Vκ Gene Repertoire and Locus Contraction Are Specified by Critical DNase I Hypersensitive Sites within the Vκ-Jκ Intervening Region

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J Immunol 2013; 190:1819-1826; Prepublished online 7 January 2013;
doi: 10.4049/jimmunol.1203127
http://www.jimmunol.org/content/190/4/1819
V\textsubscript{k} Gene Repertoire and Locus Contraction Are Specified by Critical DNase I Hypersensitive Sites within the V\textsubscript{k}-J\textsubscript{k} Intervening Region

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The processes of Ig gene locus contraction and looping during V(D)J-recombination are essential for creating a diverse Ab repertoire. However, no cis-acting sequence that plays a major role in specifying locus contraction has been uncovered within the Ig\textsubscript{k} gene locus. In this article, we demonstrate that a 650-bp sequence corresponding to DNase I hypersensitive sites HS1–2 within the mouse Ig\textsubscript{k} gene V-J intervening region binds CCCTC-binding factor and specifies locus contraction and long-range V\textsubscript{k} gene usage spanning 3.2 Mb in pre-B cells. We call this novel element Cer (for “contracting element for recombination”). Targeted deletion of Cer caused markedly increased proximal and greatly diminished upstream V\textsubscript{k} splenic Ig usage, with higher allele usage, more splenic Ig\textsuperscript{\*} B cells, and nonlinear-specific Ig\textsubscript{k} rearrangements in T cells. Relative to wild-type mice, Cer-deletion mice exhibited similar levels of V\textsubscript{k} 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 V\textsubscript{k}-J\textsubscript{k} intervening region are essential for controlling locus contraction and creating a diverse Ab repertoire. \textit{The Journal of Immunology}, 2013, 190: 1819–1826.

The Ig V gene primary Ab repertoire is generated in B lymphocytes by the process of V(D)J-recombination mediated by RAG-encoded recombinases and nonhomologous 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, misregulated or incorrect repertoire specification can trigger autoimmunity (6, 7).

The mouse Ig\textsubscript{L} L chain gene is the largest multigene family locus thus far identified, spanning 3.2 Mb on mouse chromosome 6 (8). It consists of 100 functional V\textsubscript{k} gene exons (9), four functional J\textsubscript{k} region exons, and a single C\textsubscript{k} exon (Fig. 1A). Following V(D)J-recombination of the Ig\textsubscript{H} H chain gene locus during the pro-B cell stage of development, the Ig\textsubscript{k} gene locus is poised for rearrangement in pre-B cells, whereby a V\textsubscript{k} gene becomes covalently joined to a J\textsubscript{k} region (1, 10). This recombination event results in transcriptional activation because it positions a V\textsubscript{k} 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 Ig\textsubscript{k} gene V-J joining is productively unsuccessful because of out-of-reading frame recombination junctions, then the Ig\textsubscript{k} locus becomes activated for rearrangement and expression, which, in wild type (WT) mice, accounts for production of only ∼5% of the total Ig\textsubscript{L} 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’ J\textsubscript{k}-region germline promoter, which is known to be the most significant in pre-B cells for generating germline transcripts (18), is highly detrimental to Ig\textsubscript{k} gene rearrangement in knockout mice (19). Moreover, such germline transcription requires the Ig\textsubscript{k} gene downstream enhancers, because their targeted deletion leads to a block in V\textsubscript{k}-J\textsubscript{k} joining (20, 21). More recently, intact promoters, enhancers, and transcriptional elongation were directly shown to control the binding of RAG1 to recombination signal sequences in the T\textsubscript{cra} and T\textsubscript{crb} loci at Ja and Db/Jb segments, directly validating the accessibility model (22). Furthermore, the RAG proteins were demonstrated to bind in vivo to Jh and closely linked DQ52 segments in pro-B cells, as well as to J\textsubscript{k} regions in pre-B cells (23). It was 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 V\textsubscript{k} 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 Ig\textsubscript{H} and Ig\textsubscript{k} loci exhibit contraction and looping of V genes into rosette-like structures, which juxtaposes them near Dh or J\textsubscript{k} regions in preparation for rearrangement (24–26). Furthermore, reduced contraction of Ig\textsubscript{H} loci results in a skewed repertoire, with
proximal Vh genes being preferentially used (24, 27–30), whereas persistent contraction results in greater distal Vh gene rearrangements (31). Decontraction occurs after rearrangement (24). It was proposed that germline transcription of Igk 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 Igk 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), and Ikaros (29); and the chromatin modifying enzyme, Ezh2 (35). More recently, CCCCTC-binding factor (CTCF)/cohesin proteins implicated in looping and insulation were also shown to contribute modestly to locus contraction in the Igk locus (30, 36). Furthermore, specific CTCF-binding elements in the Vh-Dh intervening sequence were 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 Igk 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 downregulation of their localized germline transcription (37, 38).

