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Association between the *Igk* and *Igh* loci mediated by the 3' *Igk* enhancer induces decontraction of the *Igh* locus in pre-B cells

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Abstract

V(D)J recombination at *Igh* and *Igk* loci takes place sequentially during successive stages in B cell development. Using 3-dimensional DNA fluorescence *in situ* hybridization (FISH), we identified a lineage- and stage-specific interchromosomal association between these two loci which marks the transition between *Igh* and *Igk* recombination. Co-localization occurred between pericentromerically located alleles in pre-B cells, and was mediated by the 3' *Igk* enhancer. Deletion of this regulatory element prevented association of the *Igh* and *Igk* loci, inhibited pericentromeric recruitment and locus decontraction of an *Igh* allele, and resulted in increased distal *V_H* gene rearrangement. Our data indicate a role for the *Igk* locus and its 3' enhancer in directing the *Igh* locus to a repressive nuclear subcompartment, and in rendering the *Igh* locus unable to contract.

Introduction

B and T cells express lineage-specific antigen receptors that mediate humoral and cellular immunity, respectively. Immunity depends upon the regulated recombination of variable (V), diversity (D), and joining (J) gene-segments at B cell receptor (BCR) and T cell receptor (TCR) loci to generate sufficient receptor diversity to enable recognition of a near-limitless array of potential antigens. Each antigen receptor consists of heavy and light chains, each of which are encoded by distinct loci. As common factors are required for V(D)J recombination at all immune receptor loci, developmentally regulated changes in locus accessibility are crucial for regulating this process ¹.

Regulation of accessibility is exerted at a number of levels to ensure lineage specificity and sequential rearrangement of gene-segments at immunoglobulin heavy chain (*Igh*) and light chain (*Igk* and *Igl*) loci ². Tight regulation is also essential for protecting the genome from these rearrangement events, which require generation of inherently unstable and potentially

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dangerous double stranded DNA breaks. Allelic exclusion, the process through which successful production of one Ig chain suppresses further rearrangement at the other allele of the same Ig locus, forms an integral part of this regulation and ensures clonality and monospecific recognition by the B cell receptor on individual lymphocytes—a basic tenet of the acquired immune response.

Recombination starts at the pro-B cell stage with D-J rearrangement of both *Igh* alleles. Synapse formation of gene segments separated by a large distance is facilitated by looping which results in locus contraction in cells undergoing rearrangement^{3,4}. Functional V-D-J rearrangement at one *Igh* allele leads to expression of immunoglobulin μ -chain as part of the pre-B cell receptor (pre-BCR). Signaling through this receptor enforces cessation of further *Igh* rearrangement, and triggers a burst of proliferation of large pre-B cells which subsequently differentiate into small pre-B cells in which *Igk* rearrangement takes place⁵. Locus contraction mediated by looping occurs prior to the onset of *Igk* germline transcription⁶. *Igk* rearrangement occurs after the onset of transcription of the unrearranged cluster of J gene-segments and depends upon well-characterized enhancers located in the J-C intron (MiEκ) and 3' of the constant region exon (3'Eκ). Deletion of the two enhancers, individually or simultaneously, diminishes or abrogates V-J *Igk* rearrangement, respectively⁷⁻⁹.

Allelic exclusion at the *Igh* locus, established at the pre-B cell stage of development by changes in chromatin accessibility¹⁰, is thought to be crucial for preventing ongoing rearrangement of the second partially assembled (DJ-rearranged) *Igh* allele when the V(D)J recombinase is re-expressed for the purpose of *Igk* rearrangement. Accumulating evidence supports a “feedback inhibition” model for establishing allelic exclusion of the *Igh* locus but the detailed molecular basis of this model has yet to be defined². However, we know that pericentromeric recruitment plays a role in establishing and maintaining allelic exclusion of all *Ig* loci^{4,11,12}. Following successful recombination of one *Igh* allele, repositioning of the second allele to pericentromeric heterochromatin, a repressive compartment of the nucleus, reduces *Igh* accessibility to the recombinase during *Igk* rearrangement⁴. In contrast, repositioning of the *Igk* allele to pericentromeric clusters occurs at the pre-B cell stage, prior to the onset of *Igk* rearrangement, and may limit recombinase accessibility to a single (euchromatic) allele¹². In addition, decontraction of the *Igh* locus occurs at the same developmental stage. This process contributes to allelic exclusion by physically separating distal and middle V_H gene segments from the proximal D-J domain of the locus, thereby preventing further synapse formation and ongoing rearrangement between these regions⁴.

Recruitment of the not-yet-rearranged *Igk* allele and the partially rearranged *Igh* allele to pericentromeric heterochromatin, and decontraction of the partially rearranged *Igh* allele occur at the same developmental stage, suggesting the existence of a coordinated event. This prompted us to examine the locations of these two loci relative to each other and to further investigate the factors required for changes in conformation that occur at the *Igh* locus during B cell development.

