Published in final edited form as:
J Immunol. 2013 February 15; 190(4): 1819–1826. doi:10.4049/jimmunol.1203127.

Vκ gene repertoire and locus contraction are specified by critical DNase I hypersensitive sites within the Vκ-Jκ intervening region[1]

Yougui Xiang[*,‡], **Sung-Kyun Park**[*], and **William T. Garrard**[*,2]

[*]Department of Molecular Biology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9148

[‡]Tianjin Research Center of Basic Medical Science, Tianjin Medical University, Tianjin 300070, P.R. China

Abstract

The processes of *Ig* gene locus contraction and looping during V(D)J-recombination are essential for creating a diverse antibody repertoire. However, no *cis*-acting sequence that plays a major role in specifying locus contraction has been uncovered within the *Igκ* gene locus. Here we demonstrate that a 650 bp sequence corresponding to DNase I hypersensitive sites HS1-2 within the mouse *Igκ* gene V-J intervening region binds CCCTC-binding factor (CTCF) and specifies locus contraction and long-range Vκ gene usage spanning 3.2 Mb in pre-B cells. We term this novel element Cer for **c**ontracting **e**lement for **r**ecombination. Targeted deletion of Cer caused markedly increased proximal and greatly diminished upstream Vκ gene usage, higher allele usage, more splenic Igκ$^+$ B cells and non-lineage-specific *Igκ* rearrangement in T cells. Relative to wild type mice, Cer deletion mice exhibited similar levels of Vκ gene germline transcription and H3K4me3 epigenetic marks but displayed a dramatic decrease in locus contraction in pre-B cells. Thus, our studies demonstrate that DNase I hypersensitive sites HS1-2 within the Vκ–Jκ intervening region are essential for controlling locus contraction and creating a diverse antibody repertoire.

Keywords

B lymphocytes; V(D)J-recombination; V gene repertoire; *Igκ* locus contraction; DNase I hypersensitive sites; CTCF

Introduction

The Ig V gene primary antibody repertoire is generated in B lymphocytes by the process of V(D)J recombination mediated by *RAG*-encoded recombinases and non-homologous end-joining proteins (1). In addition, this repertoire is further modulated by receptor editing, somatic hypermutation, transcription levels of rearranged genes, and differential mRNA stabilities (2–5). For the immune system to efficiently recognize a broad spectrum of invading pathogens, diversity in the repertoire is essential. Furthermore, mis-regulated or incorrect repertoire specification can trigger autoimmunity (6, 7).

[1]This investigation was supported by Grants GM29935 and AI067906 from the National Institutes of Health and Grant I-0823 from the Robert A. Welch Foundation to WTG, and by Grant 31270928 from the National Natural Science Foundation of China to YX.

[2]Address correspondence and reprint requests to Dr. William T. Garrard, Department of Molecular Biology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9148. Phone: 214-648-1924. FAX: 214-648-1915. william.garrard@utsw.edu.

The mouse *Igκ* light chain gene is the largest multi-gene family locus thus far identified, spanning 3.2 Mb on mouse chromosome 6 (8). It consists of 100 functional Vκ gene exons (9), four functional Jκ–region exons, and a single Cκ exon (Fig. 1*A*). Following V(D)J-recombination of the *Igh* heavy chain gene locus during the pro-B cell stage of development, the *Igκ* gene locus is poised for rearrangement in pre-B cells, whereby a Vκ gene becomes covalently joined to a Jκ-region (1, 10). This recombination event results in transcriptional activation because it positions a Vκ gene carrying its own promoter into a chromatin domain containing three powerful downstream enhancers: an intronic enhancer (Ei) within the transcription unit and two enhancers downstream of the transcription termination region, termed E3′ and Ed (11–14). If *Igκ* gene V-J joining is productively unsuccessful because of out-of-reading frame recombination junctions, then the *Igλ* locus becomes activated for rearrangement and expression, which in wild type (WT) mice accounts for production of only approximately 5% of the total IgL chains (15).

Germline transcription of the *Ig* loci has long been thought to increase locus accessibility to the recombinase apparatus and has been correlated with the process of V(D)J-recombination (16, 17). Furthermore, deletion of the most 5′ Jκ-region germline promoter (5′Gp), which is known to be the most significant in pre-B cells for generating germline transcripts (18), is highly detrimental to *Igκ* gene rearrangement in knockout mice (19). Moreover, such germline transcription requires the *Igκ* gene downstream enhancers, as their targeted deletion leads to a block in Vκ-Jκ joining (20, 21). More recently, intact promoters, enhancers, and transcriptional elongation have been directly shown to control the binding of RAG1 to recombination signal sequences in the *Tcra* and *Tcrb* loci at Ja and Db/Jb segments, directly validating the accessibility model (22). Furthermore, the RAG proteins have been demonstrated to bind *in vivo* to Jh and closely linked DQ52 segments in pro-B cells, and to Jκ regions in pre-B cells (23). It has been proposed that this stage-specific binding of the RAG proteins results in the assembly of recombination centers, which can capture the recombination signal sequences of upstream Vh and Vκ regions through the assistance of long-range chromosome reorganization events, thus creating a paired complex leading to V(D)J-recombination (1, 23).

Evidence has emerged that nuclear organization and locus contraction/decontraction of *Ig* loci contribute to repertoire specification. Results from three-dimensional DNA fluorescence *in situ* hybridization (3D DNA FISH) experiments reveal that the mouse *Igh* and *Igκ* loci exhibit contraction and looping of V genes into rosette-like structures, which juxtaposes them near Dh or Jκ regions in preparation for rearrangement (24–26). Furthermore, reduced contraction of *Igh* loci results in a skewed repertoire, with proximal Vh genes being preferentially utilized (24, 27–30), whereas persistent contraction results in greater distal Vh gene rearrangements (31). Decontraction occurs after rearrangement (24). It has been proposed that germline transcription of *Ig* gene loci may contribute to contraction in preparation for V(D)J-recombination via the assembly of proximal and distal transcribing regions into the same transcription factories (32, 33).

Specific DNA sequences and *trans*-acting factors within the *Igh* gene locus that are major determinants of contraction have been identified. These include the intronic Eμ enhancer and its associated promoter region (34), the transcription factors Pax5 (27), YY1 (28), Ikaros (29), and the chromatin modifying enzyme, Ezh2 (35). More recently, CCCTC-binding factor (CTCF)/cohesin proteins implicated in looping and insulation, have also been shown to contribute modestly to locus contraction in the *Igh* locus (30, 36). Furthermore, specific CTCF-binding elements in the Vh-Dh intervening sequence have been directly demonstrated to play a major role in dampening Dh-proximal Vh gene usage in V(D)J-recombination (37) and related elements are implicated in analogous processes in *Igκ* loci (38, 39). In these cases CTCF is thought to silence the usage of proximal V genes by creating looped domains

sequestering the downstream enhancers away from the proximal V genes' promoters, which results in down-regulation of their localized germline transcription (37, 38).

Here we characterize DNase I hypersensitive sites HS1-2 as new CTCF-binding elements in the mouse *Igκ* gene locus V-J intervening sequence and demonstrate that this 650 bp DNA segment remarkably is responsible for locus contraction and long-range Vκ gene usage spanning 3.2 Mb. We term this novel element Cer for **c**ontracting **e**lement for **r**ecombination. Deletion of Cer markedly increased Jκ-proximal Vκ gene usage and decreased middle and distal Vκ gene usage without significantly affecting localized germline transcription or a canonical positive epigenetic mark in chromatin. These results identify the first *cis*-acting DNA element that plays a major role in *Igκ* gene locus contraction and repertoire specification during Vκ-Jκ-recombination.

Materials and Methods

Mouse strains

Mice possessing a 0.65 kb deletion of HS1-2 in the endogenous Igκ locus were generated by standard embryonic stem (ES) cell targeting technology; germline transmissible mice were bred with Cre recombinase expressing MORE (40) mice to obtain HS1-2 and neo^r deletion mice (Fig. S1). Mice bearing a human Cκ knocked-in gene were kindly provided by Michel C. Nussenzweig of Rockefeller University (2). μ^+ transgenic mice and $Rag1^{-/-}$ mice were kindly provided by Mark Schlissel of UC Berkeley. All mice were used in accordance with protocols approved by the UT Southwestern Medical Center Institutional Animal Care and Use Committee (IACUC). Additional details are provided in supplemental data.

