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Cell Type–Specific Chromatin Structure Determines the Targeting of V(D)J Recombinase Activity In Vitro

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Summary

A common V(D)J recombinase that recognizes a conserved recombination signal sequence (RSS) mediates the assembly of immunoglobulin (Ig) and T cell receptor (TCR) genes in B and T cell precursors. The rearrangement of particular Ig and TCR gene segments, however, is tightly regulated with respect to cell lineage and developmental stage. Using an in vitro system, we analyzed recombinase cleavage of RSSs flanking Ig and TCR gene segments in nuclei. We found that both the lineage-specificity and temporal ordering of gene rearrangement is reflected in the accessibility of RSSs within chromatin to in vitro cleavage.

Introduction

Immunoglobulin (Ig) and T cell receptor (TCR) genes are assembled during B and T cell development through a series of site-specific recombination events referred to as V(D)J recombination (reviewed in Lewis, 1994). Strict regulation of V(D)J recombination is required to ensure the generation of functional B and T cell antigen receptors and to protect the integrity of the rest of the genome. Gene segments utilized in V(D)J recombination are flanked by tripartite recombination signal sequences (RSSs) that consist of a highly conserved heptamer, an AT-rich nonamer, and an intervening spacer of either 12 or 23 base pairs. A fundamental level of order is imposed by the restriction that recombination only occurs between gene segments flanked by RSSs with dissimilar spacer lengths (Tonegawa, 1983).

V(D)J recombinase activity is determined by the regulated expression of the recombination activating genes, *RAG1* and *RAG2* (Schatz et al., 1989; Oettinger et al., 1990). Expression of *RAG1* and *RAG2* correlates precisely with recombinase activity in cell lines and tissues. Furthermore, cotransfection of *RAG1* and *RAG2* expression constructs is sufficient to activate recombination in many nonlymphoid cell types. Therefore, the restriction of gene rearrangement to B and T lymphocyte progenitors may be entirely due to lineage- and developmental stage–specific expression of the *RAG* genes. A distinct mechanism of regulation, however, must account for the lineage specificity observed between B and T cells. Ig genes are only targeted for complete rearrangement in B lineage cells, and TCR genes are only completely rearranged in T lineage cells. Nevertheless, when artificial recombination substrates are transfected into B or T cells with recombinase activity, the constructs are rearranged without discrimination regarding the origin of the RSS elements (Yancopoulos et al., 1986).

There is also temporal regulation of gene rearrangement within a given lymphoid lineage (reviewed in Blackwell and Alt, 1989). For example, in developing B cells Ig heavy chain (IgH) genes are assembled from V, D, and J coding segments in an ordered manner: D_H-to-J_H rearrangement occurs on both heavy chain alleles before V_H-to-DJ_H rearrangement commences on either allele. Rearrangement of Ig light chain loci typically follows V_H-to-DJ_H rearrangement. The product of a fully rearranged IgH gene, μ protein, may play a role in redirecting the recombinase to the Igκ light chain locus (reviewed in Schatz et al., 1992). μ protein also mediates heavy chain allelic exclusion (Weaver et al., 1985; Nussenzweig et al., 1987; Kitamura and Rajewsky, 1992). Pre-B cells expressing μ protein continue to express the *RAG* genes and actively rearrange their light chain loci. However, remaining DJ_H alleles are not targeted for further V_H-to-DJ_H rearrangement. Recombinase discrimination between DJ_H and Igκ alleles at this stage of development is essential for maintaining the clonal distribution of antigen receptors among B cells.

Given that only a subset of the genes that are capable of being rearranged is actually targeted in a given cell with recombinase activity and that a common recombinase mediates all gene rearrangements (Yancopoulos et al., 1986), it is likely that regulation is achieved through substrate accessibility. The "accessibility hypothesis" postulates that Ig and TCR genes typically reside in a chromatin structure that is refractory to recognition by the recombinase (Yancopoulos and Alt, 1985; Alt et al., 1987). In lymphoid cells, developmental signals would result in changes in chromatin that allow the recombinase access to particular gene segments. Results showing that activation of a locus for rearrangement correlates with transcription of the germline locus support the accessibility hypothesis (Yancopoulos and Alt, 1985; Blackwell et al., 1989; Schlissel and Baltimore, 1989; Schlissel et al., 1991). However, it remains unclear whether transcription is the determinant of accessibility or a result of accessibility conferred by another mechanism.

In order to address the mechanisms that target V(D)J recombinase activity, it is necessary to understand the recombination reaction on a biochemical level. Recombination is initiated by precise cleavage at the junction between a coding gene segment and its flanking RSS to generate two broken-ended DNA intermediates, a signal end and a coding end (Roth et al., 1992a, 1992b). Signal ends are invariably blunt, 5′-phosphorylated molecules with no nucleotides lost or gained at the end (Roth et al., 1993; Schlissel et al., 1993). Coding ends are initially sealed in a hairpin structure (Roth et al., 1992b) and then processed to open-ended molecules (Ramsden and Gellert, 1995; M. S. S., unpublished data). Signal ends and coding ends are subsequently joined to generate precise signal joints and heterogeneous coding joints.

A cell-free system that catalyzes the RSS recognition and cleavage steps of V(D)J recombination has recently been described (McBlane et al., 1995; van Gent et al., 1995). In this system, either a pre-B cell nuclear extract supplemented with recombinant RAG1 protein (van Gent et al., 1995) or recombinant RAG1 and RAG2 proteins alone (McBlane et al., 1995) cleave RSS-containing plasmid or oligonucleotide substrates to generate signal and coding ends. We have modified this approach in order to study the initiation of V(D)J recombination of endogenous gene segments within chromatin.

Results

Immunoglobulin Loci in Isolated Nuclei Are Substrates for In Vitro RSS Cleavage by the V(D)J Recombinase

We prepared nuclear extract from the conditionally transformed pre-B cell line 103-bcl2/4 (103; Chen et al., 1994) and purified RAG1 core protein (rRAG1) from baculovirus-infected insect cells as described (van Gent et al., 1995). As shown previously (van Gent et al., 1995), the 103 nuclear extract supplemented with rRAG1 catalyzed cleavage of an RSS presented on a purified plasmid (see below). To determine whether the recombinase could recognize its target within native chromatin structure, we reacted intact template nuclei with nuclear extract and rRAG1 and analyzed cleavage at RSSs flanking Ig gene segments. In order to assay de novo cleavage at endogenous loci, it was critical that the nuclear template and extract used did not contain DNA with any preexisting RSS breaks. To ensure this, nuclear templates were prepared from transformed cell lines or primary cells from RAG-deficient mice (Mombaerts et al., 1992; Shinkai et al., 1992; Spanopoulou et al., 1994) that have no Ig or TCR gene rearrangements or broken-ended intermediates of rearrangement. Nuclear extract from the murine pre-B cell line 103 was useful in the analysis of cleavage at the J_H2 gene segment of the IgH locus since this cell line has deleted J_H2 in the process of rearranging its IgH alleles and therefore has no preexisting J_H2 RSS breaks. However, 103 cells actively rearrange V_κ-to-J_κ in culture (Chen et al., 1994) and have high levels of J_κ signal ends (Ramsden and Gellert, 1995; M. S. S. unpublished data). For this reason, we used extracts from newborn murine or fetal bovine thymocytes (which do not rearrange Igκ genes) for in vitro cleavage at the Igκ locus. Additionally, since our polymerase chain reaction (PCR) primers do not amplify bovine Ig or TCR sequences (data not shown), the cow extract allowed us to unambiguously analyze RSS cleavage at any murine locus.

