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Variegated Transcriptional Activation of the Immunoglobulin κ Locus in Pre-B Cells Contributes to the Allelic Exclusion of Light-Chain Expression

Hong-Erh Liang, Lih-Yun Hsu, Dragana Cado, and Mark S. Schlissel*
Division of Immunology
Department of Molecular and Cell Biology
University of California
Berkeley, California 94720

Summary

Regulated gene rearrangement is thought to underlie allelic exclusion, the observation that an individual B cell expresses only a single immunoglobulin molecule. Previous data has implicated transcriptional activation of rearranging loci in the regulation of their accessibility to the V(D)J recombinase. Using homologous recombination in ES cells, we have generated "knockin" mice which express a GFP cDNA from an unrearranged immunoglobulin κ light-chain allele. Surprisingly, we find that only a small fraction of κ alleles are highly transcribed in a population of pre-B cells, that such transcription is monoallelic, and that these highly transcribed alleles account for the vast majority of κ light-chain gene rearrangement. These data lead us to suggest that probabilistic enhancer activation and allelic competition are part of the mechanism of κ locus allelic exclusion and may be a general mechanism contributing to cellular differentiation during development.

Introduction

Developing lymphocytes use a series of site-specific DNA recombination reactions known as V(D)J recombination to assemble their antigen receptor genes from component gene segments (Jung and Alt, 2004). All rearranging gene segments are flanked by recombination signal sequences (RSSs). RSSs are recognized by the lymphocyte-specific RAG1 and RAG2 proteins that generate dsDNA breaks at the RSS-coding-segment border. These broken ends are then joined in a set of reactions which require the RAG proteins as well as proteins of the nonhomologous end joining machinery expressed by all cells.

Gene rearrangement is ordered within developing B cells such that the Ig heavy-chain locus almost invariably rearranges before the Ig light-chain loci. Successful assembly of an in-frame heavy-chain gene triggers a signaling pathway, which dramatically increases the frequency of V-to-Jκ light-chain gene rearrangement. Successful light-chain gene rearrangement leads to the ability to synthesize a complete IgM molecule, which ultimately results in inactivation of the recombinase.

Despite the autosomal nature of Ig loci, the vast majority of individual B cells express only a single Ig molecule, a phenomenon known as allelic exclusion. Previous work has shown that allelic exclusion is most often mediated at the level of regulated gene rearrangement—an individual B cell makes only one productive (in-frame) gene rearrangement at each locus, the second allele being excluded from expression. One of the outstanding questions in immunology is how productive V(D)J recombination is limited to only one allele of a locus.

Several types of evidence support the hypothesis that V(D)J recombinase activity is regulated by the accessibility of rearranging loci within chromatin structure (Schlissel, 2003). First, rearranging gene segments are transcribed prior to or coincident with their activation for rearrangement ("germline" transcripts) and treatments that enhance transcription of a locus increase the frequency of its rearrangement. Low levels of germline κ transcripts are detected in pro-B cells and much higher levels in pre-B cells correlating with increased V-to-Jκ rearrangement frequency. Second, mutations that disrupt the activity of the enhancers or promoters associated with germline transcription greatly diminish the corresponding rearrangement events. Finally, recombinant RAG proteins can recognize and cleave RSSs within any loci in purified genomic DNA templates but only recognize specific loci in purified chromatin substrates in vitro (Stanhope-Baker et al., 1996). These accessible loci correspond to those which would have undergone V(D)J recombination in vivo dependent upon the source of chromatin used.

While the accessibility hypothesis has garnered strong experimental support, it remains unclear how regulated chromatin accessibility might account for certain aspects of the allelic exclusion of Ig gene rearrangement. For example, why don't both Igκ alleles in pre-B cells occasionally undergo near-simultaneous V(D)J recombination resulting in B cells which express two functional light-chain genes? Two mechanisms have been proposed to account for this type of regulation. First, it was hypothesized that V(D)J recombination itself was inherently inefficient so that the likelihood of both alleles in a pre-B cell undergoing near-simultaneous rearrangement was remote, thus accounting for allelic exclusion (Perry et al., 1980). More recently, data has been obtained which suggests that DNA methylation may play a key role. 5-Methyl-cytosine found at CpG dinucleotides often correlates with gene inactivity and a repressive chromatin structure. The Igκ locus is highly methylated in nonlymphoid cells and undergoes demethylation during lymphoid development such that in mature B cells, one Igκ allele is methylated and the other unmethylated (Mostoslavsky et al., 1998). Recent studies have shown that in developing pre-B cells, the unmethylated allele is the preferred substrate for V(D)J recombination (Goldmit et al., 2002).

In the current report we propose and test a third hypothesis regarding the mechanism of Igκ locus allelic exclusion based on the ability of transcriptional enhancers to regulate the probability of transcriptional activation rather than the rate of transcription (Walters et al., 1995). We hypothesize that in a population of pre-B cells, the likelihood that an individual allele of the Igκ locus becomes activated is low, making the likelihood

*Correspondence: mss@uclink4.berkeley.edu

that a single pre-B cell would contain two active and recombinase-accessible alleles extremely low and thus enforcing allelic exclusion. The rare, active, and accessible κ alleles would be highly efficient substrates for the recombinase thus assuring that one allele in the cell rearranges long before the other.

To test this hypothesis, we used gene targeting in murine ES cells to generate mice that express a GFP cDNA from within the germline κ transcript. Thus, in a population of developing bone marrow B cells, we could monitor on a single-cell basis germline κ transcription and further probe the relationship between chromatin accessibility and V(D)J recombination.

Results

Generation of κ°-GFP Reporter Mice

We used a conventional cre-loxP gene-targeting approach to insert a promoterless GFP reporter gene followed by an intron and polyadenylation signal from the SV40 T antigen into the coding region of the Jκ1 gene segment within the germline Igκ locus in mouse embryonic stem (ES) cells (Figure 1A and Supplemental Figure S1 available at http://www.cell.com/cgi/content/full/118/1/19/DC1). Our strategy ensures that expression of the inserted GFP cDNA is under the control of the endogenous Igκ locus promoters and enhancers and minimizes the impact of foreign sequences on the regulation of the Igκ locus. Mice harboring only the GFP reporter insertion (designated as G allele) were interbred to produce heterozygous (+/G) mice and homozygous (G/G) animals and will be referred to here as κ^0-GFP (for germline κ locus with a knocked-in GFP reporter) mice. As we did not disrupt any of the recombination signal sequences (RSS) flanking the Jκ segments (including that on the Jκ1 gene segment itself), the targeted (G) allele should remain competent to undergo normal Vκ-to-Jκ rearrangement and to produce a functional Igκ light-chain gene when rearrangement utilizes downstream Jκ segments (Jκ2, Jκ4, or Jκ5).

