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.

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.

B cell antigen receptor genes are assembled from germ-line-encoded segments, VH, DH, and JH at the immunoglobulin heavy chain (IgH) locus and VK or VL and JK 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 loci 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 DH-to-JH rearrangement occurs first on both chromosomes before VH-to-DH rearrangement at either allele. Successful, in-frame, VH-to-DH 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 VpreB 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 (VK-to-JK) can occur before VH-to-DH rearrangement (13-15), but the frequency of VJK rearrangements is much greater at later stages of development, where cells already have a complete heavy chain gene (9, 16, 17).
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 allelic combination is stopped (19). The cellular selection model postulates that expression of IgM from both alleles is toxic and leads to the elimination of the double producing cells (20, 21). Also, with regard to the effect of IgM 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 (LM-PCR) 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 R A3-6B2 (anti-CD45R, B220), RM-2-5 (anti-CD2, LFA-2), and 11-26c.2a (anti-IgD) were purchased from PharMingen (San Diego, CA). Biotinylated and FITC-conjugated goat anti–mouse Ig was purchased from PharMingen (San Diego, CA). Biotinylated and FITC-conjugated goat anti–mouse Ig were purchased from PharMingen (San Diego, CA).

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

| B cell stage | sIgM | CD2 | sIgD | B20 | % in S-G2/M |
|--------------|------|-----|------|-----|------------|
| Pro-B cells  | +    | +   | -    | -   | 15         |
| Early pre-B cells | +    | +   | +    | -   | 9          |
| Late pre-B cells | +    | +   | +    | -   | 48         |
| Immature B cells | +    | +   | +    | +   | 22         |
| Mature B cells  | +    | +   | +    | +   | 8          |

*Percentage of all B20 + cells in adult BALB/c bone marrow represented by each subpopulation. R esults 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 R A3-6B2 (anti-
CD45R, B220) antibody was also purified in our own lab and
conjugated to biotin. Streptavidin-Q quantum red conjugate was
purchased from Sigma Chem. Co. (St. Louis, M.O.). All antibod-
ies were titrated for flow cytometric staining.

Cell Staining and Sorting by Flow Cytometry. Antigens on the
surface of cells were stained by standard methods (35). For stain-
ing of cytoplasmic IgM and DNA, we used the method described
by Schmidt et al. (36). In brief, cells were stained for surface anti-
gens in the usual manner, fixed in wax media containing 0.25% paraformaldehyde for 1 h on ice, and then permeabilized by incu-
bation 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 IgM staining, the cells were then incubated with a carefully titrated amount of FITC-conju-
gitated goat anti-mouse IgM serum on ice for 20 min and then
washed twice with PBS containing 0.2% Tween 20. For DNA sta-
ing, 7-AAD was added to the permeabilized cells in suspens-
ion to a final concentration of 15 μg/ml and the cells were incu-
bated 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 × 10⁶ sorted cells was isolated by ly-
sis in a guanidinium 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 else-
where (38). 2 μl (one-tenth) of each reverse transcription reac-
tion 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 ex-
tension step of 10 min at 72°C. One-half of the final product was
analyzed by electrophoresis on a 1% agarose, 1% NuSieve (FM C)
gel, and blotted under alkaline conditions to a nylon membrane
(Zetabind, Cuno). Blots were hybridized to the appropriate PCR
fragment labeled with [32P] by random priming (Life Technologies
kit and manufacturer's instructions) and analyzed with a Phos-
horImager using ImageQuant software (Molecular Dynamics). Due to multiple sequential re-
tactions and the large numbers of amplification cycles required to
detect signal and coding ends, these assays are not strictly quanti-
tative. Template dilution controls, however, confirmed that the
differences in intensity of the hybridization signals represent dif-
fences in the initial frequency of the corresponding broken DNA
ends (data not shown).

Primer. The primers used are listed below.