To test the system, we reacted nuclei from the RAG2-deficient A-MuLV-transformed pro-B cell line 63-12 (Shinkai et al., 1992) with 103 nuclear extract and rRAG1. Signal ends generated by in vitro cleavage at the RSS flanking J_H2 were detected using the ligation-mediated PCR (LMPCR) assay diagramed in Figure 1. DNA recovered from each in vitro cleavage reaction was ligated to a blunt, unphosphorylated linker and DNA breaks were detected by PCR with a linker-specific primer and a nested pair of locus-specific primers (Schlissel et al., 1993). We used PCR amplification of a nonrearranging genomic locus (CD14) to confirm that equivalent amounts of DNA were recovered from all in vitro reactions (Schlissel et al., 1993).

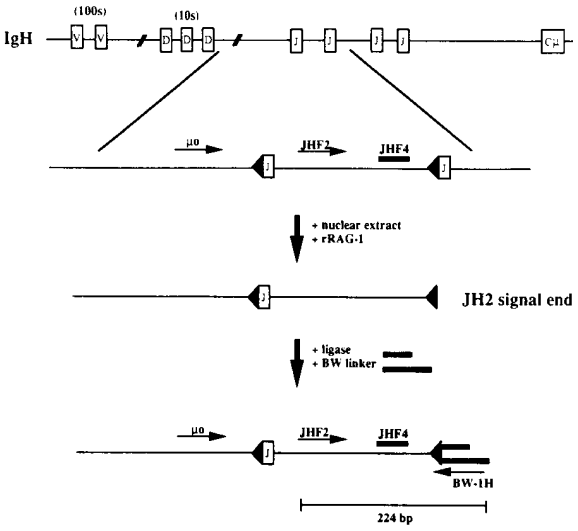

Figure 1. Diagram of the LMPCR Assay Used to Detect Signal Ends in Genomic DNA

The Ig heavy chain locus is shown with boxes representing coding gene segments and triangles representing RSSs. The positions of PCR primers are indicated by arrows; the position of the blot hybridization probe is indicated by a solid bar; and the BW linker is shown as an asymmetric pair of bold lines. Nested PCR using the anchor primer BW-1H and the locus-specific primers μo and JHF2 on DNA ligated to the BW linker will specifically amplify J_H2 signal ends. The 224 bp product is detected by Southern blotting and hybridization with the JHF4 probe. RSS breaks at other Ig and TCR loci were detected using analogous assays with different locus-specific primers and probes.

As shown in Figure 2A, we detected J_H2-associated RSS breaks in 63-12 DNA recovered from in vitro cleavage reactions incubated at 30°C, but not from those incubated on ice (compare lanes 7 and 8). Specific RSS breaks were absent from control reactions containing each component of the system alone (lanes 1–3) or lacking individual components (lanes 4–6). The amplified product comigrated with previously characterized J_H2 signal ends in murine bone marrow and thymus DNA (lane 10; Schlissel et al., 1993). By assaying serial dilutions of DNA recovered from complete in vitro cleavage reactions, we estimate that >0.2% of the input J_H2 loci were cleaved. By comparison, our LMPCR assay detects RSS breaks at ~2% of J_H2 loci in thymus genomic DNA.

To ensure that the observed in vitro cleavage at the J_H2 RSS was not peculiar to the template nuclei used, we tested nuclei from the RAG1-deficient A-MuLV-transformed pro-B cell line AH7. As shown in Figure 2C, specific cleavage at the J_H2 RSS was observed upon incubation of AH7 nuclei with 103 extract and rRAG1 (lane 7). Interestingly, rRAG1 alone was not sufficient to restore cleavage activity to the $RAG1^{-/-}$ nuclei (lane 1). This indicates that at least some of the factors critical for recognition and cleavage of RSSs within chromatin

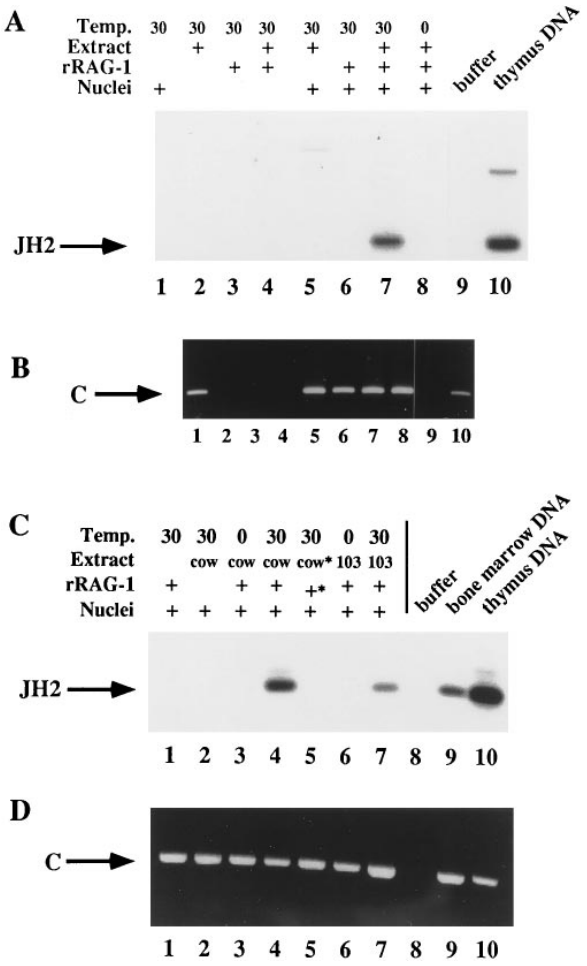

Figure 2. In Vitro Cleavage of Endogenous J_H2 RSSs in Isolated Nuclei

(A) Detection of in vitro cleavage at J_H2 by LMPCR. Nuclei prepared from the RAG2-deficient pro-B cell line 63-12 were incubated with various combinations of rRAG1 and 103 extract either on ice (0°C) or at 30°C as indicated above each lane. DNA recovered from in vitro reactions was ligated to the BW linker and assayed for signal ends at J_H2 using LMPCR as shown in Figure 1. A Southern blot of the LMPCR products hybridized with the locus-specific probe JHF4 is shown, with the J_H2 signal end product indicated by an arrow. Negative and positive controls for the PCR reaction were no DNA template (lane 9) and linker-ligated murine thymus genomic DNA (lane 10).

(B) Amplification of a nonrearranging control locus (CD14) from genomic DNA recovered from the in vitro reactions shown in (A). The specifically amplified product was detected by ethidium bromide staining of an agarose gel and is indicated by an arrow.

(C) Detection of in vitro cleavage at J_H2 by LMPCR. Nuclei from the RAG1-deficient pro-B cell line AH7 were incubated with rRAG1 and either 103 extract or bovine thymus extract (cow) on ice (0°C) or at 30°C as indicated above each lane. Recovered DNA was assayed for J_H2 signal ends as described for (A). Asterisk indicates that the extract or protein preparation was heated at 68°C for 15 min prior to addition to the template nuclei. PCR controls included no DNA template (lane 8) and linker-ligated genomic DNA from murine bone marrow (lane 9) and thymus (lane 10).

(D) Control amplification of DNA recovered from the in vitro reactions shown in (C). PCR was performed as for (B).

are provided by the added nuclear extract, rather than being retained in the template nuclei themselves. Furthermore, since we have found that nuclear extract and rRAG1 recognize and cleave RSSs flanking Ig gene segments in purified genomic DNA (see below), we conclude that chromatin structure is permissive, but not required, for this in vitro activity.