Stochastic and Infrequent Activation of the κ^0-GFP Reporter Allele in Pre-B Cells

To determine the pattern of germline κ locus activation on a single-cell basis, bone marrow cells isolated from heterozygous (+/G) mice were first stained for the pan-B cell marker B220 and then analyzed by flow cytometry (Figure 1B). Only B lineage cells expressed GFP as shown by the strict coincidence of GFP fluorescence and B220 staining. We also found that although readily detectable, κ^0-GFP reporter activity was expressed by only ~10% of total $B220^+$ cells (Figure 1B). To further dissect the dynamic pattern of germline κ transcription during B cell development, we assessed κ^0-GFP reporter activity in five successive developmental stages defined by multiparameter flow cytometry (Figure 1C). We found that few if any bone marrow pro-B cells ($B220^+$ $CD43^+$ IgM^-; Hardy et al., 1991) from heterozygous (+/G) κ^0-GFP mice expressed GFP activity while approximately 5% of small pre-B cells ($B220^+$ $CD43^-$ IgM^-) expressed the reporter (Figure 1D). We confirmed the identity of these GFP^+IgM^- B cells based on their expression of another pre-B cell marker, CD25 (data not shown). Thus, the κ^0-GFP reporter activity shows the anticipated temporal pattern of activation, but surprisingly it is activated in only a small fraction of otherwise apparently homogeneous pre-B cells.

To assess κ^0-GFP reporter activity beyond the pre-B cell stage, bone marrow cells from +/G mice were stained for the presence of IgM and IgD by flow cytometry. In contrast to pre-B cells, the GFP^+ population increased to ~7% of immature B cells (IgM^+IgD^-; Figure 1D). In the spleen, approximately 25% of the transitional B cells ($IgM^{hi}IgD^+$) were GFP^+ while about half of the mature B cells ($IgM^{lo}IgD^+$) actively expressed their κ^0-GFP reporter gene (Figure 1D). A similar distribution of GFP^+ cells (~50%, data not shown) was also seen in mature B cells ($IgM^+IgD^+B220^{hi}$) recirculating through the bone marrow. In summary, these data indicate that the pattern of κ^0-GFP reporter activity is dynamic throughout B cell development with germline κ transcription present in a low fraction of cells at the pre-B stage, which increases as cells mature.

Given the dependence of κ^0-GFP reporter activity on germline κ promoters, it was possible that the low frequency of GFP^+ pre-B cells might be due to ongoing Vκ-to-Jκ rearrangement of the targeted (G) allele which will inevitably disrupt its germline configuration, resulting in loss of the GFP signal. To address this concern, we bred our κ^0-GFP reporter allele onto the RAG1°xμ genetic background (Spanopoulou et al., 1994). In these RAG1°xμ mice, inactivation of the RAG1 gene precludes any V(D)J rearrangement while early B cell progenitors in the bone marrow can advance to the pre-B stage of development because of transgenic Ig heavy chain expression. Bone marrow cells from RAG1°xμ mice carrying one copy of the κ^0-GFP allele (RAG1°xμ, +/G) were analyzed by flow cytometry. As expected, the κ^0-GFP reporter was activated only in pre-B cells (Figure 1E, left panel). Although the integrity of the targeted allele was preserved in all cells, the frequency of GFP expression in RAG1°xμ pre-B cells was remarkably similar to that detected in the wild-type RAG1 background (+/G, pre-B gate) (Figures 1C and 1E, left). Interestingly, when we examined bone marrow pre-B cells from RAG1°xμ animals which were homozygous for the κ^0-GFP allele (RAG1°xμ, G/G) we discovered a reporter gene dosage effect. The frequency of GFP^+ pre-B cells nearly doubled (~1.8 fold, from 3.65% to 6.7%) in those cells carrying two copies of the reporter gene compared to their heterozygous littermates (Figure 1E, right panel). This gene dosage effect rules out the possibility that only a small fraction of pre-B cells are competent to activate the germline κ locus, but that those competent cells activate both loci. Furthermore, although the frequency of GFP^+ cells doubles in the G/G mice, the intensity of GFP fluorescence does not change, consistent with κ locus expression being monoallelic. Taken together, these results lead us to conclude that the activation of germline κ transcription during the pre-B stage of development is an infrequent and stochastic process in which each allele is independently regulated and that the probability of κ locus activation varies during B cell development.

The κ^0-GFP Reporter Allele Undergoes V-to-Jκ1 Rearrangement at a Normal Frequency

It was possible that although no known regulatory sequences were disrupted, the insertion of a GFP cDNA