In this article, we characterize DNase I hypersensitive sites (HS)1–2 as new CTCF-binding elements in the mouse Igk gene locus V-J intervening sequence and demonstrate that this 650-bp DNA segment is responsible for locus contraction and long-range Vk gene usage spanning 3.2 Mb. We term this novel element Cer (for “contracting element for recombination”). Deletion of Cer markedly increased Jk-proximal Vk gene usage and decreased middle and distal Vk gene usage without significantly affecting localized germline transcription or a canonical positive epigenetic mark in chromatin. To our knowledge, these results identify the first cis-acting DNA element that plays a major role in Igk gene locus contraction and repertoire specification during Vk-Jk recombination.

Materials and Methods

Mouse strains

Mice possessing a 0.65-kb deletion of HS1–2 in the endogenous Igk gene locus were generated by standard embryonic stem cell–targeting technology; germline-transmissible mice were bred with Cre recombinase expressing mice (Supplemental Table I). Mice possessing a 0.65-kb deletion of HS1–2 in the endogenous Igk1 rearrangement (40) mice to obtain HS1–2 and neor deletion mice (Supplemental Table I). Three animals of the same genetic background for cell fractionation. The three animals of the same genetic background for cell fractionation. The three animals of the same genetic background for cell fractionation. The three animals of the same genetic background for cell fractionation. The three animals of the same genetic background for cell fractionation. The three animals of the same genetic background for cell fractionation.

Flow cytometry and cell fractionation

Single-cell suspensions were prepared from bone marrow and spleens of 6–14-wk-old mice, as described (39). Single-cell suspensions were stained with Abs and analyzed using FACSCalibur with CellQuest software (BD Biosciences, San Diego, CA) or FlowJo software (TreeStar, 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 two or three animals of the same genetic background for cell fractionation. The following Abs were used: anti–mouse-Igk-PE (BD Biosciences), anti–mouse-Igα1,2,3-FTTC (BD Biosciences), anti–human-Igk-FTTC (Southern Biotech, Birmingham, AL), anti–B220-PerCP-Cy5.5 (BD Biosciences), anti-lgk–allophycocyanin (BD Biosciences), anti–CD43–PE (BD Biosciences), anti–B220-FTTC (BD Biosciences), anti–CD19-biotin (BD Biosciences), and streptavidin–allophycocyanin (Southern Biotech).

Analysis of Igk gene repertoire, Vk-Jk1 rearrangement, and germline transcription

These assays were performed as previously described (39). For analysis of Igk gene repertoire and Vk-Jk1 rearrangement, genomic DNA was purified from sorted B cell populations. For Igk gene repertoire analysis, the VkD primer and a primer in the Jk1 intron were used to amplify Vk-Jk1 rearrangements; resulting PCR products were gel purified and subcloned into the pGEM-T vector (Promega, San Luis Obispo, CA). Determined sequences of Vk genes in each clone were identified by the IgBlast program (National Center for Biotechnology Information, Bethesda, MD). For real-time PCR analysis of individual Vk-Jk1 rearrangements, forward primers specific to different Vk exons and a reverse primer complementary to the Jk1 to Jk2 intron region were used (primer sequences are listed in Supplemental Table I). Different Vk-Jk1 rearrangements were determined quantitatively using SYBR Green PCR master mix (Bio-Rad, Richmond, CA) in the 7300 real-time PCR system (Invitrogen, Carlsbad, CA). PCR was performed based on the manufacturer’s protocols, and each PCR assay was carried out in duplicate or triplicate. Relative rearrangements were calculated using the ΔCt method, according to the manufacturer’s instructions, and normalized to a β-actin genomic region. To examine Igk gene germline transcription, total RNA was extracted from 1 × 106 MoFlo-sorted pre-B cells using TRIzol reagent (Invitrogen). Then RNA was treated with DNase I and reverse transcribed into cDNA with SuperScript III Reverse Transcriptase (Invitrogen). For real-time PCR analysis of individual Vk gene’s germline transcripts, forward primers specific to different Vk genes and a reverse primer complementary to the downstream recombination signal sequence region were used (Supplemental Table I). For analysis of transcripts arising from the 5’ germine promoter upstream of the Jk1 region, a forward 5’GT-f primer annealing immediately downstream of the promoter region and a reverse Cc-r primer annealing in Cc exon were used in real-time PCR assays (Supplemental Table I). Transcript levels were calculated using the ΔCt 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 DNA FISH were prepared from bacterial artificial chromosomes (BACs). We used RP23-101G13, RP23-26A6, and RP24-387E13, which correspond to the 5’, middle, and the 3’ region of the Igk 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 plugin of ImageJ software, as described (39).