Nuclear extract prepared from fetal bovine thymus was also capable of generating J_H2 signal ends in both AH7 nuclei (Figure 2C, lane 4) and 63-12 nuclei (data not shown). These results demonstrate that the factors required for cleavage at J_H2 in pro-B cell chromatin are present in both pre-B cells and pre-T cells and are functionally conserved between mouse and cow.

Purified rRAG1 and rRAG2 Alone Are Not Sufficient for Cleavage of RSSs within Complex Templates

Previous studies have shown that rRAG1 and rRAG2 core proteins are sufficient for in vitro cleavage of RSS-containing plasmid or oligonucleotide substrates (McBlane et al., 1995). To determine whether the two proteins are also sufficient for cleavage of endogenous RSSs residing in chromatin or genomic DNA, we substituted the nuclear extract and rRAG1 used in the in vitro assays with rRAG1 and rRAG2 core proteins copurified from baculovirus-infected insect cells (R1 + R2) (McBlane et al., 1995). To confirm that the purified rRAG proteins were active, we tested whether the R1 + R2 preparation could catalyze cleavage of an RSS in the plasmid pJH200 (Hesse et al., 1987). Using an LMPCR assay similar in design to those used to detect breaks in genomic DNA, we found that R1 + R2 was at least as efficient in mediating RSS cleavage of the plasmid target as 103 extract supplemented with rRAG1 (Figure 3A, compare lanes 2 and 6). However, R1 + R2 was unable to catalyze cleavage at the J_H2 RSS in 63-12 pro-B cell nuclei (Figure 3B, lane 6) or in purified genomic DNA (data not shown). As expected, 103 nuclear extract supplemented with rRAG1 cleaved the J_H2 RSS in 63-12 nuclei (Figure 3B, lane 2), and the 103 extract also showed a low level of cleavage activity on its own (Figure 3B, lane 8). This extract could complement R1 + R2 for J_H2 RSS cleavage in 63-12 nuclei (Figure 3B, lane 4) and in genomic DNA (data not shown). We also found that nuclear extracts from fetal bovine thymus and from the transformed pro-B cell line HaftI are capable of complementing R1 + R2 to allow RSS cleavage of nuclear templates (data not shown). A HeLa cell nuclear extract failed to complement R1 + R2 in this assay, but a thymus extract depleted of native RAG2 by antibody treatment was still able to complement R1 + R2 (data not shown). These results suggest that there are factors present in lymphoid nuclear extract that are critical for recombinase activity on complex genomic targets under these conditions. We are currently fractionating nuclear extract to determine whether these factors might include full-length RAG1 or other accessory factors.

In Vitro Cleavage of Nucleoprotein Templates Reflects the Lineage-Specificity of V(D)J Recombination

While D-to-J rearrangement of the IgH and TCR β loci is not limited to cells of the corresponding lineage, V-to-

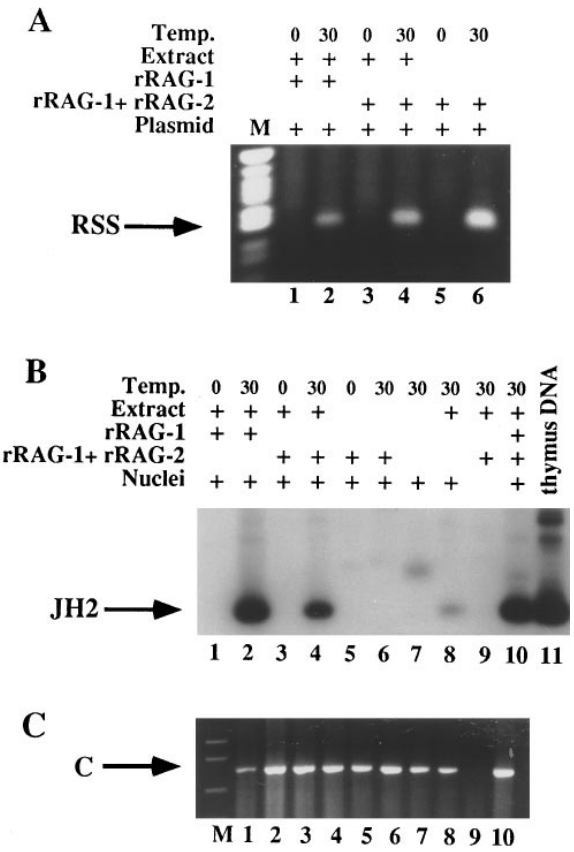

Figure 3. rRAG1 and rRAG2 Core Proteins Alone Are Not Sufficient for RSS Cleavage of Nuclear Templates

(A) Detection of RSS breaks generated in vitro on a plasmid substrate. In vitro reactions were performed using pJH200 as the template and various combinations of 103 extract, purified rRAG1, and copurified rRAG1 + rRAG2 as indicated above each lane. Following incubation, plasmid DNA was recovered, linker-ligated, and assayed for breaks at one of the two RSSs contained in the plasmid using LMPCR. The signal end product is visible on an ethidium bromide–stained gel and is indicated by an arrow. All lanes contained an equivalent amount of DNA as shown by amplification of a region of the plasmid that is not affected by RSS cleavage (data not shown).

(B) Detection of J_H2 RSS breaks generated in vitro in isolated nuclei. In vitro reactions were performed as described for (A) except that 63-12 nuclei were used as the template for cleavage. Reaction products were assayed for J_H2 signal ends as diagramed in Figure 1. A Southern blot of the LMPCR products hybridized with the JHF4 probe is shown. A positive control for amplification of J_H2 signal ends was linker-ligated murine thymus genomic DNA (lane 11).

(C) Control amplification of DNA recovered from the in vitro reactions shown in (B). PCR was performed as for Figure 2B.

DJ rearrangement of these loci, as well as rearrangement of other Ig and TCR loci, is strictly lineage specific. For example, broken-ended recombination intermediates associated with the Igκ light chain locus are present in developing B cells, but not in T cells (see Figure 4B, lanes 5 and 6). On the other hand, breaks in the TCRδ locus are observed exclusively in T cells. To test whether lineage-specific chromatin structure plays a role in determining the ability of the recombinase to discriminate between Ig and TCR loci, we analyzed in vitro RSS cleavage at various loci in nuclei from B and T lineage cells (Figure 4). We found that only a subset