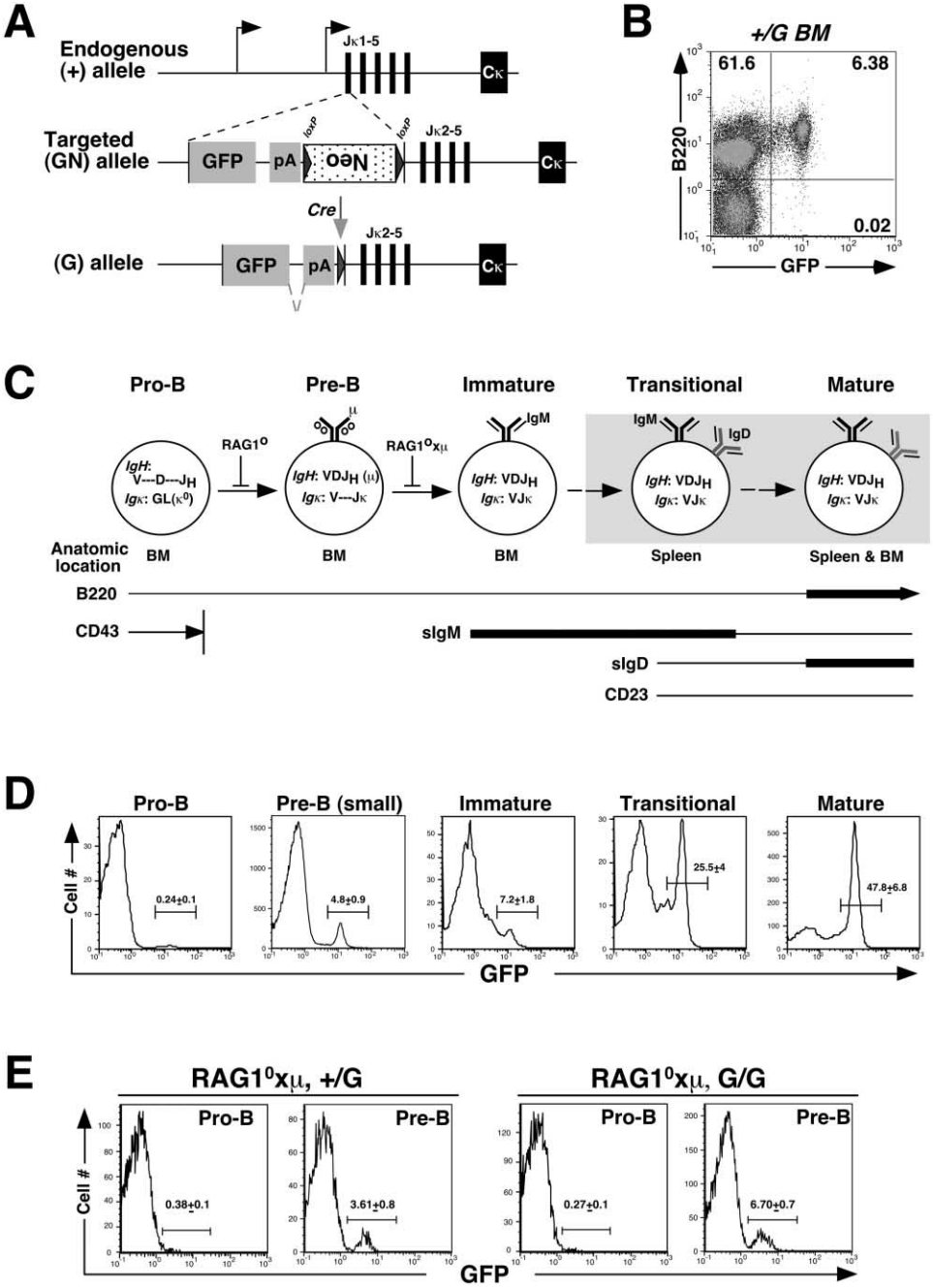

Figure 1. Expression of a GFP cDNA Targeted to the Germline κ Locus in Developing B Cells

(A) Diagrams of the unrearranged mouse Igκ locus (endogenous allele) showing the location of joining segments (Jκ1-5, bars), the κ constant region (Cκ, rectangle), and two germline transcript promoters (arrows); the targeted allele (GN) showing insertion of a GFP cDNA, small intron, polyA signal (pA) and floxed-neo^r cassette (loxP sites, filled triangles) within the Jκ1 gene segment; the targeted κ locus (G) following cre-mediated deletion of the neo cassette.

(B) Lineage specificity of κ^0-GFP reporter activity. FACS analysis of bone marrow cells from κ^0-GFP (+/G) mice after staining with PE-anti-B220; numbers indicate percentage of total live lymphocyte-gated cells.

(C) Diagram of murine B cell development. The stages of B lymphopoiesis are shown together with their anatomic localizations, cell surface markers, and Ig gene rearrangement status. Relative expression levels are indicated by line thickness.

(D) Activation of germline κ transcription during B cell development. BM and spleen cells from +/G mice were stained with antibodies against surface markers and analyzed by FACS to distinguish pro-B (IgM$^-$B220$^+$CD43$^+$), small resting pre-B (FSC^{lo}IgM$^-$B220$^+$CD43$^-$), immature (IgM$^+$IgD$^-$), transitional (IgM^{hi}IgD$^+$ or IgD$^+$CD23$^+$) and mature B (IgM^{lo}IgD^{hi} in spleen or B220^{hi}IgM$^+$ in BM) cells. Histograms displaying GFP fluorescence are shown for each of the indicated populations along with percentages of GFP$^+$ cells in each population (mean ± SD, n = 10 different mice, representative results from three independent assays).

(E) GFP expression in RAG1°xμ B cells. BM cells from RAG1°xμ, +/G (n=8) or RAG1°xμ, G/G mice (n=6) were stained for the presence of B220 and CD43. GFP histograms depict the frequency of GFP$^+$ cells within each gated population (mean ± SD).

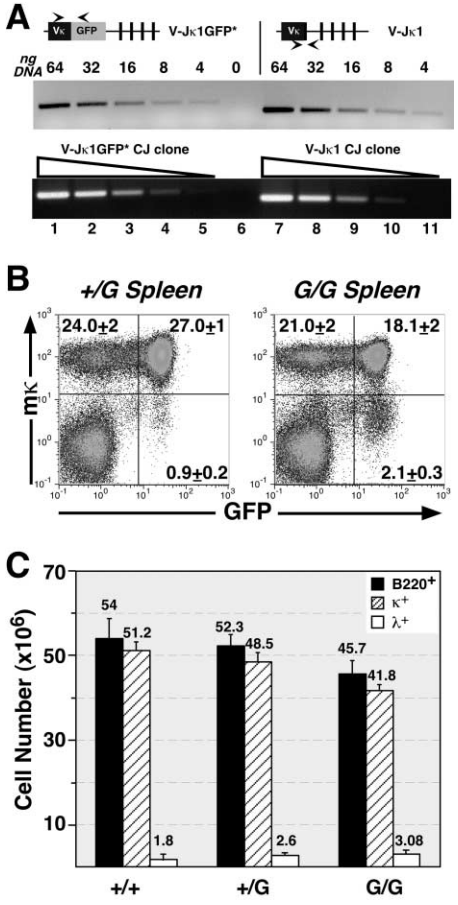

Figure 2. Comparable Frequency of V-to-Jκ1 Rearrangements on Wild-Type and κ⁰-GFP Reporter Alleles

(A) Allele-specific PCR analysis of V-to-Jκ1 coding joints was performed on the indicated amounts of genomic DNA purified from FACS-sorted total pre-B cells from +/G mice utilizing primers derived from either the GFP cDNA or the interval sequences between Jκ1 and Jκ2, individually paired with an upstream degenerate Vκ primer (arrowheads). Bottom, a 27-cycle control PCR on 200 ng RAG2-deficient 63-12 cell genomic DNA mixed with linearized pCR-XL plasmid (4-fold serial dilution starting from 1ng) containing a rearranged V-Jκ1GFP* or V-Jκ1 coding joint insert originally amplified by the corresponding primers.

(B) FACS analysis of splenocytes from heterozygous (+/G) or homozygous (G/G) mice stained with PE-antimouse Igκ. Percentages represent the means and standard deviations of live gated cells from at least six mice of each genotype.

(C) Size of spleen B cell subpopulations from wild-type (+/+), +/G, and G/G littermates. Absolute numbers of B220$^+$, κ$^+$ and λ$^+$ cells were calculated based on the relative percentages determined by flow cytometry as shown in (B). Data represent the means and standard deviations (error bars) from two independent analyses.

into Jκ1 might alter the efficiency of activation of the targeted locus. To consider this possibility, we performed allele-specific PCR assays for V-to-Jκ1 rearrangement on genomic DNA from FACS-purified total pre-B cells from +/G mouse bone marrow (Figure 2A). Control experiments showed that the two assays detected gene rearrangements with similar efficiencies. We found that the GFP-targeted allele underwent rearrangement at a frequency, which was indistinguishable from the allelic wild-type locus. We went on to compare the frequency of GFP$^+$ mature B cells in spleen from +/G and G/G mice. We found that in each case, approximately 50% of mature B cells expressed GFP (indicating that the κ°-GFP-allele remained in germline configuration, see below), again showing that the G allele undergoes rearrangement with a frequency similar to the wild-type κ locus (Figure 2B and Yamagami et al., 1999). Interestingly, we did find that G/G mice produce about 10-to-15% fewer total mature B cells and Igκ$^+$ B cells than do +/+ or +/G littermates (Figure 2C). We attribute this to the fact that rearrangements to the Jκ1 gene-segment on the G allele, which are normally found in about 50% of mature B cells (Wood and Coleclough, 1984), are invariably nonfunctional.

