Changes in Locus-specific V(D)J Recombinase Activity Induced by Immunoglobulin Gene Products during B Cell Development

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Summary

The process of V(D)J recombination is crucial for regulating the development of B cells and for determining their eventual antigen specificity. Here we assess the developmental regulation of the V(D)J recombinase directly, by monitoring the double-stranded DNA breaks produced in the process of V(D)J recombination. This analysis provides a measure of recombinase activity at immunoglobulin heavy and light chain loci across defined developmental stages spanning the process of B cell development. We find that expression of a complete immunoglobulin heavy chain protein is accompanied by a drastic change in the targeting of V(D)J recombinase activity, from being predominantly active at the heavy chain locus in pro-B cells to being exclusively restricted to the light chain loci in pre-B cells. This switch in locus-specific recombinase activity results in allelic exclusion at the immunoglobulin heavy chain locus. Allelic exclusion is maintained by a different mechanism at the light chain locus. We find that immature, but not mature, B cells that already express a functional light chain protein can undergo continued light chain gene rearrangement, by replacement of the original rearrangement on the same allele. Finally, we find that the developmentally regulated targeting of V(D)J recombination is unaffected by enforced rapid transit through the cell cycle induced by an Eμ-*myc* transgene.

B cell antigen receptor genes are assembled from germline-encoded segments, V_H, D_H, and J_H at the immunoglobulin heavy chain (IgH) locus and V_κ and J_κ or V_λ and J_λ at the light chain (IgL) loci, by a series of site-specific recombination events collectively termed V(D)J recombination (1). In addition to determining the antigen specificity of mature B cells, Ig gene products play a crucial role in guiding B cell development through a series of checkpoints based on the successful assembly of Ig genes. Thus, B cells from mice that lack the capacity to rearrange their Ig genes are arrested at an early (pro-B cell) stage in development (2, 3), but the introduction of a rearranged IgH transgene allows the cells to progress to an intermediate (pre-B cell) stage, and if both a heavy and a light chain transgene are provided the cells can reach the mature B cell stage (4, 5).

V(D)J recombination is dependent on the recombinase activating genes, *RAG-1* and *RAG-2*, whose expression is strictly limited to immature lymphoid cells (6, 7). Both gene products are necessary for recombination, and their coexpression in non-lymphoid cells is sufficient to confer the ability to recombine plasmid substrates. However, additional levels of regulation must determine which loci are targeted for recombination in developing lymphocytes, because the process shows both lineage specificity (Ig genes are fully rearranged only in B cells and T cell receptor genes only in T cells) and temporal regulation. In B cells, studies of completed rearrangements in cell lines (8) or ex vivo B lineage cells (9) representing various stages of development have shown that D_H-to-J_H rearrangement occurs first on both chromosomes before V_H-to-DJ_H rearrangement at either allele. Successful, in-frame, V_H-to-DJ_H rearrangement completes the assembly of a heavy chain gene of the μ isotype (Igμ), whose expressed product then forms a signaling complex with the surrogate light chain (SLC) proteins V_{preB} and λ5 (10-12). Signaling through this pre–B cell receptor (pre-BCR) complex mediates the checkpoint function of Igμ by effecting progression to the pre-B cell stage (4, 5), proliferative expansion of the population (13), and heavy chain allelic exclusion (see below). Light chain gene rearrangements (V_κ-to-J_κ) can occur before V_H-to-DJ_H rearrangement (13-15), but the frequency of $V_\kappa J_\kappa$ rearrangements is much greater at later stages of development, where cells already have a complete heavy chain gene (9, 16, 17).

[1]*Abbreviations used in this paper:* 7-AAD, 7-aminoactinomycin D; CBE, coding broken ends; IgH, immunoglobulin heavy chain; Igμ, μ isotype; LMPCR, ligation-mediated PCR; pre-BCR, pre–B cell receptor; RAG, recombinase activating gene; RSS, recombination signal sequence; SBE, signal broken ends; SLC, surrogate light chain.

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The simplest hypothesis for explaining these data is that the observed sequence of completed rearrangements reflects the developmental regulation of V(D)J recombinase activity by Ig gene products. According to this Ig-regulated model, the signal mediated by the product of a functional heavy chain gene inhibits rearrangement at the allelic heavy chain locus, accounting for the phenomenon of heavy chain allelic exclusion, and also activates rearrangement at the IgL loci (8, 18). However, the complexity of B cell development and the multiple selection steps that demonstrably involve Ig gene products make it difficult to determine whether completed rearrangements reflect regulation of the V(D)J recombinase or the results of selection at the cellular level after recombination. Two alternative models have been proposed for allelic exclusion. The stochastic model proposes that gene rearrangement is intrinsically inefficient and has a very low probability of producing functional heavy or light chain gene rearrangements at both alleles before all recombination is stopped (19). The cellular selection model postulates that expression of Igμ from both alleles is toxic and leads to the elimination of the double producing cells (20, 21). Also, with regard to the effect of Igμ signaling on light chain gene rearrangement, it has been proposed that it is limited to providing the proliferative signal that expands the precursor population undergoing light chain gene rearrangement as a whole, without any effect on the rate or efficiency of the recombination reaction itself (22).

Previous studies of V(D)J recombination have relied on analysis of completed rearrangements (9, 23). This approach has the disadvantages of being cumulative, and thus not able to distinguish rearrangements completed at earlier stages from recently generated ones. Analysis of completed rearrangements is also biased by selection events occurring at the cellular level after rearrangement. The definition of intermediates in V(D)J recombination provides a way to follow this process directly and to distinguish between different models of B cell development. Gene segments used in V(D)J recombination are identified by a recombination signal sequence (RSS) (24). Recent work on the biochemistry of V(D)J recombination has shown that the initial step in the reaction is precise cleavage between a coding gene segment and its flanking RSS, resulting in two species of broken-ended DNA intermediates, a pair of signal ends and a pair of coding ends (25, 26). These broken-ended DNA intermediates can be detected by means of a ligation-mediated PCR (LMPCR) assay and used as a measure of V(D)J recombinase activity at a given locus. This assay is applicable to both signal and coding ends at any of the rearranging loci by using different primers for amplification (M.S. Schlissel, manuscript in preparation and references 27–29).

We undertook to study the developmental regulation of V(D)J recombination directly, by assaying double stranded DNA breaks produced in the process of V(D)J recombination at the immunoglobulin heavy and light chain gene loci. This approach allowed us to measure rearrangement activity at individual Ig loci across stages of B cell development defined by the expression of antigenic markers and isolated by cell sorting. Furthermore, because V(D)J recombination is known to be sensitive to cell cycle status (27) and different stages of B cell development differ greatly in proliferative behavior, we addressed the question whether cell cycle regulation contributes to the developmental control of V(D)J recombination by comparing B cell development in normal and Eμ-*myc* transgenic mice. In these mutant mice, overexpression of a c-*myc* transgene targeted to the B cell lineage by the IgH (Eμ) enhancer results in vigorous proliferation of developing B cells (30). Finally, we examined the issue of V(D)J recombinase inactivation. The simplest interpretation of the Ig-regulated model would hold that V(D)J recombination is shut off as soon as a functional light chain gene rearrangement is generated, resulting in expression of the complete BCR on the cell surface at the immature B cell stage (Table 1). However, it has been observed that cells expressing IgM on their surface ($sIgM^+$ cells) are capable of further light chain gene rearrangement under special circumstances, either in vitro after IL-7 withdrawal (31), or in vivo in mice expressing a transgenic BCR with anti-self specificity (32, 33). It has not been clear, however, whether such secondary light chain gene rearrangements are a significant factor in normal B cell development. Our approach allowed us to determine directly whether light chain gene rearrangement continues to occur in $sIgM^+$ cells in normal development, and at what stage of development V(D)J recombination finally ceases.