Chromatin immunoprecipitation

For CTCF chromatin immunoprecipitation (ChIP), ~2 × 106 sorted pre-B cells (Fig. 1E) or CD19+ pre-B cells from Rag1−/−, μ−/− transgenic animals (Fig. 1F) were used for each ChIP experiment. ChIP experiments were conducted according to the protocol of Millipore. Rabbit anti-CTCF Abs (07-729) and rabbit anti-H3K4me3 Abs (07-473; both from Millipore) were used for ChIP. For real-time PCR analysis of the H3K4me3 modification levels of individual Vk gene’s recombination signal sequences, we used the same primers as those used for Vk gene’s germline transcript analysis (Supplemental Table I). Real-time PCR was performed and quantitated using the 7300 Real Time PCR System (Invitrogen) with SYBR Green, as described above, and enrichment of target regions in ChIP was normalized to actin (primer sequences are listed in Supplemental Table I).

Results

Generating mice with a targeted deletion of HS1–2

We previously showed that the Vk-Jk intervening region exhibits six DNase I hypersensitive sites (HS1–6) in cells of the B lymphocyte lineage (Fig. 1A, 1B) (14). In addition, our functional analyses of HS3–6 revealed transcription and recombination silence activity; hence, the sequence was termed Sis (“silencer in the intervening sequence”) (14, 41). Sis binds Ikaros and CTCF and is responsible for dampening Jc-proximal Vk gene usage during Vc-Jk rearrangement in pre-B cells (39, 41). In this study, we characterize the function of HS1–2 by creating a targeted
deletion of this element in the mouse germline (Fig. 1B, Supplemental Fig. 1). 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. 1C). 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. 1D). Moreover, pre-B cells from mice possessing a deletion of HS1–2 no longer exhibited localized binding of CTCF (Fig. 1E), just as we showed previously for the effect of deletion of HS3–6 (Sis) (39).

**Deletion of HS1–2 leads to increased Igk-gene-expressing 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 Igk+ B cells compared with their WT littermates or age-matched WT mice (Fig. 2A). In contrast, the percentages of splenic Igλ+ B cells were at similar levels among these groups (Fig. 2A). Hence, the splenic Igk+/Igλ+ B cell ratios were slightly increased in HS1-2–/– mice relative to controls (Fig. 2B). The percentages of Igκ+Igλ+ double-positive cells were the same between WT and HS1-2–/– mice, indicating that IgL chain isotype exclusion was still intact (Fig. 2B). To further characterize the effects of this deletion, we bred HS1-2–/– mice with a line carrying a human Cc knockin allele to obtain Igκm/m and IgκHs1–2m/m heterozygotes (2). We found that mCc and hCc alleles were used equally in Igκm/m heterozygotes, as reported previously (Fig. 2C) (2). However, in mice bearing a deletion in HS1–2, mCc alleles exhibited a modest preference to be used in the heterozygotes, both in splenic cells and bone marrow (Fig. 2C, upper and lower panels, 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 hCc alleles. We also found that the heterozygotes from these groups exhibited similar levels of mCc*hCc+ double-positive cells (Fig. 2C), suggesting that HS1–2 deletion did not affect allelic exclusion.