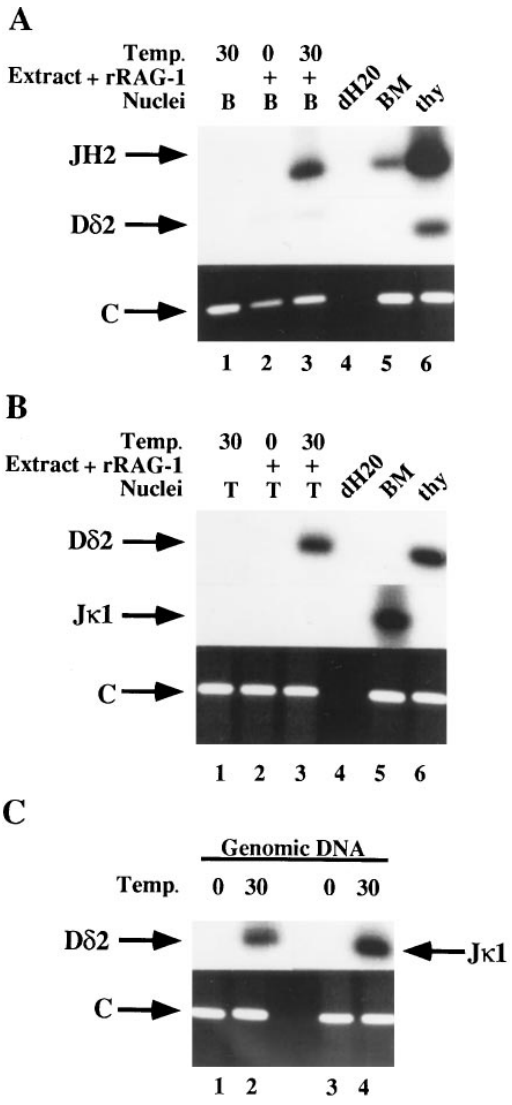

Figure 4. The Lineage Specificity of V(D)J Recombinase Activity Is Retained In Vitro

(A) In vitro cleavage of pro-B cell nuclear templates. Nuclei from the RAG1-deficient pro-B cell line AH7 were incubated with bovine thymus nuclear extract and rRAG1 at 0°C or 30°C. Reaction products were analyzed for breaks at RSSs flanking J_H2 (top gel) and $D_δ2$ (middle gel) (Roth et al., 1993) gene segments using LMPCR strategies analogous to that shown in Figure 1. Southern blots of the LMPCR products hybridized with locus-specific probes are shown, with arrows indicating the J_H2 and $D_δ2$ signal end products. Controls for broken-end amplification are no template (lane 4), and linker-ligated bone marrow (lane 5) and thymus (lane 6) genomic DNA. Control amplification of in vitro reaction products and control samples was performed (bottom gel) as for Figure 2B.

(B) In vitro cleavage of pro-T cell nuclear templates. In vitro reactions were performed as for (A) except that nuclei from RAG1-deficient thymocytes were used as the template for cleavage. Reaction products were analyzed by LMPCR for RSS breaks at $D_δ2$ (top gel) and $J_κ1$ (middle gel) gene segments. Control amplification of each sample is shown in the bottom gel.

(C) In vitro cleavage of purified genomic DNA templates. In vitro reactions contained bovine thymus extract, rRAG1, and 1.2 μg purified genomic DNA from either AH7 cells (lanes 1 and 2) or wild-type murine thymocytes (lanes 3 and 4). Recovered reaction products were analyzed by LMPCR for RSS breaks at $D_δ2$ (lanes 1 and 2, top gel) and $J_κ1$ (lanes 3 and 4, top gel). Control amplification of each sample is shown in the bottom gel.

of loci was accessible to in vitro cleavage in each type of nuclear template and that the pattern of accessibility recapitulates that observed in vivo. When AH7 pro-B cell nuclei were incubated with bovine thymus extract and rRAG1, we found that the J_H2 RSS was accessible to in vitro cleavage, but the TCR $D_\delta2$ RSS was not (Figure 4A). However, the TCR $D_\delta2$ locus was cleaved in vitro when purified genomic DNA was provided as the template (Figure 4C), suggesting that chromatin structure may play a role in preventing TCR gene rearrangement in B cells.

We next analyzed in vitro RSS cleavage in pro-T cell nuclei prepared from RAG1-deficient thymocytes. In these nuclei, RSSs in the TCRδ locus were accessible to in vitro cleavage whereas those in the Igκ locus were not (Figure 4B). The inability of the recombinase to target the Igκ locus was specific to T cell chromatin since $J_\kappa1$ signal ends were generated in vitro in pro-B cell (at a low level, data not shown) and pre-B cell (at an induced level, see below) nuclei and in purified genomic DNA (Figure 4C). These results indicate that accessibility of the Igκ and TCRδ loci to the recombinase is mediated by a stable element of chromatin structure that is determined at least in part by cell lineage.

In analyzing in vitro RSS cleavage at various loci, we have found no differences in the activity of pre-B cell and pre-T cell nuclear extracts (data not shown). This observation supports the conclusion that regulated cleavage in this in vitro system is determined by the nature of the template rather than by a component of a particular extract.

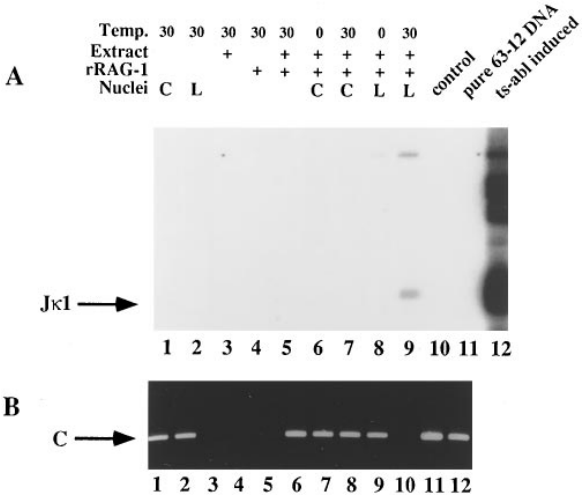

Figure 5. The Regulated Accessibility of J_κ Gene Segments Is Reflected In Vitro

(A) Detection of $J_\kappa1$ signal ends generated in vitro. In vitro cleavage reactions were performed on template nuclei prepared from 63-12 cells (C) or 63-12 cells that were cultured with LPS (L). Nuclei were incubated with rRAG1 and murine thymus extract as indicated above each lane. Reaction products were assayed by LMPCR for RSS breaks associated with $J_\kappa1$. The specific signal end product is indicated by an arrow. Controls consisting of buffer only (lane 10), 63-12 genomic DNA (lane 11), and induced 103-bcl2/4 genomic DNA (lane 12) were linker-ligated and amplified in parallel.

(B) Control amplification of the in vitro reaction products. PCR was performed as for Figure 2B.

In Vitro Cleavage of Nucleoprotein Templates Reflects the Temporal Regulation of V(D)J Recombination

Having observed that in vitro RSS cleavage reflects the lineage of the template source, we were interested in whether nuclei from B cells at different developmental stages would show differences in their patterns of locus accessibility to in vitro cleavage. In this analysis, we focused on the Igκ locus since its rearrangement is temporally regulated in vivo (Blackwell and Alt, 1989). A model system for studying the regulated activation of the Igκ locus is provided by the bacterial mitogen lipopolysaccharide (LPS). Treatment of pro-B cell lines with LPS activates both germline κ gene transcription and V_κ-to-J_κ rearrangement (Schlissel and Baltimore, 1989). We analyzed the effect of LPS treatment on in vitro cleavage at the $J_\kappa1$ RSS using nuclear templates prepared from 63-12 cells grown in the presence or absence of LPS. As shown in Figure 5A, $J_\kappa1$ RSS breaks were only generated in templates from cells treated with LPS (compare lanes 7 and 9). In some experiments, a low level of cleavage at $J_\kappa1$ was observed in templates from cells that had not been treated with LPS (data not shown); however, cleavage was invariably stimulated (at least 5-fold) by LPS-treatment. This result suggests that the enhanced accessibility of the Igκ locus conferred by LPS treatment of cultured cells is mediated by a stable change in chromatin structure.