Materials and Methods

Mice. Female 4–6-wk-old Balb/c mice were purchased from NCI (Frederick, MD). The mice used in these experiments were between 6 and 8 wk old. Eμ-*myc* transgenic mice (34) were bred in our animal facility from mice originally obtained from Dr. C. Sidman (University of Cincinnati).

Antibodies. The PE-conjugated mAb RA3-6B2 (anti-CD45R, B220), RM2-5 (anti-CD2, LFA-2), and 11-26c.2a (anti-IgD) were purchased from PharMingen (San Diego, CA). Biotinylated and FITC-conjugated goat anti–mouse Igμ antisera were purchased

Table 1. *Sequential Expression of Antigens in B Cell Development*

B cell stage	B220	cIgμ	CD2	sIgM	sIgD	% B200+*	% in S-G_2/M‡
Pro–B cells	+	−	−	−	−	15	18
Early pre–B cells	+	+	−	−	−	9	28
Late pre–B cells	+	+	+	−	−	48	9
Immature B cells	+	+	+	+	−	22	6
Mature B cells	+	+	+	+	+	8	6

*Percentage of all $B220^+$ cells in adult BALB/c bone marrow represented by each subpopulation. Results of one typical experiment are shown.

‡Percentage of cells in each subpopulation having a DNA content greater than G1 as shown by DNA staining with 7-AAD. Results of one typical experiment are shown.

from Southern Biotechnology Associates. The RA3-6B2 (anti-CD45R, B220) antibody was also purified in our own lab and conjugated to biotin. Streptavidin-Quantum Red conjugate was purchased from Sigma Chem. Co. (St. Louis, MO). All antibodies were titered for flow cytometric staining.

Cell Staining and Sorting by Flow Cytometry. Antigens on the surface of cells were stained by standard methods (35). For staining of cytoplasmic Igμ and DNA, we used the method described by Schmid et al. (36). In brief, cells were stained for surface antigens in the usual manner, fixed in wash media containing 0.25% paraformaldehyde for 1 h on ice, and then permeabilized by incubation in PBS containing 0.2% Tween 20 for 15 min at 37°C. The extent of permeabilization was monitored with a microscope by Trypan blue exclusion, and if necessary the permeabilization step was repeated. For cytoplasmic Igμ staining, the cells were then incubated with a carefully titrated amount of FITC-conjugated goat anti–mouse Igμ serum on ice for 20 min and then washed twice with PBS containing 0.2% Tween 20. For DNA staining, 7-AAD was added to the permeabilized cells in suspension to a final concentration of 15 μg/ml and the cells were incubated on ice for 30 min before analysis. Flow cytometric analysis was performed on a Becton-Dickinson FACScan® instrument using the CellQuest software package. Sorting was performed on a flow cytometer (Coulter Epics Elite, Coulter Corp., Hialeah, FL) using single laser excitation at 488 nm.

RT-PCR. RNA from 5×10^5 sorted cells was isolated by lysis in a guanidinum thiocyanate buffer in the presence of 10 μg carrier tRNA and centrifugation through a cushion of cesium chloride (37). The RNA was reverse-transcribed as described elsewhere (38). 2 μl (one-tenth) of each reverse transcription reaction was used for each PCR amplification. PCR conditions were 94°C for 1 min followed by 66°C for 2 min. 30 s, for 24 cycles (H2 transcript) or 27 cycles (cyclin D1 transcript). Primer sequences are listed below. All amplifications were completed by a long extension step of 10 min at 72°C. One-half of the final product was analyzed by electrophoresis on a 1% agarose, 1% NuSieve (FMC) gel, and blotted under alkaline conditions to a nylon membrane (Zetabind, Cuno). Blots were hybridized to the appropriate PCR fragment labeled with ^{32}P by random priming (Life Technologies kit and manufacturer's instructions) and analyzed with a PhosphorImager using ImageQuant software (Molecular Dynamics).

Purification of DNA for LMPCR. DNA purification, T4 polymerase polishing, and linker ligation were all carried out in agarose plugs, as described elsewhere (Schlissel, M.S., manuscript in preparation). In brief, 5×10^5 sorted cells from each stage were resuspended in 40 μl PBS, mixed with an equal volume of molten 1% agarose, and immediately poured into plug molds (Bio-Rad). After the plugs solidified, they were incubated overnight at 55°C (in 100 mM Tris, pH 8.0, 25 mM EDTA, 1% sarkosyl, and 400 μg/ml proteinase K), washed in TE containing 0.5 mM PMSF for 30 min, then washed three more times in TE over 24 h. The plugs were next treated with T4 DNA polymerase by adding in a 40 μl reaction mixture containing polymerase buffer, three units T4 DNA polymerase (Life Technologies), and 100 μM dNTPs, for 1 h at 37°C. Plugs were then washed two more times in excess TE over 12 h and used for linker ligation.

LMPCR Assay for Detecting Signal and Coding DNA Broken Ends. The LMPCR assays for detecting signal (27) and coding end (Schlissel, M.S., manuscript in preparation) breaks have been described elsewhere. In brief, plugs containing purified DNA from sorted cells were subjected to linker ligation in 40 μl ligation mixture containing ligation buffer (Boehringer), 40 pmol BW linker, and 2 u T4 DNA ligase (Boehringer) and incubated overnight at 16°C. The reaction was then mixed with 100 μl PCR-L buffer (10 mM Tris, pH 8.8, 50 mM KCl, 0.25% Tween-20, 0.25% NP-40) and heated to 95°C for 10 min. For PCR, the linker-ligated DNA was brought to 65°C, and 4 μl of each sample were added to 21 μl amplification mix containing the appropriate primers and Taq DNA polymerase (Life Technologies). The primary amplification consisted of 12 cycles of 94°C for 1 min and by 66°C for 2 min 30 s each. 1 μl of that reaction was used for a second amplification with a nested locus-specific primer (see below) consisting of 26 cycles (signal ends) or 29 cycles (coding ends). One half of the final product was analyzed by electrophoresis on a 1% agarose, 1% NuSieve (FMC) gel, and blotted under alkaline conditions to a nylon membrane (Zetabind, Cuno). Blots were hybridized to ^{32}P end-labeled locus-specific internal oligonucleotides and analyzed with a PhosphorImager using ImageQuant software (Molecular Dynamics). Due to multiple sequential reactions and the large numbers of amplification cycles required to detect signal and coding ends, these assays are not strictly quantitative. Template dilution controls, however, confirmed that the differences in intensity of the hybridization signals represent differences in the initial frequency of the corresponding broken DNA ends (data not shown).