All Unrearranged κ°-GFP Reporter Alleles Are Transcribed in Mature B Cells

To determine the frequency of κ locus activation in mature B cells, we paired our G allele with a mutant κ allele containing the readily distinguishable human Cκ constant region exon (hCκ) in place of the murine Cκ exon (Casellas et al., 2001). Compound heterozygous mice (IgCκ$^{h/m}$; κ^0-GFP$^{+/G}$, designated hereafter as h/G mice) carry one copy of the κ^0-GFP reporter gene (Figure 3A, G allele) and one copy of a κ allele with hCκ (Figure 3A, h allele). Flow cytometric analyses revealed that the G allele was used to produce mouse κ protein in only about 20% of mature B cells in h/G mice (10.5% of total splenocytes, 52.5% of which express surface Igκ) as compared to a wild-type murine κ locus which contributes to about 50% of mature B cells in m/h mice (Figure 3A). As noted above, this is likely due to the fact that V-to-Jκ1 rearrangement does not produce a functional κ protein from the G allele but can from the m or h alleles. Predictably, few if any mouse κ$^+$ cells were also GFP$^+$ since these cells must have deleted the κ°-GFP insertion in order to produce a functional murine VJκ rearrangement (Figure 3A and data not shown). Remarkably, only about 60% of the hCκ$^+$ cells expressed GFP. To determine whether the hCκ$^+$ GFP$^-$ cells had in fact deleted the germline G allele, we purified genomic DNA from FACS-sorted GFP$^+$ and GFP$^-$ human or murine κ expressing splenic B cells and used PCR to detect unrearranged h or G alleles (Figure 3B). These analyses confirmed the presumption that mCκ$^+$ cells had deleted the GFP insertion and importantly showed that the hCκ$^+$ GFP$^-$ cells had also deleted the GFP allele. Thus, the probability of κ°-GFP expression was nearly 100% amongst the mature B cells which had not deleted the GFP cDNA during attempted V(D)J recombination.

Monoallelic Activation of Germline κ Transcription in Pre-B Cells

To confirm that germline κ locus transcription is indeed monoallelic at the pre-B cell stage, we used RT-PCR to assay expression of the κ°-GFP allele as compared to the hCκ allele in sorted GFP$^+$ and GFP$^-$ pre-B cells from h/G heterozygous mice. The difference between the Cκ exons in these alleles allowed us to unambiguously identify the allelic origin of germline κ transcripts. Specificity of the hCκ assays was confirmed by the absence of amplified products in RNA samples obtained from cells lacking the h allele (Figure 4B) and assays for a variety

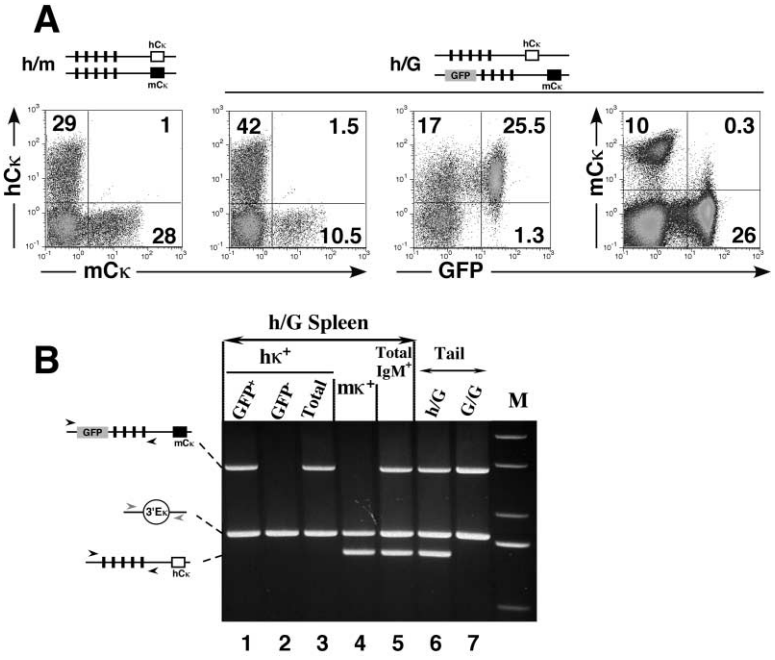

Figure 3. κ⁰-GFP Activation in Mature B Cells

(A) Flow cytometric analysis of splenocytes from IgCκ$^{h/m}$ (h/m) and compound heterozygous IgCκ$^{h/m}$; κ⁰-GFP$^{+/G}$ (h/G) mice stained using combinations of fluorochrome-conjugated antibodies with the indicated specificities. The configuration of the Igκ loci is indicated at the top of each plot. Numbers indicate percentage of total live lymphocyte-gated cells.

(B) Loss of germline G allele in mature GFP$^-$ B cells. Multiplex PCR analyses for germline κ locus sequences (either G or h allele [top or bottom] and κ 3′ enhancer [3′Eκ, as internal control]) were performed on genomic DNA purified from various FACS-sorted B cell populations as indicated. An ethidium-stained agarose gel of PCR products is shown. Note that both hκ$^+$GFP$^+$ and hκ$^+$GFP$^-$ splenocytes represent mature B cells (IgM^{lo}IgD^{hi}) as determined by anti-IgM and anti-IgD costaining with antihuman κ antibody.

of regulated control transcripts confirmed the quality of the cell sorts and RNA preparations (λ5, CD19, and MHC class I). Both κ°-GFP and wild-type hκ⁰/hκ⁰2 transcripts were present in mature CD19$^+$ splenocytes obtained from the h/G mice (Figure 4B, lane 10). Since the vast majority of mature B cells carry at least one rearranged VκJκ gene on either the h or G allele that expresses Igκ LC mRNA, biallelic transcription is inferred from the detection of germline transcripts (as κ⁰-GFP or hκ⁰/hκ⁰2) from the allelic locus.

Among multiple sorted h/G pre-B populations, we found that the distribution of κ⁰-GFP reporter transcripts and hκ⁰/hκ⁰2 transcripts was mutually exclusive (Figure 4B, compare GFP-pA with hκ⁰/hκ⁰2 messages in lanes 1, 2 and lanes 4, 5). GFP$^+$ pre-B cells expressed neither hκ transcript while GFP$^-$ cells expressed both hκ transcripts leading us to conclude that κ locus transcription is monoallelic in pre-B cells. In addition, these results suggest that activation of one κ allele interferes, perhaps through competition for limiting factors, with activation of the second allele (see Discussion).

Using real-time RT-PCR, we went on to compare the levels of κ°-GFP and hκ° transcripts in GFP$^+$ and GFP$^-$ pre-B cell RNA. RNA from mature sorted B cells from h/G mice served to calibrate these assays allowing us to calculate an approximate fraction of germline κ transcript-expressing cells. We found that the frequency of hκ°-expressing cells in the GFP$^-$ pre-B cell fraction was 7%–8% that of the GFP-expressing cells in the GFP$^+$ pre-B cell fraction (Supplemental Figure S2 available on *Cell* website). Thus, the h and G alleles are expressed in similar fractions of pre-B cells.