In vivo, ordered Ig gene rearrangement is thought to result from activation of the Igκ locus through a signal from the product of a fully rearranged IgH gene, μ protein (reviewed in Schatz et al., 1992). To test whether expression of μ protein affects the accessibility of $J_\kappa1$, we analyzed in vitro cleavage of the $J_\kappa1$ RSS in B cell nuclei from RAG1-deficient mice or RAG1-deficient mice carrying a rearranged μ heavy chain transgene (Spanopoulou et al., 1994). As show in Figure 6A, B cell development in RAG-deficient mice is arrested at the pro-B ($CD19^+$, $CD43^+$; Krop et al., 1996) stage (Mombaerts et al., 1992; Shinkai et al., 1992). Breeding a functionally rearranged μ heavy chain transgene onto the *RAG*$^{-/-}$ background results in the progression of B cells to the pre-B cell ($CD19^+$, $CD43^-$) stage of development (Figure 6B) (Spanopoulou et al., 1994; Young et al., 1994), at which point Igκ gene rearrangement is normally activated. Template nuclei were prepared from B cells purified from bone marrow using biotinylated anti-CD19 antibody (Krop et al., 1996) and paramagnetic streptavidin beads. Nuclei were also prepared from the nonselected pool of cells ($CD19^-$) that consisted primarily of granulocytes and macrophages (data not shown). Figures 6C and 6D show the purity of positively and negatively selected cells from each background. In vitro cleavage reactions contained either 103 extract or murine thymocyte extract and rRAG1. DNA recovered from the reactions was analyzed by LMPCR for signal ends associated with J_H2 or $J_\kappa1$, respectively (Figure 6E). As seen previously with 63-12 and AH7 nuclei, both RAG-deficient and RAG-deficient μ transgenic nuclei were substrates for in vitro cleavage at J_H2 (Figure 6E, lanes 9 and 11). However, signal ends associated with $J_\kappa1$ could

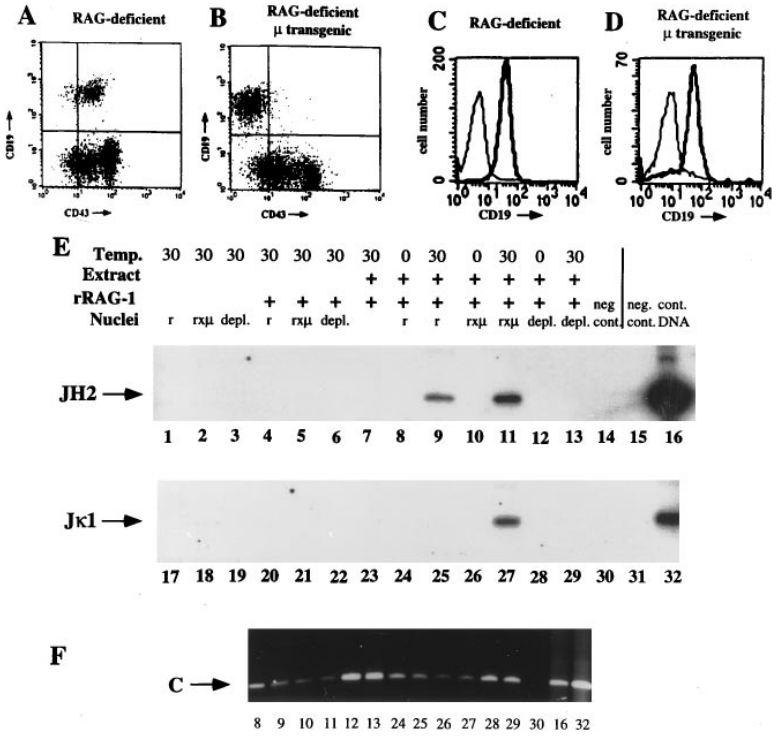

Figure 6. A Rearranged μ Heavy Chain Transgene Alters Igκ Locus Structure to Allow $J_κ1$ RSS Cleavage In Vitro

(A and B) Flow cytometric analysis of unfractionated bone marrow cells from RAG1-deficient (A) or RAG1-deficient μ transgenic (B) mice stained with anti-CD19 and anti-CD43 antibodies.

(C and D) Flow cytometric analysis of fractionated bone marrow cells from RAG1-deficient (C) or RAG1-deficient μ transgenic (D) mice. Bone marrow cells were stained with biotinylated anti-CD19 antibody and separated using streptavidin-coated paramagnetic beads into two fractions, a B-cell enriched ($CD19^+$) fraction and a B-cell depleted ($CD19^-$) fraction. The purity of the two fractions was assessed by restaining with anti-CD19 antibody. CD19 expression of the positively (heavy tracing) and negatively (light tracing) selected cells is shown.

(E) In vitro cleavage of nuclear templates. Cleavage reactions contained nuclei from B cells purified from RAG1-deficient (r) or RAG1-deficient μ transgenic (rxμ) bone marrow or from non-B cells purified from RAG1-deficient μ transgenic bone marrow (depl.). Nuclei were incubated with rRAG1 and either 103 extract (samples 1–13) or murine thymocyte extract (samples 17–29). Reaction products were assayed by LMPCR for signal ends associated with J_H2 (top gel) or $J_κ1$ (bottom gel). Southern blots of the LMPCR products hybridized with either J_H (top) or $J_κ$ (bottom) locus-specific probes are shown. Positive control DNA consisted of linker-ligated murine thymus DNA for J_H2 RSS breaks (lane 16) and linker-ligated murine bone marrow DNA for $J_κ1$ breaks (lane 32). Negative controls included no DNA template (lanes 14 and 30) and linker-ligated 63-12 genomic DNA (lanes 15 and 31).

(F) Control amplification of the in vitro reaction products assayed in (E) for broken RSS ends (only a subset of the samples is shown). PCR was performed as for Figure 2B.

only be detected in nuclei from RAG-deficient μ transgenic cells (Figure 6E, compare lanes 25 and 27). Template dilution analyses showed that in vitro RSS cleavage at $J_κ1$ was enhanced at least 30-fold in RAG-deficient μ transgenic nuclei over RAG-deficient nuclei (data not shown). Therefore, we conclude that μ protein expression has a direct effect on the Igκ locus, resulting in an alteration of chromatin structure that allows recognition by the V(D)J recombinase.

Neither J_H2 nor $J_κ1$ signal ends were generated in vitro when the template nuclei were prepared from primary nonlymphoid cells (the nonselected, $CD19^-$ fraction of RAG-deficient μ transgenic bone marrow; Figure 6E, lanes 13 and 29). Similarly, nuclei from a mastocytoma cell line, P815, were not substrates for in vitro cleavage at either the IgH or Igκ loci (data not shown). We conclude from these experiments that in nonlymphoid cells, Ig genes exist in a chromatin structure that is inaccessible to the V(D)J recombinase. This is consistent with observations that nonlymphoid cells transfected with *RAG1* and *RAG2* expression constructs rearrange artificial recombination substrates, but do not rearrange their endogenous Ig and TCR loci (Schatz et al., 1992). Therefore, expression of *RAG1* and *RAG2* is likely to be only part of the mechanism by which V(D)J recombination is activated in lymphoid precursors. Specific alterations in chromatin structure appear to be required as well.

Chromatin Structure Plays a Role in Allelic Exclusion

V(D)J rearrangement is regulated such that an individual B cell expresses only one functional heavy chain and one functional light chain, a phenomenon known as allelic exclusion (Pernis et al., 1965). While light chain allelic exclusion might be explained by inactivation of the recombinase, heavy chain allelic exclusion cannot, since cells undergoing $V_κ$-to-$J_κ$ rearrangement often (50%–90% of the time; Alt et al., 1984; Hardy et al., 1991) contain unrearranged V_H and partially rearranged DJ_H gene segments. Retargeting of the recombinase such that it ignores the second IgH allele in cells with one functionally rearranged IgH allele is dependent upon surface expression of μ protein (Kitamura and Rajewsky, 1992).