Results

Interchromosomal association between *Ig* loci

To examine the positions of the *Igh* and *Igk* loci, we performed two-color 3-dimensional DNA fluorescence in situ hybridization (FISH) using DNA probes that were generated from two bacterial artificial chromosomes (BACs)--CT7-526A21 and RP23-101G13--which map to the 5' end of the *Igh* locus on chromosome 12 and the 5' end of the *Igk* locus on chromosome 6, respectively. In each cell, the two alleles of both loci were either well separated, or one *Igh* and one *Igk* allele were found in close spatial proximity. Measurements of the distance separating the two loci were grouped into one of the following four categories: <0.5μm apart,

0.5–1µm apart, 1–1.5µm apart and >1.5µm apart (Fig. 1a). Our measurements relate to the distance separating the pair of co-localized alleles in each cell. Where no close association between *Igh* and *Igk* alleles was observed in an individual cell (>1.5µm apart), this was scored as separation of a single pair of alleles. We also used probes mapping to the *Igh* and *Igk* constant regions to rule out the possibility that deletion of distal V_H gene regions affected observations of the frequency of association of the two loci (Supplementary Figure 1, online). Using different subsets of B cells sorted from mouse bone marrow, we examined the positions of the two loci relative to pericentromeric heterochromatin as described previously⁴. Interchromosomal association occurred at low frequency in pro-B cells, but increased and peaked during the pre-B cell stage--as evidenced by the substantial increase in loci separated by <0.5µm--and declined during the late immature B cell stage (Fig. 1b, Supp. Table 1, online). Co-localization was lineage specific as we observed no significant *Igh-Igk* pairing in CD4⁺CD8⁺ thymocytes (Fig. 1b). In addition, the *Igh-Igk* association coincided temporally with pericentromeric recruitment of *Igh* and *Igk* (Fig. 1c, Supp Tables 2a, b, online).

Association on pericentromerically-recruited alleles

Co-localization between *Igh* and *Igk* occurred on one pair of alleles within each cell at a stage in development during which one allele from each locus is often repositioned at pericentromeric heterochromatin. This led us ask which alleles participated in the interchromosomal association. To address this question we carried out three-color 3-dimensional DNA FISH with *Igh* and *Igk* probes, in addition to a γ -satellite probe mapping to pericentromeric heterochromatin, in sorted pre-B cells. The most closely associated *Igh* and *Igk* alleles were positioned in heterochromatic regions (Fig. 2, Supp. Table 3, online). Where paired recruited alleles were separated by >1µm, the *Igk* allele frequently co-localized with pericentromeric heterochromatin and was scored as recruited to heterochromatic regions, whereas the *Igh* allele was positioned at a distance from the pericentromeric cluster and was not scored as recruited to heterochromatic regions (Fig. 2a, Supp. Table 3, online). This suggests that the two Ig loci do not simultaneously go to pericentromeric clusters, but that instead recruitment of *Igk* might precede that of *Igh*. To analyze this hypothesis in greater detail we compared the distance between the two loci with the orientation of *Igh* and *Igk* alleles in pericentromeric regions. When the two loci were separated by less than 1µm, either *Igh* (blue bars) or *Igk* (red bars) was positioned closer to pericentromeric heterochromatin but more often than not they were both equally close to the same pericentromeric cluster (green bars) or equally close to a different pericentromeric cluster (white bars) (Fig. 2b, c, Supp. Table 4, online). Where Ig loci were separated by >1.5µm all cells had either one *Igk* allele alone at pericentromeric heterochromatin (red bars) or both *Igh* and *Igk* at different pericentromeric clusters (white bars). *Igh* was positioned at heterochromatic regions only when it associated with *Igk* at the same pericentromeric cluster (blue and white bars) or when both Ig loci were recruited to different pericentromeric clusters (white bars). *Igh* was never seen at heterochromatic regions in pre-B cells in the absence of *Igk* recruitment. These observations suggest that pericentromeric recruitment of *Igk* was the initial event. The frequency of association declined at the immature B cell stage of development, when *Igh* and *Igk* were predominantly positioned at different pericentromeric clusters (Fig. 1b, c). These findings suggest that association takes place between the two loci at a shared pericentromeric cluster following recruitment of one *Igk* allele, and that the two loci subsequently move apart to reposition at different pericentromeric clusters. Thus pericentromeric recruitment of *Igk* may promote pericentromeric recruitment of *Igh*, perhaps via a transient association between the two loci. Thus, although Ig alleles may be constrained to a repressive subcompartment of the nucleus they do not necessarily remain static and can move from one pericentromeric cluster to another.