Primers. The primers used are listed below.

PCR primers

5′ CD14	GCTCAAACTTTCAGAATCTACCGAC
3′ CD14	AGTCAGTTCGTGGAGGCCGGAAATC
5′cycD1	CTACACTGACAACTCTATCCG
3′cycD	(C/G)A(C/A/T)(A/G)AAGGTCTG(T/C/G)GCATGCTT
5′ H2	CGATTACATCGCCCTGAACG
3′ H2	GCTCCAAGGACAACCAGAAC

Primers used in LMPCR

BW linker:
- BW-1 GCGGTGACCCGGGAGATCTGAATTC
- BW-2 GAATTCAGATC

Linker primers:
- BW-1 GCGGTGACCCGGGAGATCTGAATTC
- BW-H (inside primer in SBE amplifications) CCGGGAGATCTGAATTCCAC

Locus specific primers:

$D_{FL16.1}$ SBE
- outside (D_{FL}B): GCCTTCCACAAGAGGAGAAG
- inside (D_{FL}): GGAAGTCCCCCAGAAACAGACC
- Hybridization probe (BW-J_D): GATCTGAATTCCACA(G/C)T(G/A)GT

$J_{\kappa 1}$, $J_{\kappa 2}$ SBE
- outside (κ_{05}): GCCCAAGCGCTTCCACGCATGCTTGGAG
- inside(κ_0): TCCACGCATGCTTGGAGAGGGGGT T

$J_{\kappa 1}$ CBE
- outside (J_κB): CCTCCCTTTTGACAGGGTGTTCTCCTAGGCC
- inside(J_κB3): GTATCTTTGCCTTGGAGAGTGCCAGAAT
- Hybridization probe (J_κ1R): ACGGAAGAAAGAGACTTTGG

$J_{\kappa 2}$ CBE
- outside (J_κB): CCTCCCTTTTGACAGGGTGTTCTCCTAGGCC
- inside(J_κ1160): GTGAACAAGAGTTGAGAAGACTAC
- Hybridization probe (J_κ2R): CTTAGTGAACAAGAGTTGAG

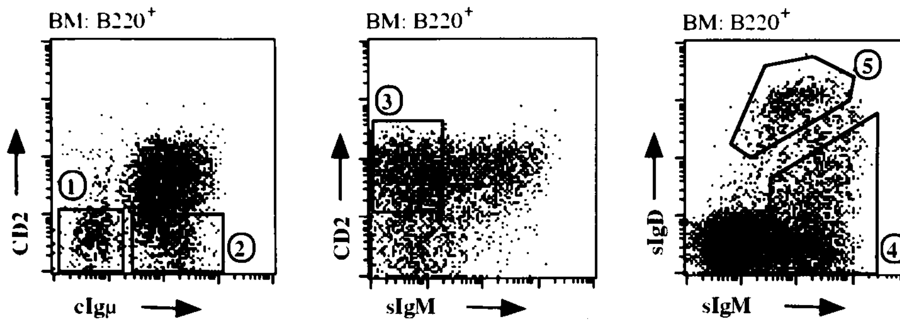
$J_{\kappa 5}$ CBE
- outside (J_κC): CGTCAACTGATAATGAGCCCTCT
- inside(J_κ5R): TGAGCCCTCTCCATTTTCTCAAG
- Hybridization probe (MD11): CATGTTAGGAGTTAAAGTC

V_κ CBE
- outside (V_κB): ACCTCTGACCCAGTCGACTTACAG
- inside (V_κS): CCGAATTCG(G/C)TTCAGTGGCAGTGG(A/G)TC(A/T)GG(A/G)AC
- Hybridization probe (V_κS)

Results

Functional Division of the B Cell Lineage. Despite the wealth of known regulated surface antigens on developing B cells (9, 39), the only markers of unambiguous functional signif-

A



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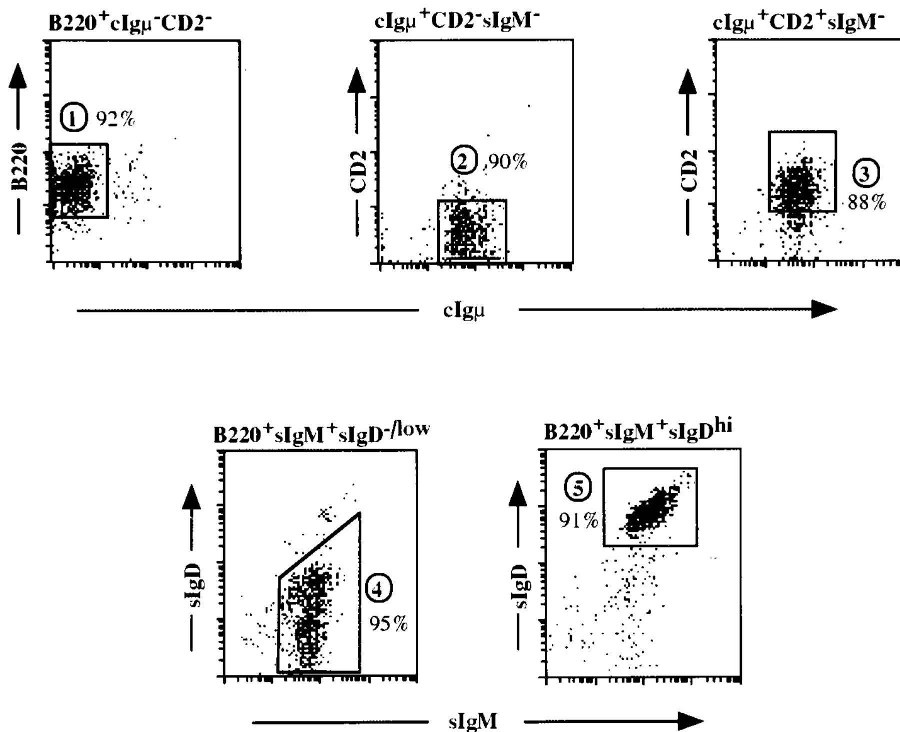
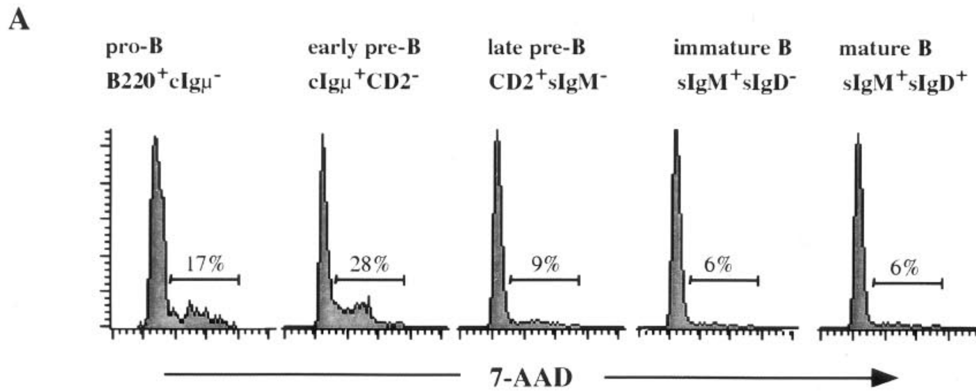
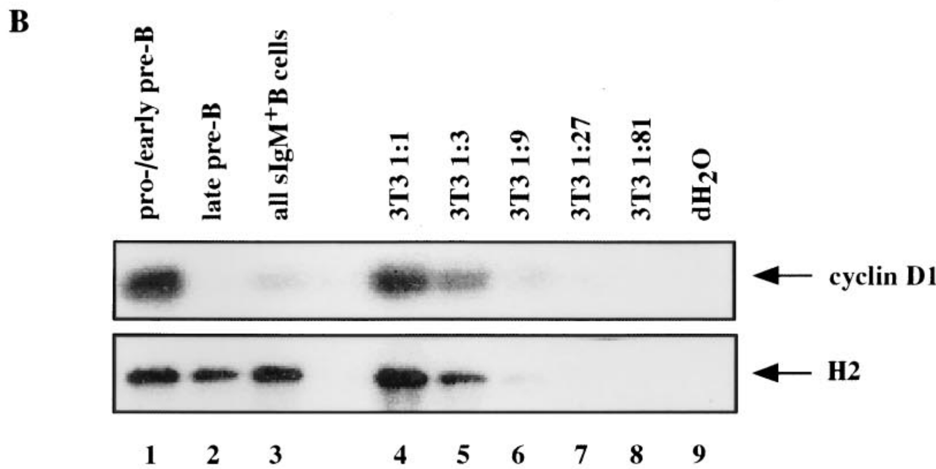