Flow cytometry and cell fractionation

Single-cell suspensions were prepared from bone marrow and spleens of 6–14 week old mice as described (39). Single-cell suspensions were stained with antibodies and analyzed using FACS Calibur with CellQuest software (BD Bioscence, San Diego, CA) or FlowJo software (Tree Star, Ashland, OR) (39). $B220^+CD43^-IgM^-$ small pre-B cells were sorted by a MoFlo flow cytometer. Splenic B cells were purified using B cell isolation kits (Miltenyi Biotec). Generally we pooled bone marrow or splenic cells from 2–3 animals of the same genetic background for cell fractionation. Antibodies used are as follows: anti-mouse-Igκ-PE (BD Bioscience); anti-mouse-Igλ1,2,3-FITC (BD Bioscience); anti-human-Igκ-FITC (Southern Biotech, Birmingham, AL); anti-B220-PerCP-Cy5.5 (BD Bioscience); anti-IgM-APC (BD Bioscience); anti-CD43-PE (BD Bioscience); anti-B220-FITC (BD Bioscience); anti-CD19-biotin (BD Bioscience); anti-B220-biotin (BD Bioscience); Streptavidin-APC (Southern Biotech).

Analysis of Igκ gene repertoire, Vκ-Jκ1 rearrangement and germline transcription

These assays were performed as previously described (39). For analysis of *Igκ* gene repertoire and Vκ-Jκ1 rearrangement, genomic DNA was purified from sorted B cell populations. For *Igκ* gene repertoire analysis, the VκD primer and a primer in the Jκ1 intron were used to amplify Vκ-Jκ1 rearrangements; resulting PCR products were gel purified and subcloned into the pGEM-T vector (Promega, San Luis Obispo, CA). Determined sequences of Vκ genes in each clone were identified by the IgBlast program (NCBI, Bethesda, MD). For real-time PCR analysis of individual Vκ-Jκ1 rearrangements, forward primers specific to different Vκ exons and a reverse primer complementary to the Jκ1 to Jκ2 intron region were used (primer sequences are listed in Supplemental Table S1). Different Vκ-Jκ1 rearrangements were determined quantitatively by using the SYBR Green PCR master mix (Bio-Rad laboratories, Richmond, CA) in the 7300 real-time PCR system (Invitrogen, Carlsbad, CA). PCR was performed based on manufacturer's protocols and each PCR assay

was carried out in duplicate or triplicate. Relative rearrangements were calculated using the ΔC_t method according to the manufacturer's instructions and normalized to an β-actin genomic region. To examine *Igκ* gene germline transcription, total RNA was extracted from 1×10^6 MoFlo sorted pre-B cells using TRIzol reagent (Invitrogen, Carlsbad, CA). Then RNA was treated with DNase I and was reverse transcribed into cDNA with SuperScript® III Reverse Transcriptase (Invitrogen, Carlsbad, CA). For real-time PCR analysis of individual Vκ gene's germline transcripts, forward primers specific to different Vκ gene exons and a reverse primer complementary to the downstream RSS region were used (Supplemental Table S1). For analysis of transcripts arising from the 5′ germline promoter upstream of the Jκ1 region, a forward 5′GT-f primer annealing immediately downstream of the promoter region and a reverse Cκ-r primer annealing in Cκ exon were used in real time PCR assays (Supplemental Table S1). Transcript levels were calculated using the ΔC_t method according to the manufacturer's instructions and normalized to the cDNA levels of the mouse β-actin gene.

3D DNA FISH

3D DNA FISH was performed as previously described (39). Probes for 3D FISH were prepared from bacterial artificial chromosomes (BACs). We utilized RP23-101G13, RP23-26A6 and RP24-387E13, which correspond to the 5′, middle, and the 3′ region of the *Igκ* locus, respectively. Probe preparation and hybridization conditions were as described previously (39). Z stacks with sections separated by 0.3 μm were analyzed by confocal microscopy using a Leica SP5 instrument and distances were measured using a pluggin of ImageJ software as described (39).

ChIP

For CTCF ChIP, about 2×10^6 sorted pre-B cells (Fig. 1*E*) or $CD19^+$ pre-B cells from $Rag1^{-/-}$, μ^+transgenic animal models (Fig. 1*D*) were used for each ChIP experiment. For H3K4me3 ChIP, sorted pre-B cells were used for each ChIP experiment. ChIP experiments were conducted according to the protocol of Millipore. Rabbit anti-CTCF antibodies (Millipore, 07-729), and Rabbit anti-H3K4me3 antibodies (Millipore, 07-473) were used for ChIP. For real-time PCR analysis of the H3K4me3 modification levels of individual Vκ gene's recombination signal sequences, we used the same primers as those used for Vκ gene's germline transcript analysis (Supplemental Table S1). Real-time PCR was performed and quantitated using the 7300 Real Time PCR System (Invitrogen, Carlsbad, CA) with SYBR green as described above and enrichment of target regions in ChIP was normalizedto actin (primer sequences are listed in the Supplemental Table S1).

Results

Generation of targeted deletions of HS1-2

We have previously shown that the Vκ-Jκ intervening region exhibits six DNase I hypersensitive sites (HS1-6) in cells of the B lymphocyte lineage (Fig. 1*A, B*) (14). In addition, our functional analyses of HS3-6 revealed transcription and recombination silencer activity and hence the sequence was termed Sis (**s**ilencer in the **i**ntervening **s**equence) (14, 41). Sis binds Ikaros and CTCF and is responsible for dampening Jκ-proximal Vκ gene usage during Vκ-Jκ rearrangement in pre-B cells (39, 41). Here we characterize the function of HS1-2 by creating targeted deletions in this element in the mouse germline (Fig. 1*B*; Figs. S1). Interestingly, an *in silico* CTCF binding site prediction tool (http://insulatordb.uthsc.edu) (42) reveals that pairs of candidate CTCF binding sites exist in both HS1-2 and HS3-6 (Fig. 1*C*). Each pair of these sites are direct repeats with a few base pair mismatches interspersed by several hundred nucleotides. Furthermore, the results of ChIP experiments demonstrate that CTCF is bound to these sites *in vivo* in pre-B cells (Fig. 1*D*).

Moreover, pre-B cells from mice possessing a deletion of HS1-2 no longer exhibited localized binding of CTCF (Fig. 1*E*), just as we have shown previously for the effect of deletion of HS3-6 (Sis) (39).

Deletion of HS1-2 leads to increased Igκ gene expression B cells

To investigate the functions of HS1-2 in B cell development, we first analyzed bone marrow and splenic cells from WT and HS1-2$^{-/-}$ mice by flow cytometry. HS1-2$^{-/-}$ mice exhibited slightly higher percentages of splenic Igκ^{+} B cells compared with their WT littermates or age-matched WT mice (Fig. 2*A*). By contrast, the percentages of splenic Igλ^{+} B cells were at similar levels among these groups (Fig. 2*A*). Hence, the splenic Igκ^{+}/Igλ^{+} B cell ratios were slightly increased in HS1-2 mice relative to controls (Fig. 2*B*). The percentages of Igκ^{+}Igλ^{+} double positive cells were the same between WT and HS1-2$^{-/-}$ mice, indicating that Ig light chain isotype exclusion was still intact (Fig. 2*B*). To further characterize the effects of this deletion, we bred HS1-2$^{+/-}$ mice with a line carrying a human Cκ knockin allele to obtain *Igκ*$^{m/h}$ and *Igκ*$^{\Delta HS1-2m/h}$ heterozygotes (2). We found that mCκ and hCκ alleles were equally used in *Igκ*$^{m/h}$ heterozygotes as reported previously (Fig. 2*C*) (2). However, in mice bearing a deletion in HS1-2, mCκ alleles exhibited a modest preference to be used in the heterozygotes, both in splenic cells and bone marrow (Fig. 2*C, upper* and *lower*, respectively). These results are consistent with the observed increase in *Igκ*$^{+}$ B cells in HS1-2$^{-/-}$ mice, and indicate that the corresponding deleted alleles gain an edge in recombination frequency compared with hCκ alleles. We also found that the heterozygotes from these groups exhibited similar levels of mCκ^{+}hCκ^{+} double positive cells (Fig. 2*C*), suggesting that HS1-2 deletion did not affect allelic exclusion.

Deletion of HS1-2 dramatically increased Jκ-proximal Vκ gene usage and decreased the usage of middle and distal Vκ genes

Previously we demonstrated that deletion of HS3-6 (Sis) caused increased Jκ-proximal Vκ gene usage during Vκ-Jκ rearrangement in pre-B cells (39). To determine whether deletion of HS1-2 also altered primary Vκ gene usage, we cloned and sequenced Vκ-Jκ1 rearrangement products from WT and mutant mice pre-B cells. As shown in Fig. 3*A*, Vκ genes within the first 0.1 Mb interval closest to the Jκ region were heavily used in pre-B cells from HS1-2$^{-/-}$ mice, accounting for 62% of the total Vκ gene usage. At same time, the % usage of Vκ genes in middle and distal *Igκ* gene regions was decreased dramatically (Fig. 3*A*). By contrast, this pattern of Vκ gene usage significantly differed from that of WT mice, or of pre-B cells from HS3-6$^{-/-}$ (Sis$^{-/-}$) mice. In the HS3-6$^{-/-}$ (Sis$^{-/-}$) samples, 25% of total Vκ gene usage occurred in the corresponding proximal region, whereas the usage of most middle Vκ genes was only decreased moderately relative to WT mice patterns (Fig. 3*A*). We also employed real-time PCR to quantitate relative Vκ-Jκ1 gene rearrangement levels in both pre-B and splenic B cell samples from WT and mutant mice for several individual Vκ genes and obtained very similar results to those described above (Fig. 3*B, C*). In addition, the total Vκ gene rearrangement levels as assayed with a degenerate Vκ gene primer (VκD) were at similar levels between WT and HS1-2$^{-/-}$ mice (Fig. 3*B, C*). We conclude that HS1-2 within the Vκ-Jκ intervening region dramatically specifies Vκ gene usage.