Sequential recruitment to pericentromeric regions

To further test the idea that the pericentromeric *Igk* allele is involved in recruitment of an *Igh* allele to pericentromeric heterochromatin, we examined nuclear positioning in mice with targeted deletions of MiEk or 3'Ek. Previous analyses of pre-B cells from these mice demonstrated a significant reduction in V_κ and J_κ germline transcription and in V_κ-J_κ recombination^{7,9}. 3-dimensional DNA FISH analysis of sorted developing B cell subsets from MiEk-KO mice revealed that pericentromeric recruitment of *Igk* occurred prematurely at the pro-B cell stage of development and, at the subsequent pre-B cell stage, nearly half the cells exhibited an atypical appearance with both *Igk* alleles located at pericentromeric heterochromatin (Fig. 3a). These results support previous data showing that the absence of the MiEk leads to silencing of *Igk* transcription⁹. Interestingly we noted no defect in *Igk* locus contraction in MiEk-KO pre-B cells (Supp. Table 5, online). As a test of our model, we determined whether increased pericentromeric recruitment of *Igk* alleles in these mutants led to an increase in co-localization with *Igh* and an increased frequency of *Igh* recruitment to heterochromatin. In pre-B cells from MiEk-KO mice, close association between the *Igh* and *Igk* loci (<0.5μm) increased slightly (Fig. 3b, Supp. Table 1, online) and this change correlated with the unusual appearance of a percentage of pre-B cells in which both *Igh* alleles were repositioned at pericentromeric heterochromatin (Fig. 3a, Supp. Table 2, online). This data further suggests that recruitment of *Igk* influences recruitment of *Igh* to pericentromeric heterochromatin. To verify this conclusion, we examined the cells in which both *Igh* alleles were repositioned at pericentromeric clusters to determine whether biallelic recruitment of *Igh* was facilitated by biallelic recruitment of *Igk*. We used three-color 3-dimensional DNA FISH to examine the position of the two loci in relation to pericentromeric heterochromatin. Analysis of the small number of cells in which both *Igh* alleles are located at pericentromeric heterochromatin revealed that, in 20/24 cells, both *Igh* and *Igk* were biallelically recruited to pericentromeric heterochromatin (Fig. 3c).

As the observed increase in frequency of closely associating (<0.5μm) alleles in MiEk-KO pre-B cells was quite subtle, to definitively assess the affect of *Igk* pericentromeric recruitment on the association between the two loci we separately analyzed the frequency of association between *Igh* and *Igk* loci in the following populations from MiEk-KO pre-B cells: (i) cells in which neither *Igk* allele was recruited; (ii) cells in which a single *Igk* allele was recruited; (iii) cells in which *Igk* was biallelically recruited (Fig. 4a). *Igh-Igk* loci association clearly increased with increased *Igk* recruitment to pericentromeric heterochromatin, and we detected negligible close association in the absence of *Igk* localization in pericentromeric regions (Fig. 4b, Supp. Table 6, online). Together these data indicate that pericentromeric localization of *Igk* promotes *Igk-Igh* association and *Igh* recruitment to pericentromeric heterochromatin

3'Ek is required for *Igh-Igk* close association

Our analysis of allelic association and pericentromeric recruitment of *Igh* in 3'Ek-KO progenitor B cells provided a strikingly different result. *Igk* loci in these cells behaved in a manner similar to the *Igk* loci in MiEk-KO developing B cells--in both cell types *Igk* was prematurely repositioned to pericentromeric clusters at the pro-B cell stage of development, and at this and later stages many cells had both alleles located within this repressive compartment (Fig. 5a). As in MiEk-KO pre-B cells we observed no defect in *Igk* locus contraction in 3'Ek-KO pre-B cells (Supp. Table 5, online). As the absence of either enhancer had a similar effect on *Igk* locus localization, we expected to find that deletion of 3'Ek would increase the frequency of association between *Igh* and *Igk* loci and increase pericentromeric recruitment of the *Igh* locus. However, we observed just the opposite (Fig. 5a–c, Supp. Tables 1, 2, online). The extent of association between the two loci did not increase beyond the pro-B cell stage and the *Igh* locus remained positioned away from pericentromeric clusters at the pre-B cell stage, and was not repositioned to pericentromeric heterochromatin until the early

immature B cell stage. Furthermore, pericentromeric recruitment of *Igh* at the immature B cell stage was not linked with an interchromosomal association, as the two loci were positioned at different pericentromeric clusters in immature B cells. We repeated these experiments using *Igh* and *Igk* constant region probes to confirm that deletion of distal V gene regions did not affect the frequency of association of the two loci (Supp. Fig. 1, online). These results indicated that the 3'Eκ is important for the association between the *Igh* and *Igk* loci and that in the absence of *Igh-Igk* association recruitment of the *Igh* locus to heterochromatin is delayed. This observation suggested that the *Igh* locus in 3'Eκ-KO mice might remain accessible to the V (D)J recombinase during the pre-B cell stage of development.