Figure 1. Flow cytometric analysis and sorting of B cell developmental stages from Balb/c bone marrow. (*A*) Triple staining for B220, cIgμ, and CD2 (*left dot plot*), B220, CD2, and sIgM (*middle dot plot*), and B220, sIgM, and sIgD (*right dot plot*). In each case, the data are gated on lymphocytes by forward and side scatter and on $B220^+$ cells by fluorescent staining. Numbered boxes identify the defined B cell subpopulations (see Table 1): 1 = pro-B cells; 2 = early pre-B cells; 3 = late pre-B cells; 4 = immature B cells; 5 = mature B cells. (*B*) Analysis of B cell populations numbered as in *A*) after sorting. The staining criteria used for sorting and the purity of each sorted subpopulation are indicated in each panel. The data are ungated.

icance with regard to V(D)J recombination are the products of the Ig genes themselves. Accordingly, we have divided the B cell lineage into pro-B cells, which do not express functional Igμ, pre-B cells, which express Igμ in their cytoplasm (cIgμ) but not on their surface, immature B cells, which express Igμ and IgL (IgM complex) on their surface, but little or no δ isotype heavy chains (IgD), and mature B cells, which express both IgM and high levels of IgD on their surface. The pre-B cell population was further subdivided based on the expression of the CD2 surface antigen into early $CD2^-$ and late $CD2^+$ pre-B cells. This marker was chosen because it is developmentally regulated to appear at a point intermediate between cIgμ and sIgM (5) (Fig. 1 *A*), and its acquisition correlates well with the cell cycle behavior of pre-B cells (see below). These populations were identified by staining bone marrow cells from adult Balb/c mice with various combinations of antibodies to the B220, cIgμ, CD2, sIgM, and sIgD antigens and analyzing them by 3-color flow cytometry (Fig. 1 *A* and Table 1).

To assess the proliferative status of cells at each developmental stage, the DNA content of these cells was determined by flow cytometry. This was done using the DNA-binding dye 7-aminoactinomycin D (7-AAD) in conjunction with staining for two antigenic markers to identify the subpopulation of interest ($B220^+cIg\mu^-$ for pro-B cells, $cIg\mu^+$ $CD2^-$ for early pre-B cells, $CD2^+sIgM^-$ for late pre-B cells, $sIgM^+sIgD^-$ for immature B cells, and $sIgM^+sIgD^+$ for mature B cells). This analysis revealed that early pre-B cells had the highest fraction of cells with DNA content greater than G_1 (Fig. 2 *A*). This population presumably comprises cells undergoing a proliferative burst in response to pre-BCR signaling. In contrast, late pre-B cells, as well as im-





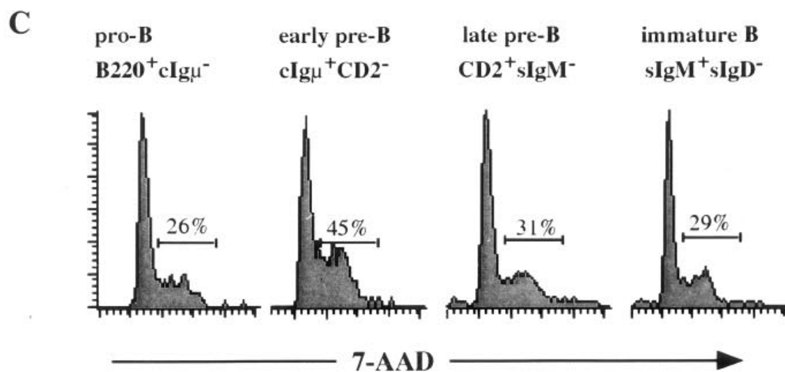


Figure 2. Proliferative behavior of B cells at different stages of development. (*A*) DNA staining of cells at different stages in the B cell lineage in wild-type Balb/c mice. Bone marrow cells were stained for two developmental markers using FITC- and PE-conjugated antibodies and counterstained with 7-AAD to determine their DNA content. The histograms represent data gated on the indicated combination of antigenic markers. The percentage of cells with DNA content greater than G_1 is indicated. (*B*) Southern blot of RT-PCR analysis of cyclin D1 gene expression in sorted B cell lineage cells. Lane *1*, pro-B and early pre-B cells ($B220^+$ $CD2^-$); lane *2*, late pre-B cells ($B220^+CD2^+sIgM^-$); lane *3*, immature and mature B cells ($B220^+sIgM^+$); lanes *4–8*, serial dilution of cDNA from 3T3 cells in exponential growth phase. The primers used in this amplification reaction span an intron, eliminating the possibility of a false signal from contaminating genomic DNA. Transcripts amplified by the H2 primers come from a non-polymorphic region of several MHC class I molecules and are expressed at constant levels. (*C*) DNA staining of cells at different stages in the B cell lineage in Eμ-*myc* transgenic mice. Staining and data acquisition were done as in *A*.

mature and mature B cells, appeared to be almost entirely quiescent, with very few cells in the S or G_2/M phases of the cell cycle (Fig. 2 *A*).