Deletion of HS1-2 increases Vκ–proximal Jκ-region usage and allows Igκ gene rearrangement in T cells

To determine whether the Jκ–region usage or tissue-specificity of rearrangement was altered in our mutant mice, we utilized a semi-quantitative PCR assay with genomic DNA isolated from pre-B cells and T cells that gives rise to four distinct bands representing V-Jκ1, V-Jκ2, V-Jκ4, and V-Jκ5 recombination products (Fig. 4*A*). Examination of the relative usage of Jκ-regions in pre-B cells revealed that the mutant mice still utilized all four Jκ regions in

Vκ-Jκ joining (Fig. 4*B*). However, quatitation of these data revealed that the usage of the Jκ1- and Jκ2-regions were increased and decreased approximately 50% and 30%, respectively, in pre-B cells from HS1-2$^{-/-}$ mice compared to WT controls, while usage of the other Jκ-regions was unaltered (Fig. 4*C*). Furthermore, we found that *Igκ* gene rearrangements were detec\ in CD4^{+}CD8^{+} double-positive T (DPT) cells from HS1-2$^{-/-}$ mice but not in their WT counterparts, and that these rearrangements strongly favored Jκ1 usage (Fig. 4*D*). To investigate further the developmental timing of rearrangement in pre-B cells, we accessed the extent of insertion of N nucleotides in Vκ-Jκ recombination junctions in pre-B cells. In contrast to P nucleotides, which become inserted naturally by the recombination mechanism, N nucleotide insertions require terminal deoxynucleotide transferase activity, which is normally expressed in pro-B but not in pre-B cells when *Igκ* genes normally rearrange (44). We found that the incorporation of N nucleotides into the Vκ-Jκ recombination junctions in pre-B samples from mice with mutant alleles was even lower than that seen in their WT counterparts, indicating that the timing of Vκ-Jκ rearrangement was not affected by deletion of HS1-2; furthermore, the frequency of P nucleotide insertions into these junctions was also lower in the mutant alleles, possibly suggesting differences in exonuclease processing events (Fig. 4*E*). Taken together with the results shown in Fig. 3, we conclude that HS1-2 within the Vκ-Jκ intervening region not only affects the choice of Vκ–regions, but also the choice of Jκ–regions, namely the usage of recombination substrates on both its 5′ and 3′ sides. In addition, HS1-2 restricts the rearrangement process to B cells. These results contrast with those observed earlier for HS3-6$^{-/-}$ (Sis$^{-/-}$) mice, whose pre-B cells or T cells did not exhibit these dramatic alterations (39).

Pre-B cells from HS1-2$^{-/-}$ and WT mice exhibit very similar patterns of Vκ gene germline transcription and H3K4me3 modification

To address whether the dramatically increased proximal Vκ gene usage correlated with increased proximal Vκ gene germline transcription, we measured by real-time PCR the levels of germline transcripts in pre-B cells arising from selected Vκ genes representing diverse physical positions in the locus and from the 5′-promoter upstream of the Jκ-region (5′GL) in samples from WT and mutant mice. We observed about 2.5-fold increases in distal Vκ2-139 and proximal Vκ21-7 gene germline transcripts in HS1-2$^{-/-}$ mice pre-B cells, whereas 5′GL transcripts and those of several other Vκ genes were at similar levels compared with those of WT (Fig. 5*A*). These results are very similar to those that we have reported previously for deletion of HS3-6 (Sis) (39), and indicate that these modest differences in germline transcription levels do not correlate with altered Vκ gene usage in pre-B cells from HS1-2$^{-/-}$ or HS3-6$^{-/-}$ (Sis$^{-/-}$) mice. Previous studies have linked the trimethylation of lysine 4 of histone H3 (H3K4me3) to localized RAG protein binding during V(D)J recombination (23). To characterize the distribution of this modification, we performed ChIP experiments and utilized real-time PCR to quantitate the levels of H3K4me3 marks in several Vκ genes in pre-B cells from WT and HS1-2$^{-/-}$ mice. Interestingly, the far upstream Vκ2-139 gene had much higher levels of H3K4me3 modification than the Jκ-proximal Vκ genes (Fig. 5*B*). However, these distal Vκ2-139 and Vκ9-132 gene H3K4me3 modifications were at similar levels in HS1-2$^{-/-}$ and WT mice pre-B cells, although the usage of these genes were decreased dramatically in HS1- 2$^{-/-}$ pre-B cells. These results indicate that neither the levels of Vκ gene germline transcription nor H3K4me3 modification correlate with either the reduced usage of distal or increased usage of proximal Vκ genes in pre-B cells from HS1-2$^{-/-}$ mice.

HS1-2 is required for Igκ gene locus contraction in pre-B cells

We hypothesized that *Igκ* locus contraction may be reduced in pre-B cells from HS1-2$^{-/-}$ mice and may account for the dramatically altered Vκ gene usage. To test this proposal, we

performed 3D DNA FISH experiments using *Igκ* gene BAC probes corresponding to 5′, middle and 3′ locations in the locus (Fig. 6*A*, probes A, B and C, respectively). As shown in Fig. 6*B*, representative confocal images generated using these probes revealed as expected that pre-B cell nuclei from WT mice exhibited looped and contracted *Igκ* gene structures in agreement with a previous report (24), while corresponding samples from HS1-2$^{-/-}$ mice exhibited less-contracted, non-looped patterns. To quantitate these results we measured the center-to-center distances between A&B, B&C, and A&C hybridization signals for several hundred alleles in these samples. As shown in Fig. 6*C*, contraction was statistically significantly decreased throughout the locus by the HS1-2 deletion. The mean distances between respective A&B, B&C, and A&C hybridization signals were 0.316-, 0.326-, and 0.279-μm for WT samples, and 0.36-, 0.405-, and 0.417--μm for HS1-2$^{-/-}$ samples. These differences correspond to 15%, 23% and 50% decreases in contraction in the respective 5′- and 3′-halves of the locus, or for the locus as a whole, for HS1-2$^{-/-}$ mice samples in comparison to those from WT mice. By contrast, our previous studies have shown that HS3-6 (Sis) is not responsible for *Igκ* locus contraction in pre-B cells (39). We conclude that HS1-2 *per se* plays a predominant role in specifying contraction throughout the *Igκ* locus in pre-B cells. Henceforth, we term this novel element Cer for **c**ontracting **e**lement for **r**ecombination.

Discussion

HS1-2 (Cer) is the first example of a *cis*-acting sequence that plays a major role in specifying locus contraction in the *Igκ* gene locus. The element is only 650 bp in length and its deletion results in a 7-fold increase in proximal Vκ gene usage along with approximately a 50% reduction in overall locus contraction. The only other sequences reported so far that play roles in contraction are in the *Igh* locus. These include the intronic Eμ enhancer and its associated promoter region (34), which play very major roles in specifying contraction, and HS5-7 that resides within the 3′ RR regulatory region, which plays only a minor role in contraction (30). The *Igκ* gene enhancers have been demonstrated to be important in specifying optimal Vκ-Jκ-recombination (20, 21), but their roles in locus contraction remain to be investigated.

We imagine that in the native locus, Cer and Sis serve complementary but functionally distinct roles in regulating Vκ gene usage. We have previously demonstrated that HS3-6 (Sis) is a recombination silencer and that its presence reduces the level of recombination 7-fold in yeast artificial chromosome-based *Igκ* mini-loci transgenes (41). Furthermore, the element exerts negative affects on the levels of Vκ gene usage in the native locus at distances up to 650 kb (39). Considering these facts it might be unexpected that HS1-2$^{-/-}$ (Cer$^{-/-}$) mice, which still possess HS3-6 (Sis), should exhibit such a dramatic increase in proximal Vκ gene usage, well beyond the level observed in HS3-6$^{-/-}$ (Sis$^{-/-}$) mice, which still possess HS1-2 (Cer). However, in the native locus Cer ensures that long-range Vκ genes will be capable of undergoing rearrangement by contracting and looping the locus, whereas Sis ensures that Jκ–proximal Vκ genes will not be over used in recombination. When Cer is deleted, the de-contracted locus has no alternative but to rearrange proximal Vκ genes, whereas when Sis is deleted in spite of the fact that the locus is still contracted and looped, rearrangement of proximal Vκ genes is no longer silenced.