Igh* decontraction mediated by association with *Igk

Locus contraction, mediated by looping, is required for rearrangement of distal and middle V_H gene segments to DJ_H segments, and subsequent decontraction at the pre-B cell stage of development is likely to contribute to allelic exclusion by preventing ongoing rearrangement of these V_H gene families^{4,13}. Decontraction and pericentromeric recruitment occur at the pre-B cell stage of development and to date observations suggest that these two processes occur simultaneously and may be linked⁴. As pericentromeric recruitment of *Igh* is delayed and occurs in early immature B cells of 3'Eκ-KO mice, independently of *Igk* association, we next asked whether *Igh* locus decontraction was also affected by the absence of the 3'Eκ. To address this question we measured the separation of the two ends of the *Igh* locus in sorted developing B cells from WT, MEκ-KO and 3'Eκ-KO mice. Two-color 3-dimensional DNA FISH was carried out using the *Igh* probes CT7-526A21 and CT7-34H6 which map to the 5' end of the *Igh* locus and the 3' *Igh* constant region, respectively (Fig. 6, Supp. Table 7, online). Separation of the two ends of the *Igh* locus was grouped into the following three categories: 0–0.5μm, 0.5–1μm and 1–1.5μm. Decontraction occurred in developing B cells from MEκ-KO mice, in which *Igh-Igk* association and pericentric repositioning of *Igh* were documented. In contrast, locus decontraction was impaired in 3'Eκ-KO cells, in which *Igh-Igk* association was impaired and no significant pericentric recruitment of *Igh* at the pre-B cell stage of development was observed. Furthermore *Igh* locus decontraction did not occur when *Igh* was repositioned to pericentromeric regions independent of *Igk* association in 3'Eκ-KO immature B cells. These results suggest that *Igh* locus decontraction depends on both repositioning to pericentromeric regions and association with *Igk*.

Enhanced distal V_H segment usage in 3'Eκ-KO cells

In our FISH analyses of WT, 3'Eκ-KO and MEκ-KO developing B cells we used the probe CT7-526A21, which hybridizes to the 3' end of the V_HJ558 genes. Deletion of this BAC signal will occur upon rearrangement of V_H genes located 5' of this region. We observed such deletion in approximately 9% of WT and 3'Eκ-KO pro-B cells and 11% of MEκ-KO pro-B cells. Deletion increased in WT and MEκ-KO pre-B cells to 21% and 17%, respectively, and in 3'Eκ-KO pre-B cells to 31% (Fig. 7a, b and Table 1.) As a control, in addition to the constant region probe, CT7-34H6, we used a probe located outside of the 5' end of the *Igh* locus, CT7-386J17. Signals from these two probes were always present even in the absence of the CT7-526A21 signal (Fig. 7c). These data indicate that distal V_H gene rearrangement was increased in pre-B cells from 3'Eκ-KO mice where both *Igh* alleles remained in a contract conformation and were positioned within euchromatic regions. This type of rearrangement would not be expected if the locus had undergone decontraction⁴.

In order to verify that the differences in rearrangement at the pre-B cell stage are seen in peripheral B cells, we analyzed the rearrangement status of the *Igh* locus in mature resting splenic B cells. PCR analyses and Southern blotting was performed to assess the relative amounts of proximal versus distal V_H-DJ_H rearrangements in 3'Eκ-KO compared to WT and MEκ-KO mice. Distal *Igh* rearrangements were increased in 3'Eκ-KO splenic B cells

compared to WT splenic B cells (Fig. 8a). Quantification of V_HJ558 rearrangements normalized to C_μ, which was used as a PCR control, demonstrated that distal V_H rearrangement to the J_H3 gene segment are increased by 50% (or 1.5-fold) in the absence of 3'Eκ (Fig. 8b). Rearrangement of the proximal V_H7183 family was slightly reduced in 3'Eκ-KO peripheral B cells, in particular those involving the J_H3 gene segment (Fig. 8d). Distal V_HJ558 rearrangements involving the J_H3 gene segment were reduced by 40% (or 0.6-fold) in MiEκ-KO splenic B cells whereas proximal V_H7183 rearrangements were largely unchanged. Rearrangements to the middle V_H gene family V_HGam3.8 demonstrate a 2-fold increase in rearrangement to the furthest J_H3 gene segment in B cells lacking 3'Eκ compared to WT B cells. These data indicate that in the absence of 3'Eκ, ongoing rearrangement at the pre-B cell stage of development changes the distribution of V_H rearrangement in mature B cells so that distal V_H gene rearrangement is increased.

Discussion

Although it is known that regulatory elements within *Igh* and *Igk* play key roles in promoting recombinase accessibility, little is known about how accessibility of partially-assembled DJ-rearranged *Igh* alleles is reduced and how they become refractory to the recombinase during the period of *Igk* rearrangement in pre-B cells. The interchromosomal associations we observe between *Igh* and *Igk* alleles suggest a mechanism for coordinating rearrangement and allelic exclusion at a stage in development when targeting of the recombinase is directed away from one locus and towards the other. Our study demonstrates a previously-unappreciated aspect of immunoglobulin gene regulation and indicates that association of two loci can induce conformational changes which could subsequently alter the function of these loci.