This transition to quiescence at the late pre-B cell stage was confirmed by RT-PCR analysis of cyclin gene transcripts from $B220^+CD2^-$ pro/early pre-B cells, $B220^+$ $CD2^+sIgM^-$ late pre-B cells, and $sIgM^+$ immature/mature B cells isolated by cell sorting from Balb/c bone marrow. As shown in figure 2B, cyclin D1 transcripts were abundant at the pro/early pre-B cell stage, but were rare or undetect-

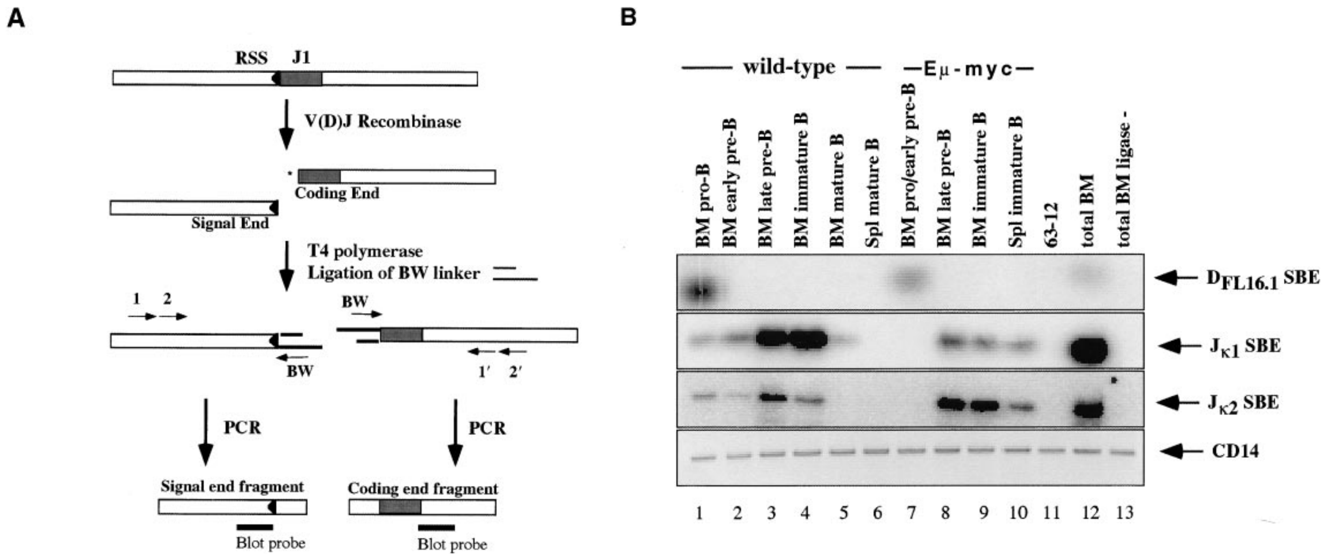


Figure 3. (*A*) Diagram of the LMPCR assay used to detect signal end and coding end double stranded DNA breaks at rearranging loci. A rearranging locus is shown, with the RSS abutting the coding portion of a rearranging gene segment (J1, *filled area*). Cleavage by V(D)J recombinase at an RSS generates two kinds of ends: a signal end and a coding end. The signal end is blunt and 5′-phosphorylated and available for linker ligation. The coding end is processed through a hairpin intermediate and the asterisk next to it signifies heterogeneity in the fine structure of the opened hairpin. Coding ends are blunted with T4 DNA polymerase and then subjected to linker ligation. Amplification is subsequently carried out using a linker-specific primer (*BW*) and a set of locus-specific primers (1, 2 or 1′, 2′). (*B*) SBE in developing B cells from wild-type and Eμ-*myc* mice. Purified DNA from sorted subpopulations of B cells from wild-type Balb/c and Eμ-*myc* mice was subjected to LMPCR to detect SBE upstream of the $D_{FL16.1}$, $J_{\kappa1}$, and $J_{\kappa2}$ segments. PCR products were analyzed by electrophoresis on agarose gels, blot transfer to a nylon membrane, and hybridization with locus-specific oligonucleotide probes. The identities of the indicated PCR products were confirmed by DNA sequence analysis (28). Labeled products were visualized with a PhosphorImager. Lanes *1–10*, B cell development stages as defined in Table 1; lane *11*, 6312, a pro-B cell line derived from a RAG-2-deficient mouse (3); lane *12*, B220$^+$ cells from bone marrow, representing all stages of B cell development; lane *13*, same as lane *12*, with no ligase added at the linker ligation step. The bottom strip shows an ethidium bromide-stained agarose gel of a control amplification of a non-rearranging locus (*CD14*), showing equivalent amounts of amplifiable DNA in all samples.

able at the subsequent late pre-B or sIgM$^+$ B cell stages. RT-PCR analysis of the expression of the cyclin D3, cyclin A, and E2F1 genes showed similar results (data not shown).

Assay for Broken-ended DNA Intermediates in V(D)J Recombination. As shown in Fig. 3 *A*, cleavage by V(D)J recombinase at an RSS results in two broken-ended species, a signal end and a coding end. Signal broken ends (SBE) are the more abundant, as they remain in the blunt-ended conformation until they are joined. This broken-ended species can persist in a resting cell for an extended period of time. However, cell cycling places a limit on the persistence of these SBE, because they are joined before the onset of DNA synthesis (27, 29). Thus, in a population of cycling cells, SBE can be a reliable measure of locus-specific V(D)J recombinase activity. This is in contrast to analysis of completed joints, which can be affected by pre-existing rearrangements or by selection at the cellular level occurring after rearrangement. Coding broken ends (CBE) are a more specific indicator of V(D)J recombinase activity, because they are rapidly processed to coding joints regardless of the cycling status of the cell (29). However, they are initially sealed in a hairpin structure and become available to linker ligation only in the interval between hairpin opening and coding joint formation (Schlissel, M.S., manuscript in preparation and 26, 29). The short half-life and the intermediate processing steps of coding ends make them much less abundant than the corresponding signal ends and more difficult to detect reliably.

To characterize the targeting of V(D)J recombinase activity at different stages of development, populations of cells within the lineage were isolated from the bone marrow of adult Balb/c mice by cell sorting based on their expression of antigenic markers. The populations isolated were pro-B cells (B220$^+$cIgμ$^-$CD2$^-$), early pre-B cells (cIgμ$^+$CD2$^-$sIgM$^-$), late pre-B cells (cIgμ$^+$CD2$^+$sIgM$^-$), immature B cells (B220$^+$sIgM$^+$sIgD$^{-/low}$), and mature B cells (B220$^+$sIgM$^+$sIgD^{hi}). Analysis of the sorted fractions showed them to be 85–95% pure (Fig. 1 *B*).