To further consider the possibility that biallelic germline transcription does occur, but perhaps at very low levels, we performed RT-PCR assays using high numbers of cycles and blot hybridization to detect weak signals (Figure 4C). Under these conditions, RNA purified from sorted CD4$^+$8$^+$ (DP) h/G thymocytes did not express any germline κ transcripts as assayed by extensive RT-PCR confirming the lineage-specificity of κ locus transcription (Figure 4C, lanes 7 and 8). However, we found that GFP$^+$ h/G pre-B cells expressed low levels of hκ⁰/hκ⁰2 transcripts (Figure 4C, lanes 1 to 3) and that GFP$^-$ h/G pre-B cells expressed low levels of GFP transcripts (Figure 4C, lanes 4–6). Real-time quantitative RT-PCR analyses showed that the difference in transcript levels was from 30–200-fold (Supplemental Figure S3 available on *Cell* website). We conclude from these experiments that both alleles of the germline κ locus undergo basal activation in pre-B cells (or perhaps even earlier in B cell development), but that only a small fraction of alleles is subjected to high-level activation as evidenced by GFP expression and that this activation is monoallelic.

Preferential Recombinase Targeting on the Highly Transcribed Allele

Given the well-known correlation between germline transcription and Igκ locus recombination (Schlissel and Baltimore, 1989), we next asked whether the highly transcribed κ°-GFP allele in GFP$^+$ cells was the preferred target for V(D)J recombination. We modified a previously described LM-PCR assay that detects recombination reaction intermediates to identify the actively rearranging alleles in genomic DNA isolated from sorted pre-B cells (Figure 5). In the initial step of V(D)J recombination, RAG proteins recognize a pair of RSSs and introduce dsDNA breaks at the RSS-coding segment borders (Figure 5A). Thus, the amounts of dsDNA breaks at Jκ gene-segments are a measure of the accessibility of the germline κ locus to the V(D)J recombinase. Coding end breaks are initially covalently closed hairpins, but these hairpins are opened by the Artemis endonuclease most often leaving a 3′-overhanging end which can be captured by ligation to an oligonucleotide linker with fully degenerate 3′-overhanging ends (Figure 5A and Schlissel, 1998).

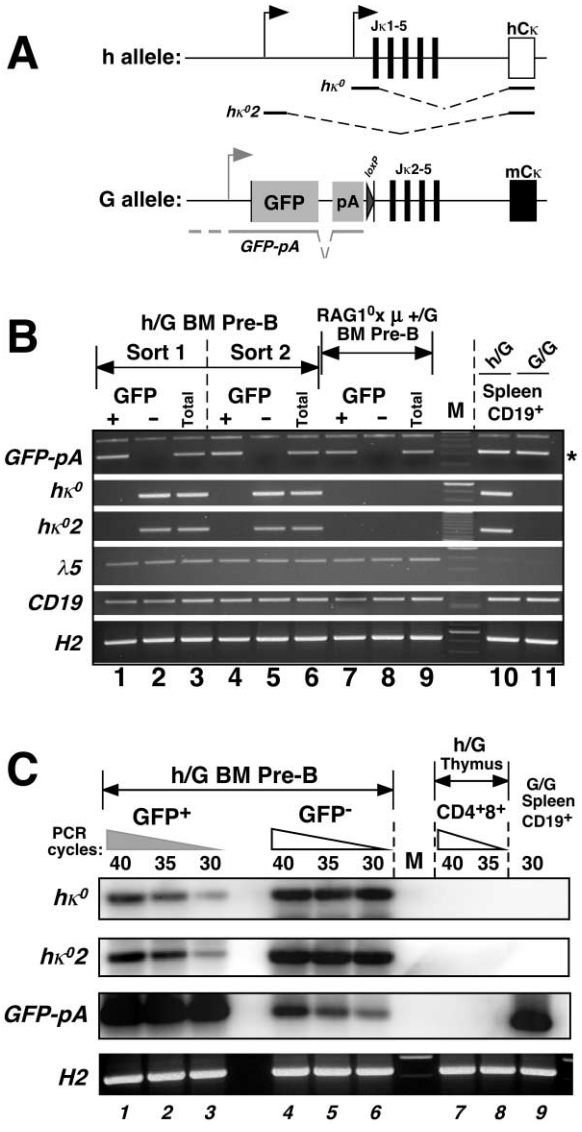

Figure 4. Monoallelic Activation of Germline κ Transcription in Pre-B Cells from h/G Mice

(A) Diagram depicting the structure of germline transcripts derived from either the h or G allele in h/G mice. $h\kappa^0$ and $h\kappa^0 2$ represent wild-type short and long germline transcripts containing hCκ sequence, respectively (Martin and van Ness, 1990).

(B) RT-PCR analysis of GFP-pA (asterisk, RT dependent products), $h\kappa^0$ and $h\kappa^0 2$ germline transcripts and pre-B-specific gene transcripts expressed in sorted GFP^+, GFP^-, or total pre-B cells purified by FACS from mice of the indicated genotypes. Sorted populations were greater than 99% pure upon reanalysis. $CD19^+$ splenocytes from compound heterozygous (h/G) and homozygous (G/G) mice (lanes 10 and 11) serve as controls for κ germline transcripts. The amounts of cDNA synthesized using total RNA purified from equivalent numbers of cells (25,000) were first normalized by real-time RT-PCR analysis of mouse HPRT transcripts. All PCR reactions were within the linear range for amplification (23–25 cycles). Digital image of an ethidium-stained agarose gel is shown with data representative of two independent experiments.

(C) Detection of rare transcripts by RT-PCR with extensive amplification followed by Southern blotting. PCR reactions with indicated cycles of amplification were performed on normalized cDNA samples from various sorted cell populations. Southern blots of PCR products are shown, each probed with an oligo specific for the indicated transcript. FACS-sorted DP thymocytes from h/G mice serve as control for lineage-specific expression of the germline transcripts. The lower panel shows control MHC class I transcript amplification, which is beyond linear range (H2; EtBr stained gel photograph).

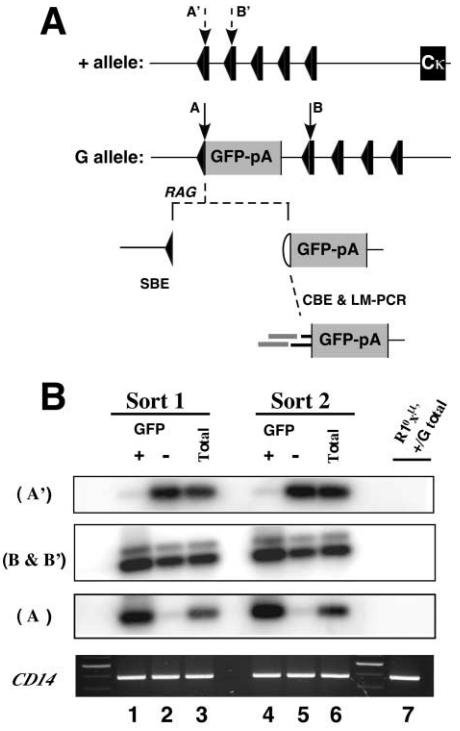

Figure 5. Germline κ Transcription and V(D)J Recombinase Targeting

(A) Diagram indicating several recombinase cleavage sites on the wild-type (+) and κ°-GFP (G) κ alleles. Reaction intermediates include signal and coding broken ends (SBE and CBE, respectively). A 3′-overhanging linker (gray lines) designed to ligate to CBE after hairpin opening was used in an LMPCR assay to detect coding ends. A, A′, B, and B′ denote specific CBE assayed below.