Sis is not conserved among human, mouse and rat. HS1-2 is conserved between mouse and rat (71% identity), but not between mouse and human. There are two separate *in silico* predicted CTCF binding sites in human *Igκ* gene V-J intervening sequence, but the sequences adjacent to these CTCF binding sites are not conserved between human and mouse. Possibly, mechanisms that regulate recombination may have evolved more recently then the time of divergence of these species.

While it seems clear that elements that bind CTCF regulate V gene choice and features of higher-order chromosome organization in *Ig* loci (30, 34, 36–39), whether CTCF functions at Cer to regulate contraction is an open question. In the *Igκ* locus, both HS1-2 (Cer) and HS3-6 (Sis) each contain 2 CTCF binding sites. However, conditional deletion of CTCF in B lineage cells leads to only minor changes in long-range chromosomal looping of Vκ gene regions and to modest increases in the rearrangement of Jκ-proximal Vκ genes without significantly compromising distal Vκ gene usage (38). Furthermore, our previous studies demonstrated that deletion of HS3-6 (Sis), which eliminates a pair of CTCF binding sites, leads to no change in locus contraction in pre-B cells and to only moderate relative increases in the rearrangement of proximal Vκ genes, again without significantly compromising usage of most upstream Vκ genes (39)(see also Fig. 3*A*). Because CTCF conditional knockouts are never complete and such cells show severe defects of pre-B cell proliferation and differentiation (38), these observations need not eliminate a central role for CTCF in mediating *Igκ* locus contraction in pre-B cells. Furthermore, once established by CTCF, higher-order chromatin structures may not be easily reversed. Nevertheless, proteins other than or in addition to CTCF may be responsible for locus contraction. Moreover, as already mentioned in the Introduction, several proteins other than CTCF are responsible for contraction in the *Igh* locus, but the roles of these proteins in the *Igκ* locus remain to be investigated. According to published ChIP-Seq data for mouse pro-B cells, HS1-2 (Cer) also binds several other transcription factors, which include E2A, PU.1 and FOXO1, but the functions of these proteins at these sites are currently unknown (45, 46). Clearly, site directed mutagenesis of CTCF and other protein binding sites will be necessary to elucidate the functions of these proteins in locus contraction in the future.

The results of our 3D DNA FISH experiments, which demonstrate reduced locus contraction in pre-B cells from HS1-2$^{-/-}$ (Cer$^{-/-}$) mice, are consistent with the interpretation that HS1-2 (Cer) is responsible for the long-range usage of Vκ genes by altering higher-order chromosome structures throughout the locus. Our results reveal further that Vκ gene germline transcription is only modestly upregulated upon deletion of either HS1-2 (Cer), or HS3-6 (Sis) in pre-B cells (Fig. 5*A* and 39). Hence, our results do not fit the simple model in which locus contraction is mediated by the co-occupation of transcribing Vκ genes with the distal transcribing Jκ-Cκ region in the same transcription factories (32, 33), a model which on the other hand can readily explain the roles of the *Ig* gene transcriptional enhancers in contributing to loci contraction and V(D)J-rearrangement. HS1-2 (Cer) and HS3-6 (Sis) each bind CTCF and both may act independently as insulator boundaries preventing the downstream enhancers Ei, E3′ and Ed from activating proximal Vκ gene germline transcription. We are currently testing the possibility that HS1-2 (Cer) and HS3-6 (Sis) may be functionally redundant in this process by creating mice with a deletion of HS1-6. Our results also demonstrate that HS1-2 (Cer) *per se* plays no obvious role in regulating H3K4me3 positive epigenetic marks in the chromatin of Vκ genes exhibiting marked changes in usage upon its deletion. By contrast, HS1-2 (Cer) plays a unique role in facilitating locus contraction, fostering long-range interactions between distal Vκ genes and the Jκ-regions. If Vκ genes form looped rosettes independent of HS1-2 (Cer), then the element may be responsible for bringing these rosettes into close proximity with Jκ-region recombination centers (1).

Why are *cis*-acting elements that regulate rearrangement specifically localized in the Vκ-Jκ or Vh-Dh intervening sequences in *Ig* loci? Once a primary rearrangement event occurs, such elements are deleted in the case of the *Igh* locus, or either deleted or inverted and moved a minimum of 265 kb upstream from their initial location in the case of the *Igκ* locus. Hence, the functions of these *cis*-elements must be only to regulate primary rearrangements. Because their engineered mutagenesis or deletion in the mouse germline results in preferential proximal V gene rearrangements (Fig. 3, 37, 39), it can be concluded further

that the functions of these intervening sequence elements is to even-out initial V region usage throughout these loci. Such evening-out processes may be related to the functions of these intervening sequences in altering higher-order chromatin structures, and in buffering the action of the downstream enhancers from hyperactivating proximal V gene chromatin accessibility to the recombination machinery. Based on the results presented here and in our previous study (39), once HS1-6 have been deleted or moved far distances by the normal recombination process, it is predicted that secondary rearrangements in the *Igκ* gene locus, such as those seen in receptor editing and revision (47), will occur using Vκ genes most proximal to those used in the primary rearrangements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are indebted to Michel C. Nussenzweig of Rockefeller University and Mark Schlissel from UC Berkeley for kindly providing mice strains.

Abbreviations

3D FISH	3D fluorescent *in situ* hybridization
ChIP	Chromatin immunoprecipitation
CTCF	CCCTC-binding factor
HS	DNase I hypersensitive site

References

1. Schatz DG, Ji Y. Recombination centres and the orchestration of V(D)J recombination. Nat Rev Immunol. 2011; 11:251–263. [PubMed: 21394103]
2. Casellas R, Shih TAY, Kleinewietfeld M, Jankovic M, Nemazee D, Rajewsky K, Nussenzweig MC. Contribution of receptor editing to the antibody repertoire. Science. 2001; 291:1541–1544. [PubMed: 11222858]
3. Longerich S, Basu U, Alt F, Storb U. AID in somatic hypermutation and class switch recombination. Curr Opin Immunol. 2006; 18:164–174. [PubMed: 16464563]
4. Casellas R, Jankovic M, Meyer G, Gazumyan A, Luo Y, Roeder RG, Nussenzweig MC. OcaB is required for normal transcription and V(D)J recombination in a subset of immunoglobulin κ genes. Cell. 2002; 110:575–585. [PubMed: 12230975]
5. Chemin G, Tinguely A, Sirac C, Lechouane F, Duchez S, Cogné M, Delpy L. Multiple RNA surveillance mechanisms cooperate to reduce the amount of nonfunctional *Igκ* transcripts. J Immunol. 2010; 184:5009–5017. [PubMed: 20357261]
6. Jankovic M, Nussenweig MC. OcaB regulates transitional B cell selection. Internat Immunol. 2003; 15:1099–1104.
7. Ishida D, Su L, Tamura A, Katayama Y, Kawai Y, Wang SF, Taniwaki M, Hamazaki Y, Hattori M, Minato N. Rap1 signal controls B cell receptor repertoire and generation of self-reactive B1a cells. Immunity. 2006; 24:417–427. [PubMed: 16618600]
8. Brekke KM, Garrard WT. Assembly and analysis of the mouse immunoglobulin kappa gene sequence. Immunogenetics. 2004; 56:490–505. [PubMed: 15378297]
9. Aoki-Ota M, Torkamani A, Ota T, Schork N, Nemazee D. Skewed primary *Igκ* repertoire and V-J joining in C57BL/6 mice: implications for recombination accessibility and receptor editing. J Immunol. 2012; 188:2305–2315. [PubMed: 22287713]