DNA from equal numbers of cells was purified, treated with T4 DNA polymerase to blunt any overhanging CBE, and subjected to ligation with a double stranded linker capable of ligating in only one orientation. Linker-ligated DNA was then used for amplification by PCR using a linker-specific primer and a pair of nested locus-specific primers (Fig. 3 *A*). Control amplification of a non-rearranging locus showed that all samples contained similar amounts of amplifiable DNA. Because heavy chain gene rearrangements occur almost exclusively by deletion, using the RSS 5′ of D_H genes for V-to-DJ_H joining (38), SBE upstream of D_H genes represent intermediates in the V_H-to-DJ_H rearrangement step. We focused on SBE upstream of $D_{FL16.1}$ because it is the most frequently used of the D_H

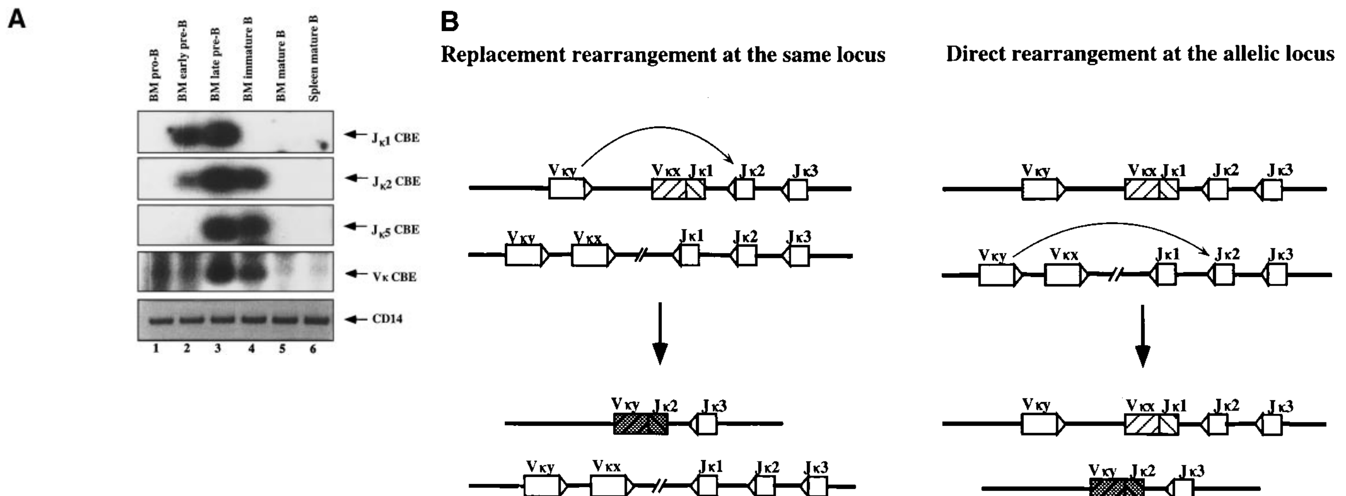


Figure 4. (*A*) CBE at the Igκ locus in B cell development. Purified DNA from sorted subpopulations of B cells from wild-type Balb/c mice was subjected to LMPCR to detect CBE associated with $J_{\kappa1}$, $J_{\kappa2}$, and $J_{\kappa5}$ and most V_κ segments. PCR products were analyzed by electrophoresis on agarose gels, blot transfer to a nylon membrane, and hybridization with locus-specific oligonucleotide probes. The identities of the indicated CBE PCR products were confirmed by DNA sequence analysis (data not shown). Labeled products were visualized with a PhosphorImager. The bottom strip shows an ethidium bromide-stained agarose gel of a control amplification of a non-rearranging locus (CD14), showing equivalent amounts of amplifiable DNA in all samples. (*B*) Diagram of possible mechanisms for secondary light chain gene rearrangements in immature ($IgM^+IgD^{-/low}$) B cells. (*Left*) replacement of the original rearrangement (*light hatched*) by joining of an upstream V_κ to a downstream J_κ segment on the same chromosome (secondary rearrangement dark hatched). (*Right*) direct rearrangement of a V_κ to a J_κ segment at the locus allelic to the original rearrangement.

segments (40). At the κ locus we measured SBE upstream of the most frequently used Jκ segments, $J_{\kappa1}$ and $J_{\kappa2}$, as indicative of V_κ-to-J_κ rearrangement (Fig. 3 *B*). To distinguish between ongoing rearrangement and the persistence of unrepaired SBE in non-cycling cells at later stages of development, we also assayed for CBE at the $J_{\kappa1}$, $J_{\kappa2}$, and $J_{\kappa5}$ segments, as well as at V_κ gene segments (Fig. 4 *A*). In each instance, the data shown is representative of that obtained from at least three independent cell sorting experiments. The identities of the various SBE and CBE fragments have been confirmed by DNA sequence analysis (Schlissel, M.S., manuscript in preparation and 27).

Retargeting of V(D)J Recombinase. The immediately striking observation that emerges from these studies is that the pro-B to pre-B cell transition is accompanied by a drastic change in the targeting of V(D)J recombinase activity, from being predominantly active at the heavy chain locus in pro-B cells to being exclusively restricted to the light chain loci in pre-B cells (Fig. 3 *B*, lanes *1–3*). The course of D_H SBE, present at high levels at the pro-B cell stage and undetectable (at least 10-fold less frequent, data not shown) at all subsequent stages, reflects the phenomenon of allelic exclusion, where a productively rearranged IgH gene (obligatory at all stages past pro-B cell) prevents subsequent rearrangement at the allelic locus, even though light chain genes are actively rearranged at later stages. This result argues that locus-specific modulation of V(D)J recombinase activity alone might be sufficient to account for the phenomenon of heavy chain allelic exclusion.

SBE upstream of $J_{\kappa1}$ and $J_{\kappa2}$ show a different pattern (Fig. 3 *B*). We consistently detected Jκ SBE in pro-B cells (Fig. 3 *B*, lane *1*, and data not shown) representing some level of active rearrangement at the Igκ locus. We interpret this signal to represent a low "constitutive" level of V_κ-to-J_κ rearrangement independent of signaling through the pre-BCR. This interpretation is consistent with evidence from mutant mice incapable of assembling a pre-BCR, showing that V_κ-to-J_κ rearrangements can occur at a detectable level regardless of the state of the IgH loci (13–15). Assembly and expression of IgH resulted in a dramatic induction of J_κ SBE levels (~10-fold as assessed by template dilution, data not shown), but interestingly, this occurred only at the late pre-B cell stage, after the proliferative burst occurring at the early pre-B cell stage (Fig. 3 *B*, lanes *2* and *3*). This suggests that signaling through the pre-BCR does induce IgL rearrangement, but as a delayed effect, not an immediate one.

Because $CD2^+sIgM^-$ late pre-B cells are quiescent and might harbor Igκ SBE for a longer time before forming a signal joint, we also assayed CBE at several J_κ segments as a more reliable indicator of ongoing recombinase activity (Fig. 4 *A*). The results confirmed that maximal induction of light chain gene rearrangement occurs at the late pre-B cell stage (Fig. 4 *A*, lanes 1–5). No CBE were detectable in pro-B cells (Fig. 4 *A*, lane *1*) possibly due to the lower sensitivity of the CBE assay.