(B) Genomic DNA purified from sorted GFP^+, GFP^- and total pre-B cells from heterozygous +/G bone marrow was linker-ligated and analyzed by LMPCR for CBEs derived from recombinase cleavage sites A, A′, B and B′ as distinguished by various locus-specific primers. Southern blots using locus-specific probes for amplified products are shown. CD14 denotes control amplifications of linker-ligated DNA with primers specific for the CD14 locus as control for DNA amount and quality.

The short half-life of these coding ends makes them an ideal measure of recombinase targeting.

We performed LMPCR assays for various coding ends on genomic DNA samples obtained from GFP^+ or GFP^- pre-B cells prepared by cell sorting using heterozygous (+/G) bone marrow (Figure 5B). Coding ends derived from recombinase cleavage involving the Jκ1 gene-segment may or may not carry the allelic polymorphism defined by the presence of GFP sequences (Figure 5A, cleavage sites A or A′) whereas identical Jκ2 coding ends can come from cleavage at Jκ2 RSS of either allele (Figure 5A, cleavage sites B and B′). We found that the Jκ1/GFP coding ends derived from the G allele existed exclusively in GFP^+ pre-B cells while the wild-type Jκ1

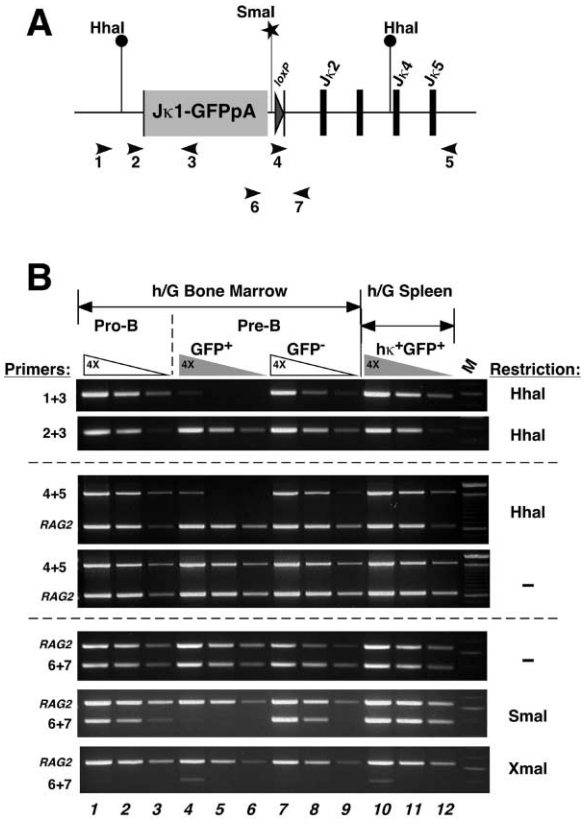

Figure 6. Germline κ Transcription and DNA Methylation

(A) A map of the Jκ region of the G allele showing the location of primers used for MSRE-PCR analysis and relevant restriction enzyme sites. The methylation sensitive SmaI site was brought in by the gene-targeting event while the two HhaI sites are present in the endogenous sequence.

(B) Genomic DNA purified from various sorted B cell populations was first digested with HhaI or SmaI and then amplified by PCR using primer pairs as indicated in the left margin. An amplicon lacking SmaI and HhaI sites from the RAG2 locus was used as a positive control in many of the assays.

coding ends could be found only in GFP$^-$ pre-B cells (Figure 5B, compare lanes 1, 2 and 4, 5). Predictably, all pre-B cell samples contained Jκ2 CBE (B and B′). We conclude from these results that the rare, highly transcribed κ alleles in a population of pre-B cells are the preferred targets for V(D)J recombination and that recombination at any given time is monoallelic.

DNA Demethylation and Activation of Germline κ Transcription in Pre-B Cells

Monoallelic demethylation of the κ locus has been suggested to play an important role in establishing κ chain allelic exclusion (Mostoslavsky et al., 1998). To further probe the relationship between κ locus demethylation and the activation of germline transcription, we carried out methyl-sensitive restriction-enzyme-dependent PCR (MSRE-PCR) analysis using DNA prepared from various sorted B cell populations from compound heterozygous (h/G) mice (Figure 6A). We found that the G allele was methylated in bone marrow pro-B cells, in GFP$^-$ pre-B cells, and in GFP$^+$ mature splenic B cells. In contrast, we found that this allele was largely unmethylated in GFP$^+$ pre-B cells (Figure 6B). This agrees with previous reports showing that demethylation of the κ locus initiates at the pre-B stage and those unrearranged alleles still present in mature Igκ$^+$ B cells remain fully methylated (Mostoslavsky et al., 1998). Thus, in pre-B cells, the rare κ alleles which are the preferred targets of the V(D)J recombinase are both highly expressed and unmethylated. These studies also suggest that distinct requirements exist for activation of germline κ transcription at different developmental stages since nearly all unrearranged κ°-GFP alleles are transcribed in mature B cells yet they remain significantly methylated.

GFP$^+$ Pre-B Cells from h/G Mice Efficiently Generate mCκ$^+$ B Cells in Short-Term Culture

As a final critical test of the hypothesis that the rare actively transcribed κ alleles efficiently generate functional κ rearrangements, we cultured FACS-purified GFP$^+$ and GFP$^-$ pre-B cells from h/G mice and assayed for the appearance of either human or mouse κ on the cell surface (Figure 7). Remarkably, 60% of sorted GFP$^+$ pre-B cells became mCκ$^+$ during the 18 hr culture period while only about 1% became hCκ$^+$. This is in contrast to the corresponding sorted GFP$^-$ pre-B cell culture in which 8% of the cells became hCκ$^+$ and 1% became mCκ$^+$. The lower yield of hCk$^+$ cells in the GFP$^-$ culture is likely due to the fact that the starting material, while depleted of cells expressing the G allele, was not specifically enriched for cells expressing the h allele (see also Supplemental Figure S2 available on *Cell* website). Selection of a rare, preexisting population during culture cannot account for these results since the cell recovery was approximately 90% in each case and proliferation was negligible (data not shown). It is worth noting that about 67% of the GFP$^+$ cells lost GFP expression during this culture period, including about 75% of the cells, which began to express light-chain from the G allele. We interpret this result to imply that the half-life of GFP in this culture system after the deletion of its cDNA by gene rearrangement is quite short. In addition, we estimate that as many as 80% of the initially GFP$^+$ pre-B cells underwent V-to-Jκ rearrangement on the G allele within 18 hr (Figure 7C). Thus, highly expressed κ alleles undergo V(D)J recombination with remarkable efficiency.