10. Schlissel MS. Regulation of activation and recombination of the murine Igkappa locus. Immunol Rev. 2004; 200:215–223. [PubMed: 15242407]
11. Parslow TG, Blair DL, Murphy WJ, Granner DK. Structure of the 5′ ends of immunoglobulin genes: a novel conserved sequence. Proc Natl Acad Sci USA. 1984; 81:2650–2654. [PubMed: 6425835]
12. Queen C, Baltimore D. Immunoglobulin gene transcription is activated by downstream sequence elements. Cell. 1983; 33:741–748. [PubMed: 6409419]
13. Meyer KB, Neuberger MS. The immunoglobulin κ locus contains a second, stronger B-cell-specific enhancer which is located downstream of the constant region. EMBO J. 1989; 8:1959–1964. [PubMed: 2507312]
14. Liu ZM, George-Raizen JB, Li S, Meyers KC, Chang MY, Garrard WT. Chromatin structural analyses of the mouse *Igκ* gene locus reveal new hypersensitive sites specifying a transcriptional silencer and enhancer. J Biol Chem. 2002; 277:32640–32649. [PubMed: 12080064]
15. Vela JL, Ait-Azzouzene D, Duong BH, Ota T, Nemazee D. Rearrangement of mouse immunoglobulin kappa deleting element recombining sequence promotes immune tolerance and lambda B cell production. Immunity. 2008; 28:161–170. [PubMed: 18261939]
16. Yancopoulos GD, Alt FW. Developmental controlled and tissue-specific expression of unrearranged VH gene segments. Cell. 1985; 40:271–281. [PubMed: 2578321]
17. Schlissel MS, Baltimore D. Activation of immunoglobulin kappa gene rearrangement correlates with induction of germline kappa gene transcription. Cell. 1989; 58:1001–1007. [PubMed: 2505932]
18. Amin RH, Cado D, Nolla H, Huang D, Shinton SA, Zhou Y, Hardy RR, Schlissel MS. Biallelic, ubiquitous transcription from the distal germline *Igk* locus promoter during B cell development. Proc Natl Acad Sci USA. 2009; 106:522–527. [PubMed: 19116268]
19. Cocea L, DeSmet A, Saghatchian M, Fillatreau S, Ferradini L, Schurmans S, Weill JC, Reynaud CA. A targeted deletion of a region upstream from the Jκ cluster impairs κ chain rearrangement in *cis* in mice and in the 103/bcl2 cell line. J Exp Med. 1999; 189:1443–1449. [PubMed: 10224284]
20. Inlay M, Alt FW, Baltimore D, Xu Y. Essential roles of the κ light chain intronic enhancer and 3′ enhancer in κ rearrangement and demethylation. Nat Immunol. 2002; 3:463–468. [PubMed: 11967540]
21. Zhou X, Xiang Y, Garrard WT. The *Igκ* gene enhancers, E3′ and Ed, are essential for triggering transcription. J Immunol. 2010; 185:7544–7552. [PubMed: 21076060]
22. Ji Y, Little AJ, Banerjee JK, Hao B, Oltz EM, Krangel MS, Schatz DG. Promoters, enhancers, and transcription target RAG1 binding during V(D)J recombination. J Exp Med. 2010; 207:2809–2816. [PubMed: 21115692]
23. Ji Y, Resch W, Corbett E, Yamane A, Casellas R, Schatz DG. The *in vivo* pattern of binding of RAG1 and RAG2 to antigen receptor loci. Cell. 2010; 141:419–431. [PubMed: 20398922]
24. Roldán E, Fuxa M, Chong W, Martinez D, Novatchkova M, Busslinger M, Skok JA. Locus ‘decontraction’ and centromeric recruitment contribute to allelic exclusion of the immunoglobulin heavy-chain gene. Nature Immunol. 2005; 6:31–41. [PubMed: 15580273]
25. Sayegh C, Jhunjhunwala S, Riblet R, Murre C. Visualization of looping involving the immunoglobulin heavy-chain locus in developing B cells. Genes Dev. 2005; 19:322–327. [PubMed: 15687256]
26. Jhunjhunwala S, van Zelm MC, Peak MM, Cutchin S, Riblet R, van Dongen JJ, Grosfeld F, Knoch TA, Murre C. The 3D structure of the immunoglobulin heavy-chain locus: implications for long-range genomic interactions. Cell. 2008; 133:265–279. [PubMed: 18423198]
27. Fuxa M, Skok J, Souabni A, Salvagiotto G, Roldan E, Busslinger M. Pax5 induces V-to-DJ rearrangements and locus contraction of the immunoglobulin heavy-chain gene. Genes Dev. 2004; 18:411–422. [PubMed: 15004008]
28. Liu H, Schmidt-Supprian M, Shi Y, Hobeika E, Barteneva N, Jumas H, Pelanda R, Reth M, Skok J, Rajewsky K, Shi Y. Yin Yang 1 is a critical regulator of B-cell development. Genes Dev. 2007; 21:1179–1189. [PubMed: 17504937]

29. Reynaud D I, Demarco A, Reddy K, Schjerven H, Bertolino E, Chen Z, Smale ST, Winandy S, Singh H. Regulation of B cell fate commitment and immunoglobulin heavy-chain gene rearrangements by Ikaros. Nature Immunol. 2008; 9:927–936. [PubMed: 18568028]
30. Volpi SA, Verma-Gaur J, Hassan R, Ju Z, Roa S, Chatterjee S, Werling U, Hou H Jr, Will B, Steidl U, Scharff M, Edelman W, Feeney AJ, Birshtein BK. Germline deletion of *Igh* 3′ regulatory region elements hs 5, 6, 7 (hs5–7) affects B cell-specific regulation, rearrangement, and insulation of the *Igh* locus. J Immunol. 2012; 188:2556–2566. [PubMed: 22345664]
31. Hewitt SI, Farmer D, Marszalek K, Cadera E, Liang HE, Xu Y, Schlissel MS, Skok JA. Association between the *Igκ* and *Igh* immunoglobulin loci mediated by the 3′ *Igκ* enhancer induces 'decontraction' of the lgh locus in pre-B cells. Nature Immunol. 2008; 9:396–404. [PubMed: 18297074]
32. Corcoran AE. The epigenetic role of non-coding RNA transcription and nuclear organization in immunoglobulin repertoire generation. Semin Immunol. 2010; 22:353–361. [PubMed: 20863715]
33. Verma-Gaur J, Torkamani A, Schaffer L, Head SR, Schork NJ, Feeney AJ. Noncoding transcription within the *Igh* distal Vh region at PAIR elements affects the 3D structure of the *Igh* locus in pro-B cells. Proc Natl Acad Sci USA. 2012; 109:17004–17009. [PubMed: 23027941]
34. Guo C, Gerasimova T, Hao H, Ivanova I, Chakraborty T, Selimyan R, Oltz EM, Sen R. Two forms of loops generate the chromatin conformation of the immunoglobulin heavy-chain gene locus. Cell. 2011; 147:332–343. [PubMed: 21982154]
35. Su IH, Basavaraj A, Krutchinsky AN, Hobert O, Ullrich A, Chait BT, Tarakhovsky A. Ezh2 controls B cell development through histone H3 methylation and *Igh* rearrangement. Nat Immunol. 2003; 4:124–131. [PubMed: 12496962]
36. Degner SC, Verma-Gaur J, Wong TP, Bossen C, Iverson GM, Torkamani A, Vettermann C, Lin YC, Ju Z, Schulz D, Murre CS, Birshtein BK, Schork NJ, Schlissel MS, Riblet R, Murre C, Feeney AJ. CCCTC-binding factor (CTCF) and cohesin influence the genomic architecture of the *Igh* locus and antisense transcription in pro-B cells. Proc Natl Acad Sci USA. 2011; 108:9566–9571. [PubMed: 21606361]
37. Guo C, Yoon HS, Franklin A, Jain S, Ebert A, Cheng HL, Hansen E, Despo O, Bossen C, Vettermann C, Bates JG, Richards N, Myers D, Patel H, Gallagher M, Schlissel MS, Murre C, Busslinger M, Giallourakis CC, Alt FW. CTCF-binding elements mediate control of V(D)J recombination. Nature. 2011; 477:424–430. [PubMed: 21909113]
38. Ribeiro de Almeida C, Stadhouders R, de Bruijn MJ, Bergen IM, Thongjuea S, Lenhard B, van Ijcken W, Grosveld F, Galjart N, Soler E, Hendriks RW. The DNA-binding protein CTCF limits proximal Vκ recombination and restricts κ enhancer interactions to the immunoglobulin κ light chain locus. Immunity. 2011; 35:501–513. [PubMed: 22035845]
39. Xiang Y, Zhou X, Hewitt SL, Skok JA, Garrard WT. A multifunctional element in the mouse *Igκ* locus that specifies repertoire and *Ig* loci subnuclear location. J Immunol. 2011; 186:5356–5366. [PubMed: 21441452]
40. Tallquist MD, Soriano P. Epiblast-restricted Cre expression in MORE mice: A tool to distinguish embryonic vs. extra-embryonic gene function. Genesis. 2000; 26:113–115. [PubMed: 10686601]
41. Liu Z, Widlak P, Zou Y, Xiao F, Oh M, Li S, Chang MY, Shay JW, Garrard WT. A recombination silencer that specifies heterochromatin positioning and Ikaros association in the immunoglobulin κ locus. Immunity. 2006; 24:405–415. [PubMed: 16618599]
42. Bao L, Zhou M, Cui Y. CTCFBSDB: a CTCF binding site database for characterization of vertebrate genomic insulators. Nucleic Acids Res. 2008; 36:D83–D87. [PubMed: 17981843]
43. Degner SC, Wong TP, Jankevicius G, Feeney AJ. Cutting edge: Developmental stage-specific recruitment of cohesin to CTCF sites throughout immunoglobulin loci during B lymphocyte development. J Immunol. 2009; 182:44–48. [PubMed: 19109133]
44. Benedict CL, Gilfillan S, Thai TH, Kearney JF. Terminal deoxynucleotidyl transferase and repertoire development. Immunol Rev. 2000; 175:150–157. [PubMed: 10933600]
45. Lin YC, Jhunjhunwala S, Benner C, Heinz S, Welinder E, Mansson R, Sigvardsson M, Hagman J, Espinoza CA, Dutkowski J, Ideker T, Glass CK, Murre C. A global network of transcription factors, involving E2A, EBF1 and Foxo1, that orchestrates B cell fate. Nat Immunol. 2010; 11:635–643. [PubMed: 20543837]