To address the mechanism of heavy chain allelic exclusion, we used LMPCR to determine the effect of IgH gene expression on V_H and DJ_H RSS cleavage in vivo (Figure 7A). We purified B cells from the bone marrow of wild-type and μ heavy chain transgenic (Nussenzweig et al., 1987) mice based on CD19 expression as described above. Genomic DNA from these cells was assayed for signal ends associated with the DFL16.1 gene segment and with gene segments of the V_H558 family. We chose to analyze DFL16.1 as a model D_H gene segment since it is frequently used in vivo, appearing in ~50% of DJ_H rearrangements in fetal liver (Chang et al.,

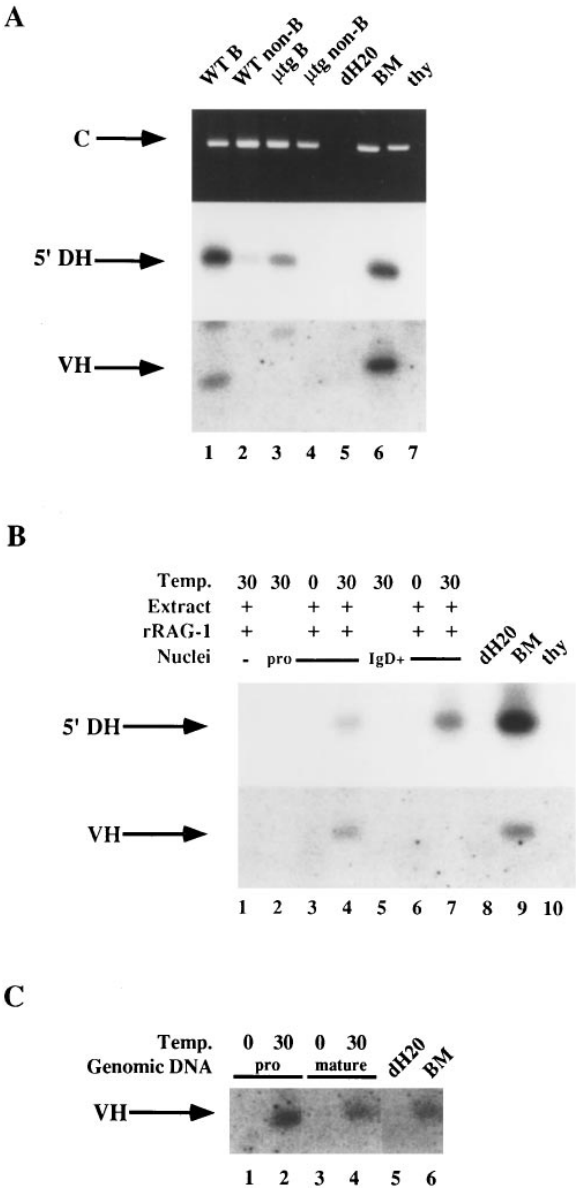

Figure 7. RSS Cleavage in Cells Displaying Heavy Chain Allelic Exclusion

(A) In vivo generated RSS broken ends. Linker-ligated genomic DNA was prepared from fractionated bone marrow of wild-type (WT) and μ heavy chain transgenic (μtg) mice. Bone marrow cells were separated based on CD19 expression into a B cell–enriched pool (WT B, μtg B) and a B cell–depleted pool (WT non-B, μtg non-B). The linker-ligated DNA was assayed for a nonrearranging control locus (arrow labeled C, top gel), and for RSS breaks upstream of DFL16.1 (5′ D_H, middle gel) and downstream of V_H558 (V_H, bottom gel) gene segments. 5′ of D_H and V_H RSS breaks were detected using LMPCR assays that are conceptually identical to that shown in Figure 1. Specifically amplified signal end products are indicated by arrows. The weak 5′ of D_H broken end signal seen in lane 2 is probably due to contaminating B cells (~5%) in the B cell–depleted population. PCR controls included no DNA template (lane 5), and linker-ligated murine bone marrow DNA (lane 6) or thymus DNA (lane 7).

(B) In vitro cleavage at RSSs flanking V_H558 and DFL16.1 gene segments in nuclei from immature and mature B cells. Nuclei from AH7 pro-B cells (pro) or from IgD^+ splenocytes (IgD^+) were used as templates for in vitro cleavage by bovine thymus extract and rRAG1. In vitro reaction products were analyzed for breaks upstream of DFL16.1 (5′ D_H, top gel) or downstream of V_H558 (V_H, bottom gel) gene segments using LMPCR. Controls include no DNA template (lane 8), and linker-ligated genomic DNA from murine bone marrow (lane 9) or thymus (lane 10). Samples 2–7 contain equivalent amounts of DNA as indicated by amplification of a control locus (data not shown).

(C) In vitro cleavage of V_H558 RSSs in purified genomic DNA. Genomic DNA from AH7 pro-B cells (lanes 1 and 2) or mature splenocytes (lanes 3 and 4) was incubated with bovine thymus extract and rRAG1 at 0°C or 30°C. Reaction products were assayed for RSS breaks at V_H558 gene segments as for (A) and (B). Controls include no DNA template (lane 5) and linker-ligated murine bone marrow genomic DNA (lane 6). Amplification of a control locus showed that samples 1–4 contain equivalent amounts of DNA (data not shown).

1992) and in A-MuLV-transformed pre-B cell lines (Reth et al., 1986). We focused on the V_H558 family since it comprises ~50% of the murine V_H repertoire. Since D_H-to-J_H rearrangement occurs nearly exclusively by deletion, breaks at the 5′ of D_H RSS, as well as breaks at V_H RSSs, are indicative of V_H-to-DJ_H rearrangement. As shown in Figure 7A, breaks at the 5′ of D_H RSS were significantly reduced in μ transgenic DNA as compared with wild-type DNA (~25–30 fold as determined by template dilution; data not shown). Breaks at RSSs flanking V_H gene segments could only be detected in wild-type DNA. Thus, allelic exclusion in the μ transgenic model is associated with a decrease in cleavage at both 5′of D_H and V_H RSSs.

To determine whether chromatin structure plays a role in the regulated cleavage of RSSs flanking DJ_H and V_H genes, we analyzed these cleavage events in vitro (Figure 7B). Nuclear substrates were prepared from the pro-B cell line AH7 or from B cells purified from murine spleen based on surface expression of IgD, a marker of B cell maturity. We used IgD^+ cells in these experiments since the structure of their IgH alleles resembles that of cells undergoing heavy chain allelic exclusion, but they no longer express *RAG1* and *RAG2* and therefore have no preexisting RSS breaks. While 5′ of D_H RSSs were accessible to in vitro cleavage in both pro-B cell nuclei and IgD^+ B cell nuclei (Figure 7B, top gel, lanes 4 and 7), RSSs flanking V_H gene segments were only cleaved in pro-B cell templates (bottom gel, compare lanes 4 and 7). The lack of detectable in vitro cleavage at V_H RSSs in IgD^+ nuclei might simply be due to a lack of substrate in cells of this developmental stage, since they contain fewer unrearranged V_H gene segments than pro-B cells. However, when purified genomic DNA from pro and mature B cells was used as the template for in vitro cleavage, we found that V_H RSSs were subject to cleavage in both types of DNA (Figure 7C). Therefore, the quantity of available V_H RSSs is not what limits in vitro cleavage at these sequences in IgD^+ B cell nuclei. These results suggest that B cell maturation is accompanied by an alteration in chromatin structure that results in selective inaccessibility of V_H gene segments.