Discussion

The steady-state level of germline κ transcripts in developing mouse bone marrow is very low (Perry et al., 1980) but increases as cells progress from the pro-B to the pre-B cell stage (Schlissel and Morrow, 1994). We showed previously that treatments which increase the overall levels of germline κ locus transcription in transformed pro-B cell lines result in correlated increases in V-to-Jκ gene rearrangement (Schlissel and Baltimore, 1989). These previous studies, however, measured germline κ transcripts in bulk cell populations. In the present study, we used homologous recombination in ES cells to modify the Jκ1 gene segment to serve as a GFP-reporter of κ locus transcriptional activation amenable to single cell analysis by flow cytometry. Surprisingly, we found that the frequency of cells expressing

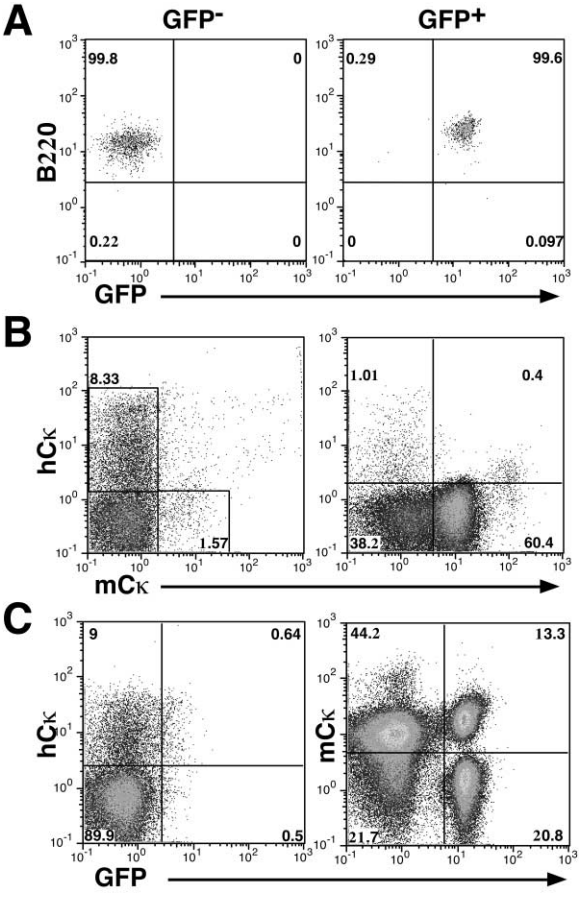

Figure 7. In Vitro Differentiation of Sorted Pre-B Cells

(A) Starting populations used in the short-term cultures. FACS-sorted pre-B cells ($IgM^-CD43^-B220^+$) from h/G mice were further distinguished by their κ^0-GFP reporter activity (GFP^- vs. GFP^+) as verified by the post sort analysis. Identical numbers of pre-B cells from each sorted population were cultured for 18 hr, collected and stained with antibodies against hκ or mκ light chain, and then analyzed by flow cytometry in conjunction with the κ^0-GFP reporter activity. Populations of cells derived from GFP^- pre-B cells (B and C, left) and GFP^+ pre-B cells (B and C, right) are shown. Percentages shown were out of the live $B220^+$ cells (retaining fluorescence from previous sorting procedure, usually exceeds 90% of the recovered cells). The figure is representative of two independent sorting/culture experiments, each consisting of three or more wells for each population.

GFP from germline κ transcripts varies during B cell development from undetectable in pro-B cells to 5% of pre-B cells to approaching 100% of mature B cells in a pattern, which likely makes a significant contribution to allelic exclusion.

The pre-B cell population is a complex mixture of cells in the process of assembling and editing Ig light-chain genes. Previous work has shown that as many as ~20% of pre-B cells express cytoplasmic Igκ (Pelanda et al., 1996). While it is possible that we have underestimated the frequency of κ locus activation due to the existence of a fraction of rearranged κ alleles within the pre-B cell compartment, we found that the frequency of κ locus activation was independent of recombinase activity (Figure 1D and 1E). In addition, our observation that GFP^- pre-B cells generate sIgM+ cells in short term culture with only 8% efficiency argues that previous studies may have overestimated the frequency of pre-B cells expressing cytoplasmic Igκ (Figure 7). Finally, the fact that 60% of GFP^+ pre-B cells generate a productive κ rearrangement in short term culture despite lacking the Jκ1 gene segment leads us to conclude that as many as 80% of pre-B cells may lack any κ rearrangements at all thereby validating our estimate of the frequency of germline κ locus transcription. Further work may be necessary to more accurately measure this frequency.

In interpreting these results, one must consider whether the developmental regulation of the GFP-targeted allele is an accurate indicator of the regulation of wild-type κ alleles. The proximal germline κ transcript initiates 50 nucleotides 5′ of Jκ1 (Martin and van Ness, 1990). The GFP insertion in κ°-GFP was targeted to the coding region of Jκ1, a location not previously found to contain regulatory sequences. It remains possible, however, that the GFP cDNA itself, or its associated SV40 splice and poly-A sequence, may in fact alter the regulation of the targeted allele. We believe that such an effect, if present at all, is unlikely to alter the interpretation of these results for four reasons. First, we found that the GFP-targeted allele undergoes V-to-Jκ1 rearrangement in pre-B cells with an efficiency similar to that of a wild-type κ allele (Figure 2A). Second, approximately 50% of both +/G and G/G splenic B cells are GFP^+ (Figure 2B). This indicates that half of these B cells have rearranged both κ alleles and is in agreement with measurements performed by others using single-cell PCR on wild-type mature B cells (Yamagami et al., 1999). If the G allele was particularly inefficient in its ability to undergo V-to-Jκ rearrangement, we would expect there to be a much higher fraction of GFP^+ G/G B cells. Third, GFP^+ pre-B cells fail to express significant levels of germline transcript from the allelic wild-type κ locus (Figure 4). Finally, the targeted allele, when expressed, efficiently generates productive gene rearrangements (Figure 7). Despite these findings, in heterozygous h/G mice, the GFP allele expresses a functional murine κ light chain in ~20% of mature B cells while 80% of the cells express a human Cκ. In wild-type mice, each of the two κ alleles are functionally rearranged and expressed in roughly equal fractions of splenic B cells (Casellas et al., 2001). We propose that expression of functional light-chain from the G allele in h/G mice is diminished because of the effect of the GFP cDNA knockin on the utility of the Jκ1 gene segment. In wild-type mice, Jκ1 is the most frequently used gene segment in initial gene rearrangements accounting for approximately 50% of all κ rearrangement in splenic B cells (Nishi et al., 1985; Victor et al., 1994; Wood and Coleclough, 1984). Rearrangement to Jκ1 on the κ^0-GFP allele, however, cannot code for functional light chain. Thus, the κ^0-GFP allele might be expected to generate a functional κ light-chain gene less frequently than the wild-type allele since its most effective gene segment has been eliminated from productive use. Furthermore, our data show that mCκ expression underestimates the frequency of rearrangement on the κ°-GFP allele since our molecular studies showed that the GFP cDNA had been deleted in 40% of hCκ-expressing mature B cells from h/G mice. While we believe this data to be compel-

ling, we still cannot rule out the possibility that the variegation we see in κ°-GFP expression is in some part a consequence of gene targeting.