46. Heinz S, Benner C, Spann N, Bertolino E, Lin YC, Laslo P, Cheng JX, Murre C, Singh H, Glass CK. Simple combinations of lineage-determining transcription factors prime *cis*-regulatory elements required for macrophage and B cell identities. Mol Cell. 2010; 38:576–589. [PubMed: 20513432]

47. Nemazee D. Receptor editing in lymphocyte development and central tolerance. Nat Rev Immunol. 2006; 6:728–740. [PubMed: 16998507]

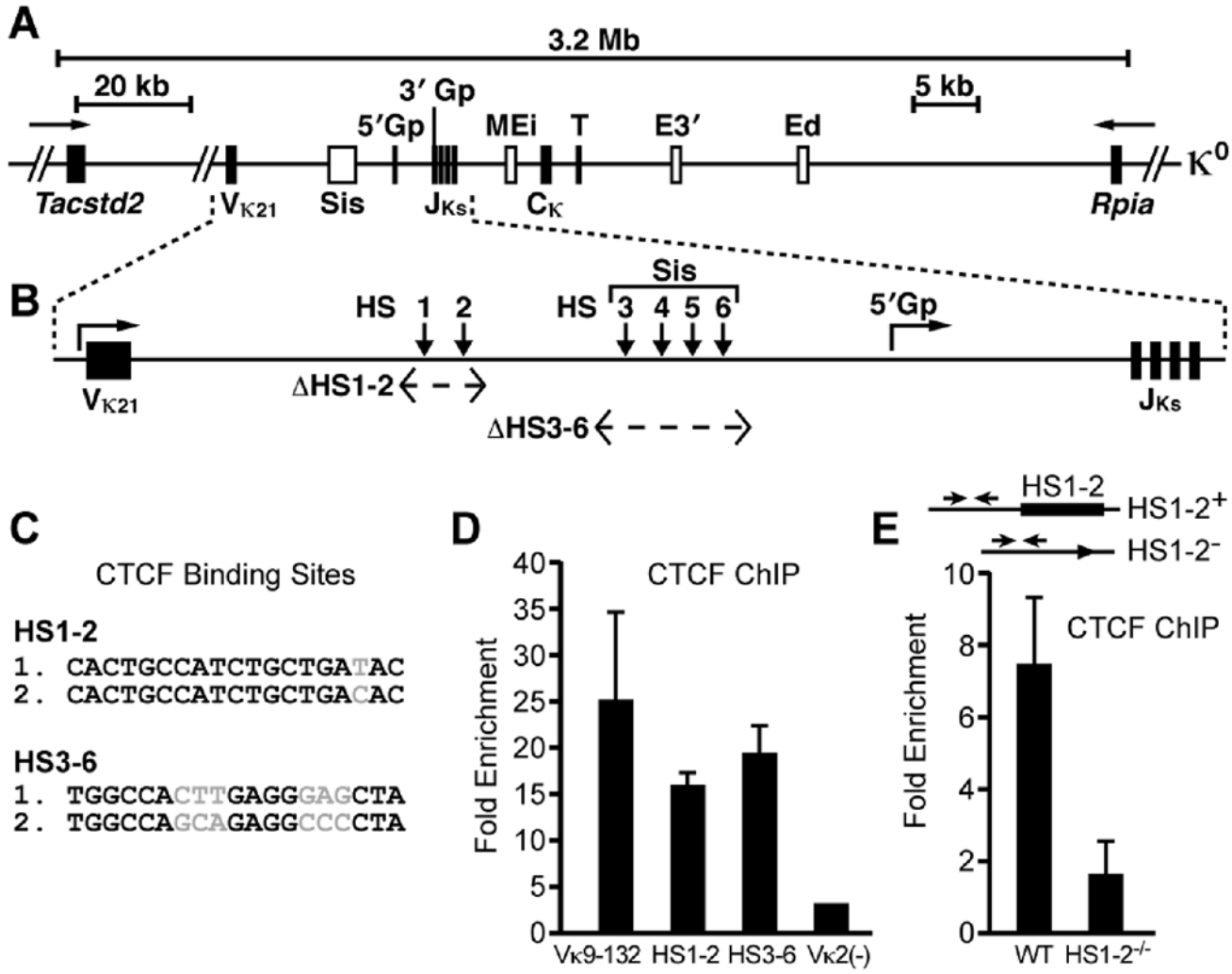

FIGURE 1.

Map of the mouse *Igκ* gene locus, highlighting features of the Vκ–Jκ intervening region. *A,* Schematic diagram of the *Igκ* gene locus, with exons indicated as closed rectangles, and *cis*-acting sequences indicated as open rectangles, which include Sis, 5′ and 3′ germline promoters (Gp), and downstream enhancers (E). T indicates the termination region of transcription. The arrows at the locus boundaries indicate the directions of transcription of flanking housekeeping genes. *B,* Schematic diagram of the Vκ–Jκ intervening region indicating the location of DNase I hypersensitive sites 1 to 6 (vertical arrows). The bracketed horizontal dashed lines indicate the positions of deletion mutants. The horizontal arrows indicate the directions of transcription from a Vκ21 gene and the 5′ germline promoter (5′Gp). *C, In silico* predicted CTCF binding sites within HS1-2 and HS3-6 (Sis). The scores for these predicted CTCF binding sites were larger than 10. Usually a sequence with a score >3.0 is a suggestive match for a CTCF binding site (42). Base mismatches between the site pairs are depicted in grey. *D,* CTCF enrichment in HS1-2 and HS3-6 in pre-B cells from $Rag1^{-/-}\mu^{+}$ mice as assayed by ChIP. Vκ9-132 is known to possess CTCF binding positive sites and served as a positive control, whereas Vκ2(−) lacks such sites and served as a negative control (43). Data are the means ± SD of 3 independent experiments. *E,* HS1-2 deletion eliminates localized CTCF binding in pre-B cells. Real-time PCR ChIP assays of CTCF occupancy for WT and HS1-$2^{-/-}$ samples. Map of the HS1-2 element and

upstream PCR primers used in the ChIP assay common to alleles possessing or lacking HS1-2 (upper, small arrows). Results are the means ± SD of 3 independent experiments.