**Deletion of HS1–2 dramatically increased Jk-proximal Vk gene usage and decreased the usage of middle and distal Vk genes**

Previously, we demonstrated that deletion of HS3–6 (Sis) caused increased Jk-proximal Vk gene usage during Vk-Jk rearrangement in pre-B cells (39). To determine whether deletion of HS1–2 also altered primary Vk gene usage, we cloned and sequenced Vκ-Jκ rearrangement products from WT and mutant mice pre-B cells. As shown in Fig. 3A, Vκ genes within the first 0.1-Mb interval closest to the Jk region were heavily used in pre-B cells from HS1-2–/– mice, accounting for 62% of the total Vκ gene usage. At the same time, the percentage usage of Vκ genes in middle and distal Igk gene regions was decreased dramatically (Fig. 3A). In 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. 3A). We also used real-time PCR to quantitate relative Vκ-Jκ 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. 3B, 3C). 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. 3B, 3C). We conclude that HS1–2 within the Vκ-Jκ intervening region dramatically specifies Vκ gene usage.

**Deletion of HS1–2 increases Vk-proximal Jk-region usage and allows Igk gene rearrangement in T cells**

To determine whether the Jk-region usage or tissue specificity of rearrangement was altered in our mutant mice, we used 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-Jk1, V-Jk2, V-Jk4, and V-Jk5 recombination products (Fig. 4A). Examination of the relative usage of Jk regions in pre-B cells revealed that the mutant mice still used all four Jk regions in Vκ-Jκ joining (Fig. 4B). However, quantitation of these data revealed that the usage of the Jk1 and Jk2 regions was increased and decreased ~50 and 30%, respectively, in pre-B cells from HS1-2–/– mice compared with WT controls, whereas usage of the other Jk regions was unaltered (Fig. 4C). Furthermore, we found that Igκ gene rearrangements were detectable in CD4+CD8+ double-positive T cells from HS1-2–/– mice but not their WT counterparts and that these rearrangements strongly favored Jk1 usage (Fig. 4D). To investigate further the developmental timing

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**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 filled rectangles, and cis-acting sequences indicated as open rectangles, which include Sis, 3′ and 3′′ germline promoters (Gp), and downstream enhancers. 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 1 HS1–6 (vertical arrows). The bracketed horizontal dashed lines indicate the positions of deletion mutants. The horizontal arrows indicate the directions of transcription from a Vκv21 gene and the 5′ germline promoter. (C) In silico-predicted CTCF binding sites within HS1–2 and HS3–6 (Sis). The scores for these predicted CTCF binding sites were >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 gray. (D) CTCF enrichment in HS1–2 and HS3–6 in pre-B cells from Rag1–/–μκ+ 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 three 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 three independent experiments.
of rearrangement in pre-B cells, we assessed 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 deoxynucleotidyl 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 cell 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. 4E). Taken together with the results shown in Fig. 3, we conclude that HS1–2 within the Vκ-Jκ intervening region affects the choice of Vκ genes, as well as the choice of Jκ regions (i.e., 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 used real-time PCR to measure 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 ~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 levels similar to those of WT mice (Fig. 5A). These results are very similar to those that we 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 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 used 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 did the Jκ-proximal Vκ genes (Fig. 5B). 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 was 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 Vk 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. 6A, probes A, B, and C, respectively). As shown in Fig. 6B, 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), whereas corresponding samples from HS1–2−/− mice exhibited less-contracted, nonlooped patterns. To quantify these results, we measured the center-to-center distances between A and B, B and C, and A and C hybridization signals for several hundred alleles in these samples. As shown in Fig. 6C, contraction was statistically significantly decreased throughout the locus by the HS1–2 deletion. The mean distances between respective A and B, B and C, and A and C hybridization signals were 0.316, 0.326, and 0.36, 0.405, and 0.417 μm, respectively, for WT samples and 0.36, 0.405, and 0.417 μm, respectively, for HS1–2−/− mice compared with those of WT mice in which Jκ-region usage was set as 100% (depicted as dashed lines). Data are means ± SD of three independent experiments. The distances of these Vk genes from the Jκ1 region are Vκ2-139: 3085 kb; Vκ9-132: 2854 kb; Vκ9-122: 2740 kb; Vκ19-15: 311 kb; Vκ21-7: 111 kb; and Vκ21-1: 18 kb (8). (B) Usage of different Vk genes in pre-B cells from HS1–2−/− mice. (C) Usage of different Vk genes in splenic B cells from HS1–2−/− mice.