Discussion

The experiments presented demonstrate in vitro V(D)J recombinase activity on physiologically relevant sub-

strates: endogenous gene segments within intact nuclei. Nuclear extracts from sources with high levels of *RAG* gene expression supplemented with rRAG1 core protein were capable of catalyzing precise cleavage of nuclear substrates. This approach has allowed us to address issues of developmental regulation that cannot be addressed by in vitro systems that use artificial recombination substrates. As discussed below, we found that recombinase activity on nucleoprotein targets is fundamentally different from its activity on purified DNA targets of low complexity. Amounts of recombinant RAG1 and RAG2 core proteins that are adequate for cleavage of artificial substrates cannot catalyze cleavage of RSSs within chromatin or genomic DNA, suggesting that additional factors may be required. Furthermore, RSSs within chromatin are differentially accessible to cleavage depending upon the lineage and developmental stage of the chromatin source.

The Role of the Nuclear Extract in RSS Cleavage of Nucleoprotein Substrates In Vitro

While the nuclear extract does not determine the regulated targeting of the recombinase in these in vitro experiments (see above), it does however provide an activity that is essential for recognition and cleavage of RSSs within genomic DNA and chromatin. We found that purified recombinant RAG1 and RAG2 core domains, while sufficient for cleavage of RSSs on artificial substrates (McBlane et al., 1995; see above), cannot cleave RSSs within purified genomic DNA or intact nuclei. Nuclear extracts from lymphoid cells complemented the recombinant proteins to allow cleavage of these complex targets, suggesting that initiation of V(D)J recombination in vivo may require factors in addition to the core RAG proteins. These factors might be involved in increasing the specificity of RSS recognition or in stabilizing a protein complex or protein–DNA interaction that is important for recombinase activity. Such factors might be crucial for cleavage of endogenous gene segments in genomic DNA or chromatin, but not required for cleavage of plasmid substrates in vitro or in vivo, situations in which the complexity and concentration of the target are very different. It is possible that these factors might include native RAG1 or RAG2. However, thymus extract depleted of native RAG2 by antibody treatment retained its ability to complement core rRAG1 and rRAG2 (data not shown), and extracts active in nuclear RSS cleavage were greatly stimulated by core rRAG1 (Figures 2 and 3). By fractionating active nuclear extracts, it should be possible to identify the factors that are required to complement rRAG1 and rRAG2 core domains for efficient cleavage of endogenous RSSs.

The Role of Chromatin Structure in the Developmental Stage-Specificity of V(D)J Recombination

Through comparisons of in vitro RSS cleavage activity on nuclear templates from B cells of various developmental stages, we have investigated the role of chromatin structure in the regulated pattern of locus activation and inactivation that characterizes B cell development. We show that templates from cells that have reached the pre-B cell stage of development by expression of a rearranged heavy chain transgene show enhanced cleavage at J_κ gene segments in vitro. In vivo, the coincident effects of heavy chain expression on germline transcription of the Igκ locus and its rearrangement suggest that μ protein provides a signal that results in altered accessibility of the locus (Reth et al., 1987; Schlissel and Morrow, 1994). By analyzing isolated nuclei in vitro, we have directly demonstrated that the signal sent by μ protein has a specific effect on the structure of the Igκ locus that enhances its accessibility to the recombinase. We conclude that μ protein activates Igκ gene rearrangement by directly affecting the Igκ locus in a cell-autonomous manner rather than by promoting the proliferation of pre-B cells within a heterogeneous population.

In cells undergoing heavy chain allelic exclusion, as modeled by μ transgenic B cells, V_H-to-DJ_H rearrangement is specifically suppressed (Weaver et al., 1985; Nussenzweig et al., 1987). This is accompanied by decreased transcription through the germline V_H locus (but not the DJ_H locus) and decreased levels of RSS breaks associated with DJ_H alleles (Schlissel and Morrow, 1994). We have shown that down-regulation of V_H-to-DJ_H rearrangement in μ transgenic B cells is associated with a failure of the recombinase to target RSSs flanking both V_H gene segments and DJ_H genes. The reduced levels of V_H and 5′ of D_H breaks in μ transgenic B cells as compared with wild-type B cells cannot be attributed to lower levels of recombinase activity in the transgenic cells, since cells from the two backgrounds have equivalent levels of J_H breaks (data not shown). Furthermore, the reduction of these breaks is not due to a lack of substrate in the μ transgenic cells since they contain at least as many DJ_H alleles as the wild-type cells (Schlissel and Morrow, 1994; data not shown).

Analysis of in vitro cleavage of V_H and DJ_H genes shows that the accessibility of V_H gene segments is developmentally regulated. In nuclei from mature IgD^+ B cells, RSSs flanking V_H gene segments were not cleaved when recombinase was provided. However, the V_H locus was susceptible to in vitro cleavage when the substrate consisted of either intact pro-B cell nuclei or purified genomic DNA from pro- or mature B cells. Therefore, the accessibility of V_H RSSs within nuclei to in vitro cleavage is determined by the maturity of the cells that the nuclei are isolated from. This suggests that a developmentally regulated alteration in chromatin structure at the V_H locus might play a role in heavy chain allelic exclusion. Since pairwise cleavage of a 12 bp spacer RSS and a 23 bp spacer RSS appears to be required in vivo (Roth et al., 1992a), inaccessibility of V_H RSSs could result in the observed suppression of cleavage at both V_H and DJ_H gene segments.

While remaining V_H gene segments in IgD^+ nuclei were not accessible to in vitro RSS cleavage, unrearranged Igκ alleles were (data not shown). The finding that added recombinase can access and cleave J_κ RSSs in mature B lymphocyte chromatin suggests that cessation of Igκ recombination in vivo is primarily due to inactivation of RAG1 and RAG2 rather than to an alteration in chromatin structure. This is consistent with the possibility of receptor editing in mature B cells that have reactivated *RAG*

gene expression (Gay et al., 1993; Tiegs et al., 1993). Importantly, reactivation of *RAG* gene expression in mature lymphocytes could also lead to aberrant rearrangement events involving Ig or TCR loci and proto-oncogene loci (Korsmeyer, 1992).

In Vitro Cleavage of Endogenous RSSs Provides a Direct Assay for Locus Accessibility

Our results confirm the role of locus accessibility in targeting gene rearrangement that has been suggested by many previous correlative studies. Most of these studies have relied upon transcription in the vicinity of a locus as a definition of accessibility (reviewed in Schatz et al., 1992). Here we directly measure accessibility in terms of RSS recognition and cleavage. Since active transcription is not occurring on the substrates used in this in vitro system (NTPs are not provided), we conclude that regulated accessibility is not determined by the act of transcription, but by a stable element of chromatin structure. This structure appears to be dependent upon both the lineage and developmental stage of the cell.