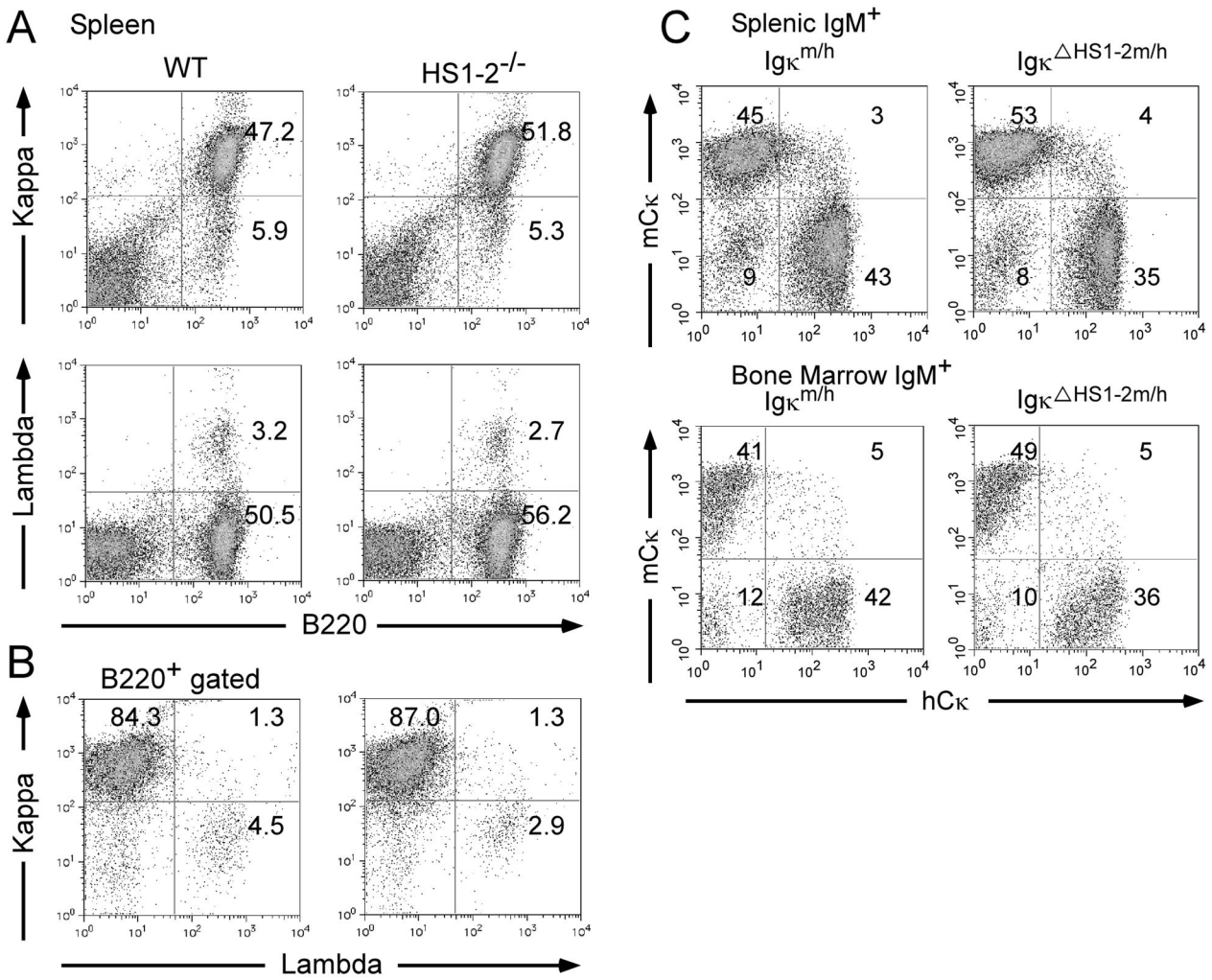

FIGURE 2.

HS1-2$^{-/-}$ mice exhibit increased Igκ^{+} B cells. *A,* FACS analysis of cell surface Ig expression in WT and HS1-2$^{-/-}$ mice splenic cells. Single cell suspensions from spleen were stained with anti-B220 and anti-Igκ antibodies (upper); or anti-B220 and anti-Igλ antibodies (lower). Only cells residing in the live lymphocyte gate were analyzed. Percentages of cells residing in various windows are shown in the figure sub-panels. Data are representative of independent FACS analyses from at least 10 mice of each genotype. For B220^{+}Igκ^{+} cells: HS1-2$^{-/-}$ vs WT, p=0.004; (Student's t-test). *B,* Igκ vs Igλ usage and IgL isotype exclusion in WT and HS1-2$^{-/-}$ splenic cells were analyzed by FACS. *C,* FACS analysis of the effect of HS1-2 deletion on mCκ allele usage and Igκ allelic exclusion.

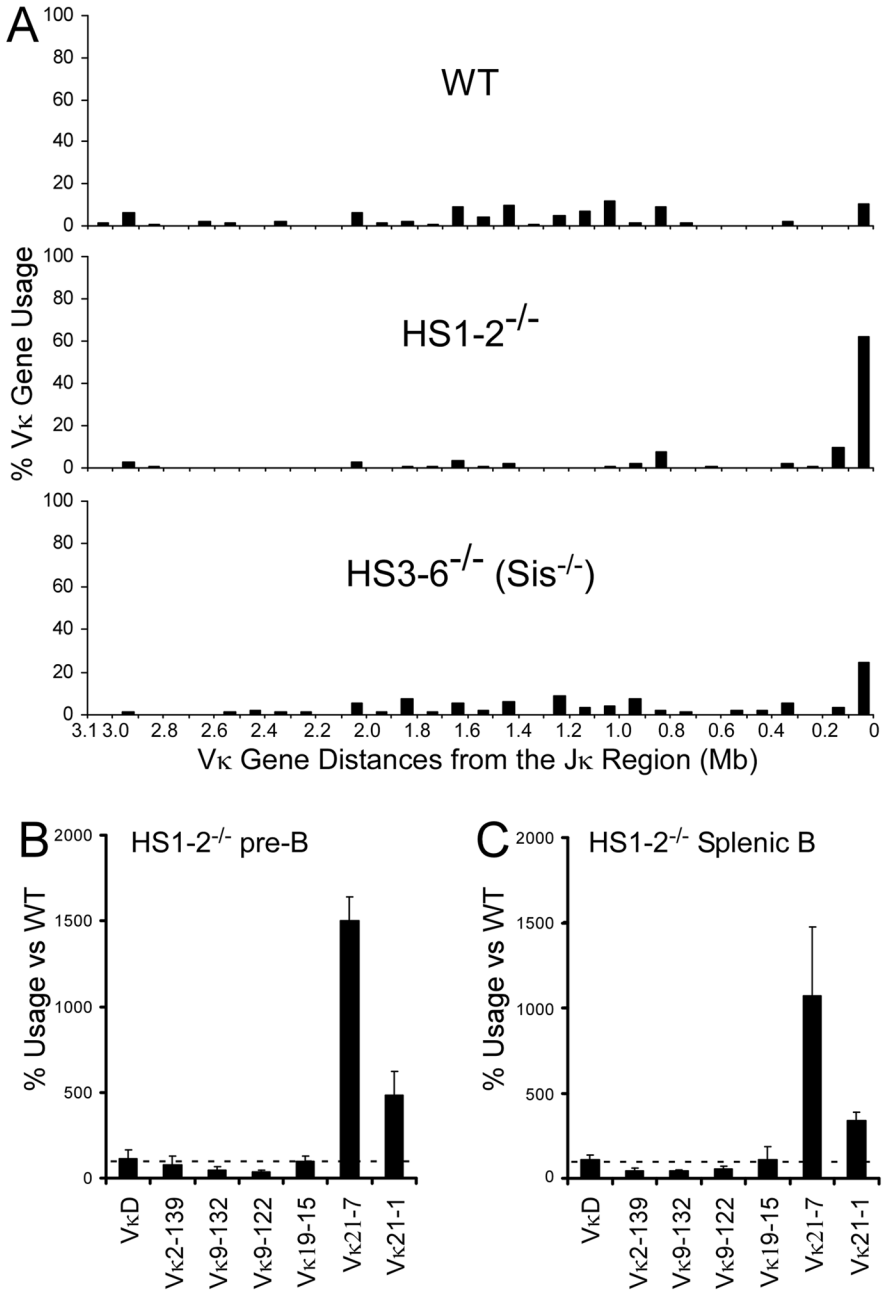

FIGURE 3.

HS1-2$^{-/-}$ mice exhibit dramatically altered Vκ gene usage. *A,* The Vκ-Jκ1 rearrangement products of pre-B cells from WT, HS1-2$^{-/-}$ and HS3-6$^{-/-}$(Sis$^{-/-}$) mice were amplified from genomic DNA by PCR and cloned into the pGEM-T vector. More than 100 independently determined Vκ gene sequences from each group were identified by IgBlast. Their percent usage relative to the total Vκ gene usage as 100% are presented in 0.1 Mb interval distances from the Jκ-region along the *Igκ* gene locus. *B and C,* Analysis of relative Vκ gene usage by real-time PCR assays. Vκ gene specific primers and a primer downstream of Jκ1 were used to assay for specific Vκ-Jκ1 rearrangements in genomic DNA samples. The % usage of Vκ genes was compared with those of WT mice in which the % usage was set as 100%

(depicted as dashed lines). Data are means ± SD of 3 independent experiments. The distances of these Vκ genes from the Jκ1-region are: Vκ2-139, 3,085 kb; Vκ9-132, 2,854 kb; Vκ9-122, 2,740 kb; Vκ19-15, 311 kb; Vκ21-7, 111 kb; Vκ21-1, 18 kb (8). *B,* Usage of different Vκ genes in pre-B cells from HS1-2$^{-/-}$ mice. *C,* Usage of different Vκ genes in splenic B cells from HS1-2$^{-/-}$ mice.