FIGURE 3. HS1–2−/− mice exhibit dramatically altered Vk gene usage. (A) The Vk–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 Vk gene sequences from each group were identified by IgBlast. Their percentage usage relative to the total Vk gene usage as 100% are presented in 0.1-Mb-interval distances from the Jκ gene locus. (B and C) Analysis of relative Vk gene usage by real-time PCR assays. Vk gene–specific primers and a primer downstream of Jκ1 were used to assay for specific Vk–Jκ1 rearrangements in genomic DNA samples. The percentage usage of Vk genes was compared with those of WT mice in which the percentage usage was set as 100% (depicted as dashed lines). Data are means ± SD of three independent experiments. The distances of these Vk genes from the Jκ1 region are Vκ2-139: 3085 kb; Vκ9-132: 2854 kb; Vκ9-122: 2740 kb; Vκ19-15: 311 kb; Vκ21-7: 111 kb; and Vκ21-1: 18 kb (8). (B) Usage of different Vk genes in pre-B cells from HS1–2−/− mice. (C) Usage of different Vk genes in splenic B cells from HS1–2−/− mice.

B, B and C, and A and C hybridization signals for several hundred alleles in these samples. As shown in Fig. 6C, contraction was statistically significantly decreased throughout the locus by the HS1–2 deletion. The mean distances between respective A and B, B and C, and A and C hybridization signals were 0.316, 0.326, and 0.279 μm, respectively, for WT samples and 0.36, 0.405, and 0.417 μm, respectively, 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 with those from WT mice. In contrast, our previous studies showed 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.

FIGURE 4. Analysis of Jκ-region usage and the developmental timing and tissue specificity of Vk–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′ 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 Vk joining to the different Jκ regions. (B) Jκ-region usage analysis. The Vk–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 is shown as ratios for pre-B cell samples from HS1–2−/− mice compared with those of WT mice in which Jκ-region usage was set as 1. (D) Vκ-Jκ rearrangement products of thymus double-positive T cells from the indicated genetic lines of mice were amplified by PCR, 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 Vk-Jκ1–Jκn junction regions. The Vk-Jκ1 rearrangement products were amplified by PCR 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 nucleotides in >100 sequenced samples from each genetic mouse line were analyzed.
FIGURE 5. Levels of \( \kappa \) gene germline transcription and H3K4me3 modification in pre-B cells. (A) Real-time PCR assays were used to measure I\( \kappa \)k gene germline transcripts arising from the 5' promoter (5'GL) and from the indicated \( \kappa \) genes in pre-B cell samples from WT and HS1-2\(^{-/}\) mice. Data are mean \( \pm \) SD of three independent experiments. (B) Real-time PCR ChIP assays of H3K4me3 levels in \( \kappa \) gene recombination signal sequence regions in pre-B cells of WT and HS1-2\(^{-/}\) mice. Data are mean \( \pm \) SD (\( n = 3 \)). The distances of these \( \kappa \) genes from the Jk1 region are given in Fig. 3.

Henceforth, we term this novel element Cer (“contracting element for recombination”).

Discussion
To our knowledge, HS1–2 (Cer) is the first example of a \( cis \)-acting sequence that plays a major role in specifying locus contraction in the I\( \kappa \)k gene locus. The element is only 650 bp in length, and its deletion results in a 7-fold increase in proximal \( \kappa \) gene usage along with \( \sim 50\% \) reduction in overall locus contraction. The only other sequences reported to play roles in contraction are in the I\( g \h \)h locus. These include the intrinsic E\( q \) enhancer and its associated promoter region (34), which play very major roles in specifying contraction, as well as HS5–7 that resides within the 3' RR regulatory region, which plays only a minor role in contraction (30). The I\( \kappa \)k gene enhancers were demonstrated to be important in specifying optimal \( \kappa \)-J\( k \) 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 \( \kappa \)k gene usage. We 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 I\( \kappa \)k mini-loci transgenes (41). Furthermore, the element exerts negative affects on the levels of \( \kappa \)k 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 \( \kappa \)k 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 \( \kappa \)k genes will be capable of undergoing rearrangement by contracting and looping the locus, whereas Sis ensures that Jk-proximal \( \kappa \)k genes will not be overused in recombination. When Cer is deleted, the decontracted locus has no alternative but to rearrange proximal \( \kappa \)k genes, whereas when Sis is deleted, despite the fact that the locus is still contracted and looped, rearrangement of proximal \( \kappa \)k 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 in silico–predicted CTCF binding sites in the human I\( g \h \)k gene V-J intervening sequence, but the sequences adjacent to these CTCF binding sites are not conserved between mouse and human. Possibly, mechanisms that regulate recombination may have evolved more recently than the time of divergence of these species.