Taken together, these results show that the known progression of rearrangement products in B cell development (8, 9, 16, 41) can be explained by the developmentally regulated targeting of the recombinase alone, and that the developmental stages at which IgH and IgL rearrangements predominantly occur are sharply demarcated and separated by a highly proliferative stage during which little rearrangement occurs at either locus. These data also show that IgH rearrangement is suppressed at the same time that pre-B cells are actively rearranging Igκ, arguing against the stochastic and the cellular selection models of allelic exclusion (19–21).

Rearrangement and Proliferation. The observation that Igμ expression maximally induced IgL gene rearrangement

only after the cells had become quiescent (Figs. 3 *B* and 4 *A*, compare lanes *2* and *3*) led us to consider a causal relationship between proliferation and the delayed induction of light chain gene rearrangement. To study the effect of proliferation on the regulation of V(D)J rearrangement, we examined this process in the B lymphocyte lineage cells from Eμ-*myc* transgenic mice (34). B cell development in these animals is grossly normal up to the immature B cell stage, with the exception that the forced overexpression of the c-*myc* transgene in the B cell lineage results in vigorous proliferation at all stages of development (Fig. 2 *C*; unpublished data; and 30). If induction of IgL gene rearrangement requires that pre-B cells stop proliferating, then it should be abrogated in Eμ-*myc* mice, because B cells at all stages of development are cycling rapidly. However, in sorted cells from Eμ-*myc* mice, SBE upstream of $D_{FL16.1}$, $J_{\kappa 1}$ and $J_{\kappa 2}$ showed the same pattern of developmental regulation of V(D)J rearrangement as wild-type cells, even though the absolute level of the signal was decreased (Fig. 3 *B*). One point of difference between wild-type and Eμ-*myc* mice is the absence of J_κ SBE in pro/early pre-B cells of the latter genotype (Fig. 3 *B*, lanes *1* and *2* vs. lane *7*). Levels of several SBE are reduced in Eμ-*myc* mice, possibly as a consequence of reduced RAG-2 levels in cycling cells (42–44). It is possible that a signal that was weak in wild-type pro-B and early pre-B cells dropped below the limits of detection by this assay in Eμ-*myc* mice because of the general decrease in SBE levels associated with this genotype. Another point of difference concerns the high level of J_κ SBE, especially at $J_{\kappa 2}$, found in cells from Eμ-*myc* spleen (Fig. 3 *B*, lane *6* vs. *10*). This is probably due to the fact that B cells from wild-type spleen are almost exclusively of the mature $sIgM^+IgD^{hi}$ phenotype and were isolated by that criterion, whereas B lineage cells from Eμ-*myc* spleens are largely pre-B and immature $sIgM^+IgD^-$ B cells and were isolated on the basis of sIgM expression alone. A previous report (45) demonstrated that $sIgM^+$ cells from N-myc transgenic mice fail to inactivate RAG expression, a factor which might also contribute to a relatively higher level of $J_{\kappa 2}$ SBEs.

These data indicate that quiescence is not a requirement for induction of Igκ rearrangement, and that other differences between early and late pre-B cells must determine the level of recombinase activity at the Igκ locus.

Replacement Rearrangements in sIgM⁺ Cells. B cells at later stages of development are largely quiescent, making SBE levels a potentially misleading indicator of ongoing V(D)J recombination due to the long half-life of signal ends in non-cycling cells (29). Thus, it is possible that the SBE detected in immature B cells may have been generated at the pre-B cell stage. In order to assess the inactivation of the V(D)J recombinase, we focused our analysis on the short-lived coding ends associated with rearrangement at the $J_{\kappa 1}$, $J_{\kappa 2}$, and $J_{\kappa 5}$ gene segments, and at V_κ gene segments recognized by a set of degenerate primers (46). As shown in Fig. 4 *A*, $J_{\kappa 1}$ coding ends are abundant in late pre-B cells, but become undetectable in either immature ($sIgM^+D^{-/low}$) or mature ($sIgM^+D^{hi}$) B cells (*top panel*, lanes *3–5*), even though ~40% of Igκ alleles remain in germline configuration (9). However, rearrangements to the downstream $J_{\kappa 2}$ and $J_{\kappa 5}$ gene segments do occur in immature, but not mature, B cells, as shown by our ability to detect $J_{\kappa 2}$, $J_{\kappa 5}$, and V_κ CBE (Fig. 4 *A*, *bottom three panels*). Negative samples contained at least 5–10-fold fewer CBE than any positive sample in these assays (determined by template dilution, data not shown). This demonstrates ongoing rearrangement at the Igκ locus in immature B cells, but not in mature B cells from bone marrow or spleen and implies that the signal mediating allelic exclusion at the IgL loci is more complex than simple expression of a functional gene product in the sIgM complex. Also, the absence of CBE at $J_{\kappa 1}$ is of significance in determining the mechanism by which these rearrangements occur and remain allelically excluded (see Discussion).

Discussion

Fractionating Bone Marrow B Cells Based on Heavy Chain Expression. Several labs have reported strategies for fractionating bone marrow B cells according to developmental stage (9, 44). Each of these schemes relies on the differential expression of surface markers whose expression is correlated with various regulated events. Because our goal was to assess the effects of immunoglobulins on targeting of the V(D)J recombinase, we chose to rely on Ig heavy-chain rather than CD25 (44) or CD43 (9) as a definitive marker for pre-B cells. We incorporated the membrane protein CD2 into this scheme because it was shown previously to correlate with pre-B cell cycle activity (5). Other workers have used cell size (as assessed by forward light scatter) to identify rapidly cycling pre-B cells. Because cell cycle stage correlates with cell size, this approach tends to overestimate the fraction of pre-B cells with greater than 2n DNA content (60–70%), while ignoring cells which are actively cycling but are in the G1 phase of the cycle (44). Using our approach, which does not involve cell size as a criteria, we found that 28% of $cIg\mu^+CD2^-$ had a greater than 2n DNA content. We believe our fractionation strategy more completely identifies the population of early, cycling pre-B cells.

The Pre-B Cell Receptor and Recombinase Retargeting. The dramatic change in the loci undergoing rearrangement across the pro-B to pre-B cell transition supports the hypothesis that V(D)J recombinase targeting is a major mechanism by which Igμ controls B cell development. Allelic exclusion has been previously shown to depend on the membrane-embedded form of Igμ and on the surrogate light chain λ5 (13, 14) leading to the conclusion that it is a consequence of pre-BCR signaling. More recently, it has also been shown that heavy chain allelic exclusion is already established at the early pre-B cell stage in wild-type mice (23, 47). However, studies of completed heavy chain gene rearrangements in developing B cells cannot exclude the possibility of bias due to selection at the cellular level, because both positive and negative selection based on Igμ expression can occur in early development (22, 48, 49). Interestingly, the few mature B cells that accumulate in $\lambda5^{-/-}$

mice are allelically excluded (13), whereas their newly generated early pre-B cell progenitors are not (47), implying that selective processes after rearrangement do contribute to allelic exclusion under certain conditions. The complete absence of DNA breaks associated with heavy chain gene rearrangement after the pro-B cell stage seen in this study demonstrates that in normal development allelic exclusion at the IgH locus is mediated at the level of V(D)J recombination.