We know from previous studies that dynamic movement of immunoglobulin loci exerts control of recombination at a number of levels. Enrichment of these loci at the nuclear periphery is important for restricting recombination to the B cell lineage³. Relocation to the center of the nucleus occurs at the pro-B cell stage of development and correlates with increased accessibility of the two loci^{3,13,14}.

Conformational changes involving contraction of the two loci occur following relocation. At the *Igh* locus, contraction, mediated by looping, correlates with recombination of distal and middle V_H gene rearrangement and may function to facilitate long range RSS interactions⁴. It is now known that all antigen receptor loci undergo similar conformational changes during recombination¹⁵. However, the mechanisms underlying locus contraction are not understood, although Pax5 and another unknown factor are required at the *Igh* locus¹³. It may be that the underlying mechanism regulating contraction of the *Igk* locus is similar to that of the *Igh* locus and Pax5 could have a common role in this context. Our data from the two *Igk* enhancer knockout mice indicated that, individually, MiEκ and 3'Eκ are not required for locus contraction to occur, as in the absence of either enhancer element, *Igk* loci adopt conformations similar to those seen in WT developing B cells. However, as the two enhancer elements have overlapping roles, we cannot rule out that an absence of both enhancer elements could affect this process.

At the pre-B cell stage of development, pericentromeric recruitment of one allele of *Igh* and *Igk* has been implicated in establishing allelic exclusion¹⁶. The data we report here indicate that recruitment of the *Igk* locus is the initiating event, and influences subsequent recruitment of the *Igh* locus. The *Igk* locus exerts its effect on the *Igh* locus through transient association at pericentromeric regions. *Igh-Igk* close association adds a further dimension to the control exerted by nuclear organization in regulating the process of recombination at these two loci. Our data indicate that *Igh* locus decontraction is dependent on association with the *Igk* locus at pericentromeric regions and that pericentromeric repositioning in the absence of *Igk*

association does not induce decontraction. This conclusion is supported by our finding that locus decontraction did not occur in 3'Ek-KO immature B cells, in which delayed pericentromeric repositioning of *Igh* occurred, independent of association with *Igk*. Thus, the association we observe in our FISH analysis may indeed be a physical interaction. It could be that repressive factors bound to the pericentromeric *Igk* allele are shared with the associated *Igh* allele and this induces changes in chromatin that disrupt contraction. Such possibilities add a further layer of complexity to immunoglobulin gene regulation and indicate that association of the two loci brings about an epigenetic change to alter locus conformation.

Our microscopy-based investigations provide us with information on events that occur within individual cells from sorted developmental populations. As we use fixed cells, our analysis can only provide us with an incomplete picture of the dynamic movements of these loci. However, it is clear from our current studies that the immunoglobulin loci are not static. After they have repositioned to pericentromeric regions at the pre-B cell stage of development, they do not remain in the same nuclear location as associated alleles, but move apart to different pericentromeric clusters at the immature B cell stage. The snapshot in time that we analyze is therefore likely to be a low estimate for the frequency with which *Igh-Igk* close association occurs. This could also explain why only 60–70% of pre-B and immature cells have one *Igh* and one *Igk* allele repositioned to pericentromeric regions. In the remaining cells from these populations, where no pericentromeric recruitment is seen, we have observed that one allele is always located at the periphery (J.S., unpublished observations). It may be that alleles shuttle between the periphery and pericentromeric heterochromatin, two environments which are thought to be repressive.

Our analysis of cells lacking 3'Ek or MiEk identified a shared role for these regulatory elements in diminishing pericentromeric repositioning of the *Igk* locus, in line with their overlapping roles in activating transcription and rearrangement^{7,8}. It is likely that binding of transcription factors to the two enhancers at the pre-B cell stage of development prevents association with centromeres, and that a limited supply of activating factors within the cell could result in relocalization of one *Igk* allele to heterochromatic regions. Analysis of developing B cells from 3'Ek-KO mice identified a role for this regulatory element in mediating an interchromosomal *Igh-Igk* association which alters the conformation of the *Igh* locus at the pre-B cell stage of development. The absence of activating factors or the presence of repressive factors at the 3'Ek region on the pericentromeric *Igk* allele may facilitate binding of repressive factors at the *Igh* locus. Sharing of common repressive factors could mediate association of pericentromeric *Igk* with the non-productive or DJ-rearranged *Igh* allele, thereby reducing accessibility and altering the conformation of the latter.

If this model is correct then locus decontraction should only take place on the pericentromeric *Igh* allele, mediated by interaction with 3'Ek. This is difficult to conclude from an analysis of contracted and decontracted alleles in developing B cells, as pericentromeric recruitment of immunoglobulin loci is a transient process, the percentage of alleles found at these regions declines at later stages of development, and no pericentromeric recruitment of immunoglobulin loci is observed in mature resting cells¹¹. As a result, although contracted loci are mostly identified within euchromatic regions, decontracted alleles are also present in these locations.