**Primer used in LMPCR**

| Primer (BW) | Primer (BW-H) |
|-------------|---------------|
| GCGTGACCCGCGGAAGATCATACTGAA | GCGTGACCCGCGGAAGATCATACTGAA |
| GACATCCAAGCAGAGAGAGAGAGAGAC | GACATCCAAGCAGAGAGAGAGAGAGAC |

**Locus specific primers:**

| Primer (BW) | Primer (BW-H) |
|-------------|---------------|
| GCGTGACCCGCGGAAGATCATACTGAA | GCGTGACCCGCGGAAGATCATACTGAA |
| GACATCCAAGCAGAGAGAGAGAGAGAC | GACATCCAAGCAGAGAGAGAGAGAGAC |

**Primer used in LMPCR**

| Primer (BW) | Primer (BW-H) |
|-------------|---------------|
| GCGTGACCCGCGGAAGATCATACTGAA | GCGTGACCCGCGGAAGATCATACTGAA |
| GACATCCAAGCAGAGAGAGAGAGAGAC | GACATCCAAGCAGAGAGAGAGAGAGAC |

Results

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

611 Constantinescu and Schlissel
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 IgM, pre-B cells, which express IgM in their cytoplasm (cIgM) but not on their surface, immature B cells, which express IgM 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 cIgM 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, cIgM, 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, cIgM, CD2, sIgM, and sIgD) and analyzing them by 3-color flow cytometry (Fig. 1 A and Table 1).

Figure 1. Flow cytometric analysis and sorting of B cell developmental stages from Balb/c bone marrow. (A) Triple staining for B220, cIgM, 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 is indicated in each panel. The data are un gated.
mature and mature B cells, appeared to be almost entirely quiescent, with very few cells in the S or G2/M phases of the cell cycle (Fig. 2A).

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

A assay for Broken-ended DNA Intermediates in V(D)J recombination. As shown in Fig. 3A, 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 (Schlisel, 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<sup>+</sup> cIg<sup>m1</sup> CD2<sup>+</sup> ), early pre-B cells (clg9<sup>m2</sup> CD2<sup>-</sup> cIgM<sup>-</sup> ), late pre-B cells (clg9<sup>m2</sup> CD2<sup>-</sup> cIgM<sup>-</sup> ), immature B cells (B220<sup>+</sup> splGm<sup>-</sup> splGd<sup>-/low</sup> ), and mature B cells (B220<sup>+</sup> splGm<sup>-</sup> splGd<sup>+</sup> ). Analysis of the sorted fractions showed them to be 85–95% pure (Fig. 1B).

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. 3A). Control amplification of a non-rearranging locus (CD14), showing equivalent amounts of amplifiable DNA in all samples.
might be sufficient to account for the phenomenon of heavy chain-specific modulation of V(D)J recombinase activity alone actively rearranged at later stages. This result argues that loss of the signal joint, even though light chain genes are silenced at the allelic locus, even though light chain genes are specifically expressed in pre-B cells (Fig. 4 A). Figure 4. (A) CBE at the Igk locus in B cell development. Purified DNA from sorted subpopulations of B cells from wild-type Balb/c mice was subjected to LM-PCR to detect CBE associated with J\(k_1\), J\(k_2\), and J\(k_3\), and most V\(k\) 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\(k\) to a downstream J\(k\) segment on the same chromosome (secondary rearrangement dark hatched). (Right) direct rearrangement of a V\(k\) to a J\(k\) segment at the locus allelic to the original rearrangement.

