to the centre in pro-B cells [24]; altered localisation of the lambda locus has not been reported but if it occurs later, the ability of PU.1/PIP to interact with $E\lambda_{3\text{-}1}$ could be reduced.

In conclusion, by taking advantage of the ability to isolate short-lived populations of B cells at distinct stages of development, we find a strict correlation between hypersensitive site formation and locus activation. Future studies will define more precisely the time frames involved.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.08.171.

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