As we observed pericentromeric repositioning of the *Igh* locus at the immature B cell stage in 3'Ek-KO mice, it is likely that at later stages of development additional mechanisms enforce silencing of the *Igh* locus. An alternative pathway is likely to involve a reduction in interleukin 7 (IL-7), and subsequent deacetylation, a modification known to reduce accessibility at the *Igh* locus¹⁷. Early repression of *Igh* by *Igk* at the pre-B cell stage of development is important for inducing locus decontraction. IL-7R signaling may act as an important back-up mechanism

to reduce accessibility of the *Igh* locus preventing ongoing rearrangement of distal V_H segments on the second DJ_H-rearranged *Igh* allele during *Igk* rearrangement.

Both enhancers play overlapping roles and individually have been shown to be quantitatively important, but not essential, for *Igk* rearrangement. Prior to our study, the only clear functional difference identified between the two enhancers was a role for MiEk in regulating monoallelic demethylation of *Igk*⁸. Our studies indicate that absence of the two enhancers have substantially different effects on the *Igh* locus.

Our data showing increased deletion of the V_HJ558 associated signal in 3'E_K-KO but not MiE_K-KO cells suggests that the *Igh* locus can continue to undergo distal V_H gene rearrangement only when the *Igh* locus remains in a contracted conformation within euchromatic regions of the nucleus. Despite the alterations in *Igh* locus conformation observed in the *Igk* enhancer mutant pre-B cells, flow cytometric analyses of allotypically marked *Igh* alleles failed to detect evidence of allelic inclusion (data not shown). Thus, additional mechanisms must exist to enforce *Igh* allelic exclusion.

Several recent studies have shown that transcription of genes located on one chromosome can be controlled by regulatory elements located on a different chromosome. These include an association between the promoter of the T helper type 1 (T_H1) cytokine gene, *Ifnγ* and the locus control region (LCR) of the T_H2 cytokine genes¹⁸. In addition, a nonallelic association has been reported between the imprinting control region of the *Igf2/H19* locus and the *Wsb1/Nf1* gene, mediated by CTCF, a factor that is involved in DNA methylation¹⁹. Interchromosomal pairing has also been demonstrated between the two X chromosomes, and this association is implicated in silencing of the genes on one X chromosome during X-inactivation^{20,21}. This contrasts with the interchromosomal associations involved in olfactory receptor choice which may be important for promoting gene expression²². All these interchromosomal associations, including the one that we have identified, involve coordinating decisions involving changes in gene accessibility and/or expression. It could be that physical association of loci or alleles within the nucleus has an essential role in ensuring that the timing of coordinated changes in gene expression is correctly regulated. Previous analyses of interchromosomal associations have not examined association relative to pericentromeric heterochromatin. Our analysis indicates that association between *Igh* and *Igk* is a transient event that is important for early silencing of one *Igh* allele in pre-B cells and for changing the conformation of this allele. This is the first demonstration of an interchromosomal association occurring at pericentromeric heterochromatin. The challenge now is to define the precise regions of *Igh* which mediate association with *Igk* and to identify the proteins which mediate this association.

Methods

FACS sorting and analysis

The following fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), allophycocyanin (APC), PE-Cy7 (from BD Biosciences) or PE-Alexa610 (Invitrogen)-coupled antibodies were used for flow cytometry: CD19 (1D3), CD25 (PC61), CD117 (2B8), IgM (II/411), IgD (11-26c.2a), CD4 (RM4-5), CD8α (53-6.7) and Thy1.2 (53-2.1). Isolated bone marrow cells were stained with the appropriate antibody combination, and pro-B cells were isolated on a Becton Dickinson FACS Aria or a Dako MoFlo Flow Cytometer as CD19⁺c-Kit⁺ IgM⁻ cells, pre-B cells as CD19⁺ CD25⁺ IgM⁻ cells, early immature B cells as CD19⁺ IgM^{low} IgD⁻ and late immature B cells as CD19⁺ IgM^{high} IgD⁻ cells. The purity of the sorted cells was verified by FACS reanalysis.

3D DNA-FISH and confocal analysis

FACS-sorted cells were washed three times in PBS and then fixed onto poly-L-lysine-coated slides for two- and three-color 3D DNA-FISH analysis as described previously in detail¹³. Probes were directly labeled by nick translation with ChromaTide® Alexa Fluor® 488-5-dUTP, ChromaTide® Alexa Fluor® 594-5-dUTP (Molecular Probes/Invitrogen) or dUTP-Cy5 (GE Healthcare). The γ -satellite probe was prepared from a plasmid containing eight copies of the γ -satellite repeat sequence¹¹ and was directly labeled with dUTP-FITC (Roche/Enzo Biochem) or dUTP-Cy5. Cells were analyzed by confocal microscopy on a Leica SP2 and Leica SP5 AOBS (Acousto-Optical Beam Splitter) system. Optical sections separated by 0.3 μ m were collected, and only cells with signals from both alleles (typically 90%) were analyzed.