The segregation of heavy and light chain gene rearrangements to distinct stages of development separated by a highly proliferative stage is consistent with the hypothesis that the proliferative signal transduced by the pre-BCR initially stops all recombination, possibly through the cell cycle–dependent degradation of RAG-2 protein (42, 43). This hypothesis is supported by the absence of RAG-2 protein noted previously in highly proliferative early pre-B cells (44). A separate mechanism would then be responsible for maintaining heavy chain allelic exclusion once RAG protein levels build up again, by limiting recombination to light chain loci. The differential accessibility of heavy and light chain loci in intact nuclei to in vitro cleavage by V(D)J recombinase has recently been shown to be mediated at the level of chromatin conformation (50). It is therefore tempting to speculate that one or more rounds of DNA replication after signaling through the pre-BCR are necessary in order to reprogram the chromatin conformation at Ig loci, making it inaccessible at the heavy chain locus and more accessible at the light chain loci. The increase in SBE and CBE levels at multiple gene segments in the Igκ locus across the pro-B to pre-B cell transition shows that the role of pre-BCR signaling is not limited to expanding the population undergoing light chain gene rearrangement (22), but actually increases the activity of V(D)J recombination at the Igκ locus.

V(D)J Rearrangement and the Cell Cycle. The cell cycle regulation of V(D)J recombination is evidenced by the restriction of recombination-associated, double stranded DNA breaks to the G_0 and/or G_1 phases of the cell cycle (27). A satisfying, but unproven explanation for this phenomenon is the cell cycle–dependent degradation of the RAG-2 protein (42, 43). The concordance of induction of light chain gene rearrangement and quiescence suggests the possibility that an actively cycling state is not permissive for light chain gene rearrangement, with the effect that differentiation is linked to cell cycle control. There is a parallel in the 103 pre-B cell line, which is transformed with a temperature-sensitive allele of the v-*abl* gene. When shifted to the nonpermissive temperature, this cell line simultaneously arrests in G_0, increases expression of the RAG genes, and induces rearrangement at the Igκ locus (51). However, the correct developmental regulation of V(D)J rearrangement at both heavy and light chain loci in Eμ-*myc* mice (Fig. 3 *B*, lanes 7–10) indicates that quiescence is not a requirement for the induction of light chain gene rearrangement. The decreased levels of SBE at several loci seen in Eμ-*myc* mice compared to wild-type can be explained either by rapid joining linked to cell cycle progression or by lower overall efficiency of the recombinase due to a shorter average G_1 phase and the consequent lower levels of RAG-2 protein.

Secondary Rearrangements in Immature B Cells. We demonstrate in this study that DNA breaks indicative of light chain gene rearrangement continue to occur in immature, but not mature B cells (Fig. 4 *A*, lanes *4* and *5*). Because a functional light chain gene is necessary for cell surface expression of the IgM complex, these breaks must reflect secondary rearrangements in cells that have already generated one functional light chain gene. The generation of secondary rearrangements represents a potential violation of the principle of allelic exclusion, which ensures that only one immune specificity is generated per cell.

Two mechanisms can be envisioned for these secondary light chain gene rearrangements in immature B cells, depending on the allele of the Igκ locus on which they occur. At the locus containing the initial rearrangement, an upstream $V_κ$ segment can be joined to a downstream $J_κ$ segment, deleting the initial rearrangement (replacement mechanism, Fig. 4 *B*). It should be noted that the $J_{κ1}$ segment cannot participate in this kind of secondary rearrangement, because its RSS is obligatorily deleted by the initial rearrangement. If the Igκ locus allelic to the initial rearrangement is in germline configuration, any $V_κ$ can be joined to any $J_κ$, leaving the original rearrangement intact (direct mechanism, Fig. 4 *B*). This mechanism predicts prominent involvement of $J_{κ1}$ in secondary rearrangements, matching its over-representation in completed rearrangements at all stages of development, the abundance of $J_{κ1}$ CBE in pre-B cells, and the availability of $J_{κ1}$ segments in germline configuration (9, 52, 53; Fig. 4 *A*; and data not shown). The observed exclusion of the $J_{κ1}$ segment from secondary rearrangements indicates that these must occur by replacement of the original rearrangement on the same chromosome. Replacement rearrangement probably accounts for the relatively high level of Jκ2 SBEs in myc-transgenic B cells as well (Fig. 3 *B*, lanes *8* and *9*). This preference for restricting secondary rearrangements to the same "active" allele could be due to the original rearrangement deleting the large segment of DNA between the $V_κ$ and $J_κ$ loci, bringing upstream $V_κ$ segments into proximity to the actively transcribed J-Cκ locus. These $V_κ$ segments might then be favored in secondary rearrangements because of their proximity to $J_κ$ segments, or because they are transcriptionally activated by the nearby κ enhancers (46). It should also be noted that this mechanism of replacement rearrangement limited to one active allele maintains light chain gene allelic exclusion in the setting of continued expression of RAG proteins and rearrangement capability.

Secondary light chain gene rearrangements have been hypothesized to allow rescue of cells that generate a self-reactive BCR (32, 33). In our studies, the specificity of cells undergoing secondary rearrangements during normal development could not be determined; it is possible that they were autoreactive. However, it is equally plausible to envision a model where replacement rearrangement occurs at an intrinsic rate independent of the original specificity, and is in competition with the processes of negative selection

and recruitment to the mature IgD^{hi} population, which are determined by the nature or strength of the BCR signal.

Inactivation of V(D)J Recombinase. The mechanism by which V(D)J recombination is inactivated in B cells has remained unclear. Comparison with T cell development may be relevant, because T cells also continue to undergo V(D)J rearrangement after the expression of the antigen receptor on the cell surface, at the CD4^{+}CD8^{+}TCR^{low} stage (54–56). In T cells, it has been shown that high-level surface expression of the TCR and cessation of *RAG-1* and *RAG-2* gene expression and of TCRα gene rearrangement are correlated with a positive selection step dependent on TCR–MHC interaction (54, 55). Furthermore, the changes in *RAG* gene expression could be mimicked in vitro by cross-linking of the TCR on the cell surface (56).

Direct evidence of a positive selection step for B cells has been more elusive, and support for this notion has come mainly from the disparity in the representation of different V_H gene families between immature and mature B cells (57, 58). More direct evidence of the involvement of BCR signaling in V(D)J recombinase inactivation in B cells is provided by sIgM^{+} B cell lines derived from tumors induced by an Eμ-N-*myc* transgene. These cell lines constitutively express *RAG-1* and *RAG-2* transcripts, but their expression is shut off by cross-linking of IgM on the cell surface (45). Our observation that CBE are generated at the Igκ locus in immature but not mature cells (Fig. 4 *A*, lanes *4* and *5*) is consistent with the observation that RAG-2 protein is present in immature but not mature cells (44) and indicates that V(D)J recombination is inactivated before cells attain the mature phenotype characterized by high expression of IgD. In view of the preceding observations, this suggests that V(D)J recombinase inactivation and recruitment into the mature IgD^{hi} B cell pool are correlated and may be the result of a positive selection signal mediated by the BCR.

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