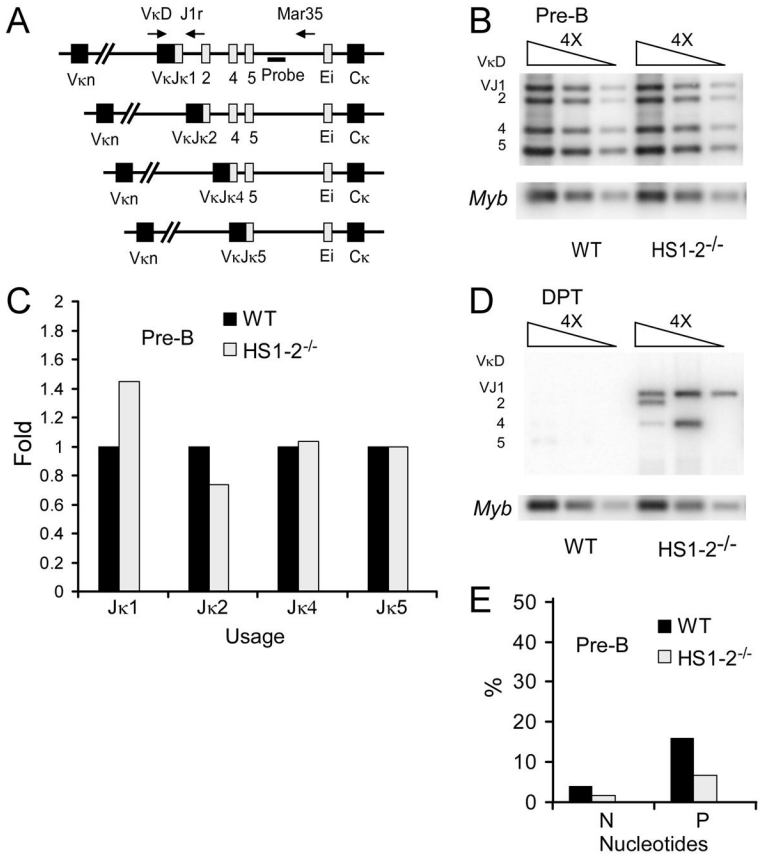

FIGURE 4.

Analysis of Jκ-region usage, and the developmental timing and tissue-specificity of Vκ–Jκ rearrangement. *A,* Schematic diagrams (not to scale) of the PCR assays used for determining Jκ-region usage or N and P nucleotide insertions. The top map shows the positions of a degenerate VκD gene 5′ primer, and J1r and Mar35 3′ primers along an *Igκ* gene germline locus. Also shown is the position of a probe (solid bar) used in Southern blotting. Below are shown the four possible recombination products resulting from Vκ joining to the different Jκ–regions. *B,* Jκ-region usage analysis. The Vκ-Jκn rearrangement products of pre-B cell samples were PCR amplified using VκD and MAR35 primers. Reaction products were separated by electrophoreses on agarose gels, and the intensities of Vκ-Jκ1 to Vκ-Jκ5 bands were visualized by PhosphorImaging of Southern blots. The PCR amplifications of *c-myb* are shown at the bottom, which were used as loading controls for the amount of genomic DNA template in the PCR reactions. *C,* The relative usage of the indicated Jκ-regions determined by quantitation of Phosphorimages are shown as ratios for pre-B cell samples from HS1-2$^{-/-}$ mice compared with those of WT in which Jκ–region usage was set as 1. *D,* Vκ-Jκ rearrangement products of thymus double positive T (DPT) cells from the indicated genetic lines of mice were PCR amplified and the intensities of Vκ-Jκ1 to Vκ-Jκ5 bands were visualized by PhosphorImager analysis of Southern blots. *E,* Analysis of N and P nucleotides in Vκ-Jκ1 junction regions. The Vκ-Jκ1 rearrangement products were PCR-amplified using VκD and J1r primers from genomic DNA of pre-B cell samples from WT and HS1-2$^{-/-}$ mice and cloned into the pGEM-T vector. N and P nucleotide in more than 100 sequenced samples from each genetic mouse line were analyzed.

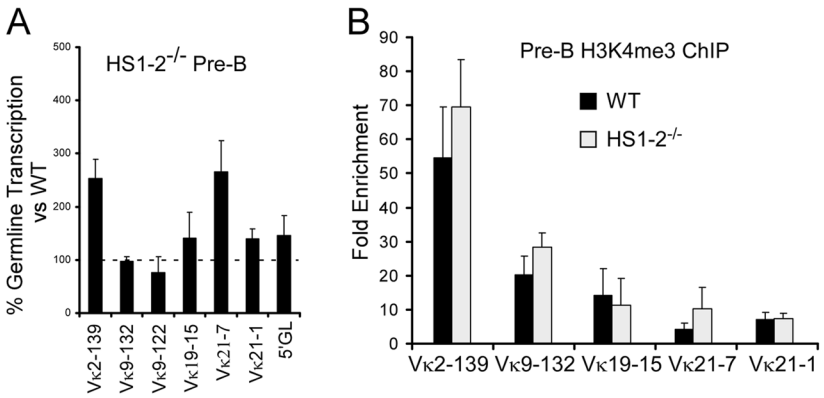

FIGURE 5.

Levels of Vκ gene germline transcription and H3K4me3 modification in pre-B cells. *A,* Real-time PCR assays were used to measure *Igκ* gene germline transcripts arising from the 5′ promoter (5′GL) and from the indicated Vκ genes in pre-B cell samples from WT and HS1-2$^{-/-}$ mice. Data are means ± SD of 3 independent experiments. *B,* Real time PCR ChIP assays of H3K4me3 levels in Vκ gene RSS regions in pre-B cells of WT and HS1-2$^{-/-}$ mice. Data are presented as means ± SD (n=3). The distances of these Vκ genes from the Jκ1-region are given in Fig. 3.

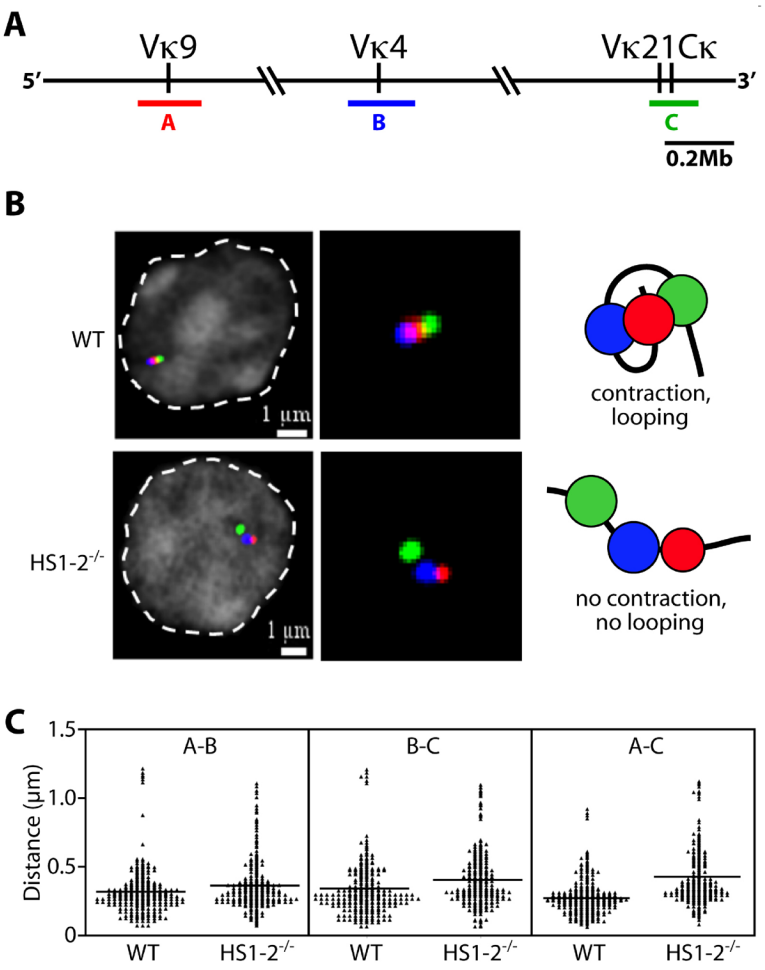

FIGURE 6.

HS1-2$^{-/-}$ pre-B cells exhibit reduced *Igκ* gene locus contraction. *A,* Map of the *Igκ* locus indicating the positions of color-coded BAC probes (A: RP23-101G13, red; B: RP23-26A6, blue; and C: RP24-387E13, green). The center-to-center distances between these probes in naked DNA are: A-B, 1,128 kb; B-C, 1,780 kb; and A-C, 2,908 kb. *B,* The left panels show representative 3D DNA FISH confocal optical sections from WT and HS1-2$^{-/-}$ mice pre-B cell nuclei, outlined by white dashed lines, as identified by DAPI DNA staining (white). The center panels represent enlargements of the corresponding hybridization images. The right panels depict schematic interpretations of the looping patterns of the loci based on the probe hybridization patterns. *C,* Dot plots representing measured center-to-center distances between the indicated hybridizing signals of the *Igκ* locus BAC probes. Data from 263 WT and 222 HS1-2$^{-/-}$ pre-B cell alleles were accumulated from measurements in several independent experiments. Mean distances are indicated by the horizontal lines, which are significantly different between WT and HS1-2$^{-/-}$ samples (Student's t-test, A-B, p=0.004; B-C, p=2.6e^{-5}; A-C, p=1.8e^{-17}).