Statistical analyses of separation of locus probe signals or subnuclear location of probe signals was carried out by applying the χ^2 test to observed and expected frequencies. The original data of cell numbers were used rather than percentages. Expected frequencies were calculated according to standard methods²³ and χ^2 probabilities were calculated in Microsoft Excel.

Activation of splenic B cells

Splenic B cells were purified by CD43 depletion and activated in culture with 10 μ g/ml anti-CD40 as described previously¹¹.

V(D)J recombination analysis

Splenic B cells were isolated by CD19+ MACS selection and digested with proteinase K. DNA was isolated by phenol extraction and ethanol precipitation. PCR analyses of immunoglobulin genes were performed with published primers^{13,24}. PCR cycle numbers were adjusted to be in the linear range, based on the analysis of serially diluted DNA. PCR products were separated on agarose gels, transferred to a Hybond-XL membrane and analyzed by Southern blotting using a published upstream J_H3 oligonucleotide probe or purified C μ PCR product^{13,24}. Quantification of hybridization products was carried out using a Phosphor imager and Quantity One software (Bio-Rad). DNA amounts were normalized to C μ . The average of the two highest dilutions of DNA is represented.

Mice

3'E κ -KO mice were provided by F. Alt (Harvard University)⁷⁹. All animal experiments were done in accordance with the guidelines of the IACUC of the NYU School of Medicine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Interchromosomal association between one *Igh* and one *Igk* allele coincides with pericentromeric recruitment of the two loci. **(a)** Top, confocal sections showing distances (0.5μm, 1μm and 1.5μm) separating the 5' ends of the *Igh* and *Igk* alleles. Bottom, schematic representation of the position of probes used for detecting *Igh* and *Igk*. **(b)** Distance (in μm) separating the 5' ends of the *Igh* and *Igk* loci was evaluated in indicated subsets of sorted bone marrow cells and thymocytes. The percentage and sample sizes are shown in Supplementary Table 1, online. Cells in which no close association between *Igh* and *Igk* alleles was observed (>1.5μm) were scored as separation of a single pair of alleles. **(c)** Pericentromeric location of *Igh* and *Igk* alleles during B cell development. Cells at the indicated developmental stages were

analyzed by three-colour 3-D DNA FISH with the *Igk* probes RP23-101G13 (V κ 24) and RP24-387E13 (*Igk* constant region) in combination with a γ -satellite probe and separately with the *Igh* probes CT7-526A21 (V $_H$ J558) and CT7-34H6 (C $_H$) in combination with a γ -satellite probe. The data represent the percentage of cells showing monoallelic association of *Igh* (blue bars) and *Igk* (red bars) with pericentromeric clusters. The percentages and sample sizes of this data are shown in Supplementary Table 2, online. Data are representative of three experiments.

Figure 2.

Association between *Igh* and *Igk* occurs on the pericentromerically recruited allele. **(a)** Pre-B cells were analyzed by three-color 3-dimensional DNA FISH with the *Igk* probe described in Fig. 1. The data represent the localization *Igh* and *Igk* alleles at different distances apart (<0.5 μ m, 0.5–1 μ m, 1–1.5 μ m and >1.5 μ m) relative to pericentromeric heterochromatin. The percentage and sample sizes are shown in Supplementary Table 3, online. **(b)** Evaluation of the orientation of *Igh* and *Igk* alleles at different distances apart (0.5, 0.5–1, 1–1.5 and <1.5 μ m) relative to pericentromeric heterochromatin. The percentage and sample sizes are shown in Supplementary Table 4, online. **(c)** Confocal sections demonstrating different facets of

interacting loci relative to pericentromeric heterochromatin. Data are representative of three experiments.

Figure 3.

Increased pericentromeric recruitment of *Igk* promotes an increased frequency of interchromosomal association between *Igh* and *Igk* and increased pericentromeric recruitment of *Igh* dependent on 3'Eκ. **(a)** Pericentromeric location of *Igh* and *Igk* alleles. Cells sorted from WT and MiEk-KO bone marrow at the indicated developmental stages were analyzed by three-color 3-dimensional DNA FISH as described for Fig. 1. The percentages and sample sizes of this data are shown in Supplementary Table 2, online. **(b)** The distance (in μm) separating the 5' ends of the *Igh* and *Igk* loci was evaluated in pro-B, pre-B, IgM^{low} (early immature B) and IgM^{high} (late immature B) cell subsets of sorted bone marrow cells from WT and MiEk-KO mice. No IgM^{high} cells were present in the mutant mice. The percentage and sample sizes are shown in Supplementary Table 1, online. **(c)** Top, confocal sections showing biallelically recruited *Igh* and *Igk* in pre-B cells from MiEk-KO mice. Bottom, schematic representation of

the position of probes used for detecting *Igh* and *Igk*. Data are representative of three experiments.