Segments (40). At the \(k\) locus we measured SBE upstream of the most frequently used J\(k\) segments J\(k_1\) and J\(k_2\), as indicative of V\(k\)-to-J\(k\) 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\(k_1\), J\(k_2\), and J\(k_3\) segments, as well as at V\(k\) 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 post-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 locusspecific 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\(k_1\) and J\(k_2\) show a different pattern (Fig. 3 B). We consistently detected J\(k\) SBE in pro-B cells (Fig. 3 B, lane 1, and data not shown) representing some level of active rearrangement at the Igk locus. We interpret this signal to represent a low "constitutive" level of V\(k\)-to-J\(k\) 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\(k\)-to-J\(k\) 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\(k\) 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\(^{+}\)IgM\(^{low}\) late pre-B cells are quiescent and might harbor Igk SBE for a longer time before forming a signal joint, we also assayed CBE at several J\(k\) 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 each locus. These data also show that IgH rearrangement is suppressed at the same time that pre-B cells are actively rearranging Igk, arguing against the stochastic and the cellular selection models of allelic exclusion (19–21).

Rearrangement and Proliferation. The observation that Igk 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 Dfl, Jk, and J2 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 Jk 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 Jk SBE, especially at Jk2, 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 slgM*1gDhi phenotype and were isolated by that criterion, whereas B lineage cells from Eμ-myc spleens are largely pre-B and immature slgM*1gDlo-B cells and were isolated on the basis of slgM expression alone. A previous report (45) demonstrated that slgM* cells from N-myc transgenic mice fail to inactivate RAG expression, a factor which might also contribute to a relatively higher level of Jk2 SBEs.

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

Replacement rearrangements in slgM+ 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 Jk, Jk2, and Jk5 gene segments, and at Vk, gene segments recognized by a set of degenerate primers (46). As shown in Fig. 4 A, Jk coding ends are abundant in late pre-B cells, but become undetectable in either immature (slgM*Dlod) or mature (slgM*Dhi) B cells (top panel, lanes 3-5), even though ~40% of Igk alleles remain in germline configuration (9). However, rearrangements to the downstream Jk2 and Jk5 gene segments do occur in immature, but not mature, B cells, as shown by our ability to detect Jk2, Jk5, and Vk, 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 Igk 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 Igk loci is more complex than simple expression of a functional gene product in the slgM complex. Also, the absence of CBE at Jk2 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 slgM*CD2hi 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 Igk locus 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 Igk controls B cell development. Allelic exclusion has been previously shown to depend on the membrane-embedded form of Igk 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 Igk expression can occur in early development (22, 48, 49). Interestingly, the few mature B cells that accumulate in λ5−/−
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 configuration (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 configuration at Ig loci, making it inaccessible at the heavy chain locus and more accessible at the light chain locus. The increase in SBE and CBE levels at multiple gene segments in the Igk 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 Igk 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 G0 and/or G1 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 G0, increases expression of the RAG genes, and induces rearrangement at the Igk 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 G1 phase and the consequent lower levels of RAG-2 protein.

Secondary R 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 Igk locus on which they occur. At the locus containing the initial rearrangement, an upstream Vκ segment can be joined to a downstream Jκ segment, deleting the initial rearrangement (replacement mechanism, Fig. 4 B). It should be noted that the Jκ segment cannot participate in this kind of secondary rearrangement, because its RSS is obligatorily deleted by the initial rearrangement. If the Igk locus allelic to the initial rearrangement is in germline configuration, any Vκ can be joined to any Jκ, leaving the original rearrangement intact (direct mechanism, Fig. 4 B). This mechanism predicts prominent involvement of Jκ in secondary rearrangements, matching its over-representation in completed rearrangements at all stages of development, the abundance of Jκ CBE in pre-B cells, and the availability of Jκ segments in germline configuration (9, 52, 53; Fig. 4 A; and data not shown). The observed exclusion of the Jκ 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 Vκ and Jκ loci, bringing upstream Vκ segments into proximity to the actively transcribed J-Cκ locus. These Vκ segments might then be favored in secondary rearrangements because of their proximity to Jκ 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 T cell development may be related, 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 TCR α 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 VH gene families between immature and mature B cells (57, 58). Moreover, recent evidence of the involvement of BCR signaling in V(D)J recombination inactivation in B cells is provided by sIgM^+ B cell lines derived from tumors induced by the Eμ-N-myctransgene. 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 Igk 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 recombination 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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