Although it seems clear that elements that bind CTCF regulate \( \kappa \) gene choice and features of higher-order chromosome organization in I\( g \) loci (30, 34, 36–39), whether CTCF functions at Cer to regulate contraction is an open question. In the I\( g \)k locus, HS1–2 (Cer) and HS3–6 (Sis) each contain two CTCF binding sites.

FIGURE 6. HS1–2\(^{-/}\) pre-B cells exhibit reduced I\( \kappa \)k gene locus contraction. (A) Map of the I\( \kappa \)k 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: 1128 kb; B–C: 1780 kb; and A–C: 2908 kb. (B) Representative 3D DNA FISH confocal optical sections from WT and HS1–2\(^{-/}\) mice. Data are mean \( \pm \) SD of three independent experiments. The center-to-center distances between these probes in the indicated hybridizing signals of the BAC probes (A: RP23-101G13, red; B: RP23-26A6, blue; and C: RP24-387E13, green). The center-to-center distances between these probes in the indicated hybridizing signals of the BAC probes (A: RP23-101G13, red; B: RP23-26A6, blue; and C: RP24-387E13, green). The center-to-center distances between these probes in the indicated hybridizing signals of the BAC probes (A: RP23-101G13, red; B: RP23-26A6, blue; and C: RP24-387E13, green). The center-to-center distances between these probes in the indicated hybridizing signals of the BAC probes (A: RP23-101G13, red; B: RP23-26A6, blue; and C: RP24-387E13, green). The center-to-center distances between these probes in the indicated hybridizing signals of the BAC probes (A: RP23-101G13, red; B: RP23-26A6, blue; and C: RP24-387E13, green). The center-to-center distances between these probes in the indicated hybridizing signals of the BAC probes (A: RP23-101G13, red; B: RP23-26A6, blue; and C: RP24-387E13, green). The center-to-center distances between these probes in the indicated hybridizing signals of the BAC probes (A: RP23-101G13, red; B: RP23-26A6, blue; and C: RP24-387E13, green). The center-to-center distances between these probes in the indicated hybridizing signals of the BAC probes (A: RP23-101G13, red; B: RP23-26A6, blue; and C: RP24-387E13, green).
proximal Vk genes, again without significantly compromising usage of most upstream Vk genes (39) (Fig. 3A). Because CTCF conditional knockouts are never complete and such cells show severe defects in pre-B cell proliferation and differentiation (38), these observations need not eliminate a central role for CTCF in mediating Igk 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 mentioned above, several proteins other than CTCF are responsible for contraction in the Igk locus, but the roles of these proteins in the Igk 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 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 Vk genes by altering higher-order chromosome structures throughout the locus. Our results further reveal that Vk gene germline transcription is only modestly upregulated upon deletion of either HS1–2 (Cer) or HS3–6 (Sis) in pre-B cells (Fig. 5A) (39). Hence, our results do not fit the simple model in which locus contraction is mediated by the co-occupation of transcribing Vk genes with the distal transcribing Jc-Cx region in the same transcription factories (32, 33), a model that, conversely, can easily explain the roles of the Igk 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 Vk gene germline transcription. We are currently testing the possibility that HS1–2 (Cer) and HS3–6 (Sis) are 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 Vk genes exhibiting marked changes in usage upon its deletion. In contrast, HS1–2 (Cer) plays a unique role in facilitating locus contraction, fostering long-range interactions between distal Vk genes and the Jk regions. If Vk genes form looped rosettes independent of HS1–2 (Cer), then the element may be responsible for bringing these rosettes into close proximity with Jk-region recombination centers (1).

Why are cis-acting elements that regulate rearrangement specifically localized in the Vv-Jk or Vb-Dh intervening sequences in Igk loci? Once a primary rearrangement event occurs, such elements are deleted in the case of the Igk locus or either deleted or inverted and moved a minimum of 265 kb upstream from their initial location in the case of the Igk locus. Hence, the functions of these cis-elements must only be to regulate primary rearrangements. Because their engineered mutagenesis or deletion in the mouse germline results in preferential proximal V gene rearrangements (37, 39) (Fig. 3), 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 in this article 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 Igk gene locus, such as those seen in receptor editing and revision (47), will occur usingVk genes most proximal to those used in the primary rearrangements.

**Acknowledgments**

We thank Michel C. Nussenzweig of Rockefeller University and Mark Schlissel of the University of California, Berkeley for kindly providing mouse strains.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