Figure 4.

Association of *Igh* and *Igk* increases with increasing *Igk* recruitment to pericentromeric regions. **(a)** Percentage of pre-B cells with monoallelically and biallelically pericentromeric *Igk* alleles, and cells where neither *Igk* allele is pericentromeric, in MiEκ-KO mice. The pre-B cells analyzed here were from a different batch of mice as those analyzed in the experiments shown in Fig. 3. **(b)** Analysis of *Igh* and *Igk* association in cells with monoallelically and biallelically pericentromeric *Igk* alleles, and in cells where neither *Igk* allele is pericentromeric. The percentage and sample sizes are shown in Supplementary Table 5, online. Data are representative of three experiments.

Figure 5.

3'Eκ mediates association of *Igh* and *Igk*, and *Igh* pericentromeric recruitment at the pre-B cell stage in development. **(a)** Cells sorted from WT and 3'Eκ-KO bone marrow at the indicated developmental stages were analyzed by three-color 3-dimensional DNA FISH as described for Fig. 1. The percentages and sample sizes of this data are shown in Supplementary Table 2, online. **(b)** The distance (in μm) separating the 5' ends of the *Igh* and *Igk* loci in pro- and pre-B cell subsets of sorted bone marrow cells from WT and 3'Eκ-KO mice. The percentage and sample sizes are shown in Supplementary Table 1, online. **(c)** Confocal sections showing the location of *Igh* alleles relative to pericentromeric clusters in pre-B cells from WT, MiEκ-KO and 3'Eκ-KO mice. The *Igh* probes used were CT7-526A21 (V_HJ558) and CT7-34H6 (C_H)

in combination with a γ -satellite probe. Individual alleles from the same cell are shown in different confocal sections. Data are representative of three experiments.

Separation of V_HJ558 and C_H gene segments

a.

b.

Figure 6.

Association of *Igh* and *Igk* mediates *Igh* locus decontraction. The distance (in μm) separating the V_HJ558 and C_H gene segments in cells sorted from WT, MiEκ-KO and 3'Eκ-KO bone marrow at the indicated developmental stages were analyzed by two-color 3-dimensional DNA FISH. The *Igh* probes used were CT7-526A21 (V_HJ558) and CT7-34H6 (C_H). Data are representative of three experiments.

Figure 7. Deletion of the V_HJ558 CT7-526A1 probe occurs more frequently in 3'Eκ-KO than in WT or MiEκ-KO pre-B cells. 3-dimensional DNA FISH was performed using the *Igh* probes CT7-526A21 (V_HJ558) and CT7-34H6 (C_H) in combination with a probe located outside of the 5' region of the *Igh* locus, RP24-386J17. (a) Graph showing frequency of deletion of V_HJ558 CT7-526A1 in WT, 3'Eκ-KO and MiEκ-KO pre-B cells. Table 1. showing frequency of deletion of the V_HJ558 CT7-526A1 and RP24-386J17 signal in WT, 3'Eκ^{-/-} and MiEκ^{-/-} pro-B and pre-B cells. (b) Top, confocal sections showing the presence of both CT7-526A21 (V_HJ558) and CT7-34H6 (C_H) signals on both *Igh* alleles (left) and in a different nucleus, the absence of the V_HJ558 CT7-526A21 signal on one allele (right). Bottom, position and colors of the probes used. (e) Top, confocal section showing the presence of CT7-526A21 (V_HJ558), CT7-34H6 (C_H) and RP24-386J17 signals on one *Igh* allele and the absence of the V_HJ558 CT7-526A21 signal on the other allele. Bottom, position and colors of the probes used. Data are representative of three experiments.

Figure 8.

An altered spectrum of proximal versus distal V_H gene segment rearrangements in $3'E\kappa$ -KO splenic B cells. **(a)** PCR and Southern blot analysis of *Igh* rearrangements in $CD19^+$ splenic B cells. Genotypes are indicated beneath each set of serial three-fold-diluted DNA samples. PCR primers were chosen to detect V_H558 (top), $V_HGam3.8$ (middle) and V_H7183 (bottom) family rearrangements to DJ_H 1, 2, and 3 segments. Input DNA was normalized by amplification of a PCR fragment from the *Igh* $C\mu$ region and DNA of stromal ST2 cells was used as a negative control. The numbers to the left of bands indicate rearrangements involving J_H1 , J_H2 and J_H3 gene segments. **(b–d)** Quantification of rearrangements shown in **(a)**. The recombination signal is shown as a proportion of $C\mu$ amounts for each DNA dilution and as an average across the two highest dilutions of DNA.

Figure 9.