Although the correlation between transcription and activation of V(D)J recombination has been convincingly substantiated, there are potential mechanisms for the regulation of locus accessibility that do not necessarily rely upon transcription. In a simplistic model, loci packaged in chromatin are generally inaccessible for interaction with proteins (Workman and Buchman, 1993). In certain cell types, chromatin surrounding particular gene segments might be remodeled as a result of transcription or another unrelated mechanism to allow recombinase access. It is also possible that chromatin composition at certain loci actively targets gene rearrangement. Tightly bound factors might recruit the recombinase through protein–protein interactions or by presenting the RSS in a modified chromatin structure. In this way transcriptional enhancer elements might serve a second and separate role as recombination enhancers (Fernex et al., 1995). Alternatively, specific factors associated with a locus might act in a negative fashion to prevent recombinase access (Hiramatsu et al., 1995). The approach that we have described provides a direct assay for locus accessibility and should enable us to define features of chromatin required for regulated V(D)J recombinase activity.

Experimental Procedures

Components of the In Vitro Cleavage System

Nuclear extracts were prepared as described (Parker and Topol, 1984) and stored in aliquots at −80°C. The 103 extract was prepared from 1 liter 103-bcl2/4 cells (a gift from N. Rosenberg) that had been grown at the nonpermissive temperature (39°C) for 10 hr to induce *RAG* gene expression and recombinase activity (Chen et al., 1994). The cells were maintained at 33°C, 5% CO_2 in RPMI 1640 supplemented with 10% fetal calf serum, 20 mM glutamine, 500 IU/ml penicillin, 500 μg/ml streptomycin, 50 μM β-mercaptoethanol, and 0.5 mg/ml G418. The protein concentration of the 103 extract was ~1 μg/μl (MicroBCA assay, Pierce). The bovine thymus extract was prepared from a third trimester fetal calf thymus (Antech). The protein concentration of the bovine extract was ~1 μg/μl. The murine thymus extract was prepared from 10-day-old wild-type mice and had a protein concentration of ~250 ng/μl.

Nuclear substrates were prepared as described (Parker and Topol, 1984) and were shown to be intact by microscopy. Nuclei were resuspended at a final concentration of 50,000/μl in 20 mM HEPES (pH 7.6), 2 mM $MgCl_2$, 70 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 25% glycerol and frozen in aliquots at −80°C. Sources of nuclei included the A-MuLV-transformed cell lines 63-12 (derived from RAG2-deficient mice, a gift from F. W. Alt) and AH7 (derived from RAG1-deficient mice, a gift from C. Roman and D. Baltimore), the murine mastocytoma cell line P815 (a gift from A. Huang and D. Pardoll), and primary murine cells from either fractionated bone marrow or spleen (see below) or thymus. For nuclei prepared from LPS-treated 63-12 cells, the cells were grown for 12 hr in 10 μg/ml LPS (Difco) prior to harvest. Purified genomic DNA substrates were prepared using either standard methods (Ausubel et al., 1987) or a Puregene kit (Gentra). The plasmid substrate pJH200 (Hesse et al., 1987) was prepared using a Qiagen kit.

Recombinant RAG proteins were purified essentially as described (van Gent et al., 1995) from insect cells infected with recombinant baculoviruses AcD23 and AcD25 (gifts from D. van Gent and M. Gellert) that express the core domains of RAG1 (amino acids 384–1008) and RAG2 (amino acids 1–387), respectively. The purity and concentration of each protein preparation was estimated from Coomassie-stained SDS–PAGE gels.

In Vitro Cleavage Reactions and LMPCR Analysis

Reactions were performed in a volume of 20 μl with 100,000 nuclei or the indicated amount of genomic or plasmid DNA as template. Reactions contained ~0.6 μg rRAG1 and ~2 μg nuclear extract. Reaction conditions (including all components) were: 40 mM HEPES (pH 7.6), 2.5 mM Tris–Cl (pH 8.0), 110 mM KCl, 1 mM MnCl2, 2.5 mM DTT, 0.2 mM $MgCl_2$, 0.05 mM phenylmethylsulfonyl fluoride, 0.025 mM EDTA, 7% glycerol (v/v). Cleavage reactions using copurified rRAG1 and rRAG2 (R1 + R2) contained ~0.9 μg R1 + R2. Reactions were incubated for 30 min on ice followed by 60 min either on ice or at 30°C. Nucleic acid was recovered from in vitro cleavage reactions as described (van Gent et al., 1995) and ligated (Schlissel et al., 1993) to 20 pmol BW linker (Mueller and Wold, 1989). Control DNAs ligated in parallel typically included genomic DNA from wild-type murine bone marrow and thymus and from 63-12, AH-7, and 103-bcl2/4 cell lines. Following ligation, an equal volume of PCR-lysis buffer (10 mM Tris–HCl [pH 8.3], 50 mM KCl, 0.45% NP-40, 0.45% Tween-20) was added and samples were denatured at 95°C for 15 min. Typically, 1/12 of each sample was analyzed in each PCR reaction.

Nested PCR amplification of broken ends in genomic DNA was performed using the anchor primer BW-1H and a pair of locus-specific primers essentially as described (Schlissel et al., 1993). PCR products were run on an agarose gel, transferred to a nylon membrane (Zetabind), and hybridized with a third locus-specific oligonucleotide. The sequences of locus-specific PCR primers and probes were derived from published Ig and TCR sequences and will be furnished upon request. Images were generated using a phosphorimager (Molecular Dynamics) and quantitated using ImageQuant software (version 4.1). The identity of signal end LMPCR products was confirmed either by sequencing (Schlissel et al., 1993) or by restriction enzyme sensitivity (van Gent et al., 1995) as described. The relative amounts of specific broken DNA ends in different samples were estimated by serially diluting linker-ligated DNA and determining the dilution at which identical hybridization signals were obtained. All experiments were repeated at least three times generating essentially identical results.

Broken ends generated by cleavage at the 12 bp spacer RSS of pJH200 (Hesse et al., 1987) were amplified using a single round of PCR (27 cycles) with primers BW-1H and oJH200-3 (5′-AACAATTTC ACACAGGAAACAGC-3′). The identity of the signal end was confirmed by two methods: one, comigration with a broken-ended product generated by restriction enzyme digestion of the plasmid at a site corresponding to the coding segment/RSS border, and two, hybridization with an internal oligonucleotide, oJH200-4 (5′-GGGCT GCAGG TCGACGGATCCGCGCTAAGG-3′). PCR with a pair of primers that amplify plasmid sequences unaffected by RSS cleavage was used to confirm that equivalent amounts of plasmid DNA were recovered from all in vitro reactions.

Purification of Primary Cells

RAG-deficient thymocytes: thymocytes were prepared from month-old RAG1-deficient mice (Spanopoulou et al., 1994). Thymuses were dissected into PBS, disrupted by shearing between frosted microscope slides, and filtered through nylon mesh.

$CD19^+$ B cells: B cells were purified from the bone marrow of RAG1-deficient, RAG1-deficient μ heavy chain transgenic (Spanopoulou et al., 1994), wild-type, and μ transgenic (Nussenzweig et al., 1987) mice using biotinylated monoclonal anti-CD19 antibody and streptavidin paramagnetic beads (MiniMacs system, Milltenyi Biotech; A. L. S. and M. S. S., unpublished data). The purity of the selected populations was assessed by flow cytometry after restaining the cells with anti-CD19 antibody followed by a phycoerythrin-conjugated secondary antibody. Enriched B cell fractions were >90% $CD19^+$ and depleted fractions were <10% $CD19^+$.

IgD^+ B cells: mature IgD^+ B cells were purified from wild-type mouse spleen using biotinylated monoclonal anti-IgD antibody and streptavidin paramagnetic beads as described above. The positively selected pool of splenic B cells was typically >95% IgD^+.

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