Evidence for Allelic Competition

We detected much higher levels of hκ° transcripts in GFP⁻ as compared to GFP⁺ pre-B cells sorted from h/G mice (Figure 4). Since sorting on GFP expression should do nothing to enrich or deplete hκ°-expressing cells, both fractions were expected to contain similar levels of hκ° transcripts. The fact that we observed so little hκ° transcript in GFP⁺ cells suggests that κ alleles might actually compete with one another for activation resulting in a scenario where the activation of one allele prevents the activation of the other allele in the same cell. Our observation that the frequency of dsDNA breaks involving the wild-type Jκ1 gene segment is much greater in GFP⁻ as compared to GFP⁺ pre-B cells also suggests allelic competition (Figure 5).

κ Locus Activation Occurs at Several Levels

We found GFP to be a relatively insensitive indicator of transcriptional activity in our experiments since high cycle number RT-PCR combined with blot hybridization revealed that GFP⁻ pre-B cells actually express low levels of GFP transcript and that GFP⁺ cells also express low levels of wild-type germline κ transcript (Figures 4 and Supplemental Figure S3 available on *Cell* website). This confirms recent work from another group which used single-cell RT-PCR to show that both germline κ alleles are transcriptionally active in pre-B cells (Singh et al., 2003). These assays, however, used greater than 90 cycles of amplification to reveal κ transcripts. Consistent with these results, we reported previously that both the intronic and 3′ κ enhancers were similarly occupied with factors in both primary pro-B and pre-B cells (but not in thymic T cells) as detected by in vivo footprinting even though much higher levels of germline κ transcript can be detected in pre-B cells (Shaffer et al., 1997). In addition, mice harboring mutations in components of the pre-BCR arrest development at the pro-B cell stage, but nonetheless display low levels of germline κ transcription and rearrangement, again suggesting some level of κ locus accessibility at the pro-B cell stage (Muljo and Schlissel, 2000).

Taken together, these results lead us to propose a model in which transcription factors begin to associate with Igκ locus regulatory elements early during B cell development, marking most if not all κ alleles for activation. This results in chromatin structural changes associated with low levels of germline transcription and very modest recombinase accessibility on both alleles at the pro-B cell stage and beyond. At the pre-B cell stage, cells express heterogeneous levels of various κ locus transcription factors. Pre-B cells which express high levels of one or more of these factors have a greater probability of further activating individual κ alleles to express high levels of germline transcript and to serve as very efficient substrates for V-to-Jκ rearrangement. We have already obtained preliminary data in favor of this aspect of our model (H.-E.L. and M.S.S., unpublished data). This may involve cooperativity in the binding of factors to adjacent sites in gene-regulatory elements as has been observed by others. The allelic bias in accessibility causes a pre-B cell to rearrange one κ allele before the other, allowing sufficient time to test the functional capacity of the first rearranged allele before the second allele undergoes a rearrangement event and in so doing, contributes to the allelic exclusion of Igκ gene expression. As development progresses, this competition may lessen, allowing activation of the allelic locus. Receptor editing may also contribute to biallelic κ locus rearrangement. Casellas et al. (2001) reported that up to 25% of the B cell repertoire had undergone light-chain receptor editing during a several hour delay in maturation due to autoreactivity. Our finding that κ locus activation becomes more frequent as development progresses likely contributes to the fact that about half of all B cells have rearrangements on both κ alleles, one being out-of-frame or deletional and the other in-frame (Yamagami et al., 1999). Our data predict that at any given time, most pre-B cells do not express high levels of transcripts from either κ allele. Light-chain rearrangement in these cells would be very rare, a situation which would also lead to allelic exclusion due to the low probability that rare events would occur coincidently within the same cell, as suggested previously (Perry et al., 1980). Other workers have also observed that low levels of germline transcription are often inadequate to promote recombinase accessibility (Casellas et al., 2002). Upon productive κ rearrangement and formation of a self-tolerant BCR, recombination ceases (Melamed et al., 1998). The inefficiencies introduced by these mechanisms are consistent with earlier observations that only a small fraction of pre-B cells transit to the mature B cell compartment (Osmond, 1990).

A Role for DNA Methylation?

Elegant work from Bergman and colleagues has revealed another factor which may lead to an allelic bias in recombinase accessibility, DNA methylation. The Igκ locus is heavily methylated in nonlymphoid cells but was shown to undergo monoallelic demethylation during B cell development (Mostoslavsky et al., 1998) and the unmethylated allele was shown to be the preferred substrate for V-to-Jκ rearrangement (Goldmit et al., 2002). In the present study, we found that the GFP-modified κ allele was unmethylated in GFP⁺ pre-B cells and highly methylated in GFP⁻ cells, consistent with these earlier observations. The precise role of methylation remains uncertain, however, since the κ°-GFP allele, when still present, is invariably transcribed but largely methylated in splenic B cells (Figures 4 and 6). It is possible that the importance of DNA methylation varies depending upon the relative levels of various key transcription factors at specific developmental stages and that DNA demethylation influences, but does not determine, transcriptional activation.

A Probabilistic View of Gene Expression and Its Implications for Development

Model system studies previously showed that transcriptional enhancers may in some instances function by altering the probability of gene expression in a population of cells rather than by modulating the rate of transcription per allele (Walters et al., 1995) and that occu-

pancy of individual transcription factor binding sites may influence that probability (Boyes and Felsenfeld, 1996). In this study, we found that the probability of κ locus activation varies with development. Because enhanceosome assembly is likely to be highly cooperative, increased levels of only one or two transcription factors may be sufficient to increase the probability of complete assembly and gene activation. These observations provide support for the hypothesis that one function of transcriptional enhancers may be to establish discordant cell fates from a precursor population in a stochastic but quantitatively predictable fashion. An additional example of this type of gene regulation is provided by studies of odorant receptor genes. There are greater than 1000 such genes in the mammalian genome, but a given olfactory neuron expresses only one of them. The decision of which gene to express is stochastic and exhibits potential allelic competition in a fashion similar to what we report here for the Igκ locus (Shykind et al., 2004). Thus, the mechanism revealed in our studies of allelic exclusion might prove to be of more general importance to other cell fate and gene regulation decisions during animal development.

Experimental Procedures

Mouse Strains

Details of targeting vector construction, and the generation of κ^0-GFP knockin ES cells and mice are provided as Supplemental Data (available on *Cell* website).

Flow Cytometry

Single-cell suspensions depleted of RBC by buffered NH_4Cl lysis or lympholyte-M sedimentation (Cedarlane) were first incubated with FcR blocker (2.4G2) then stained with FITC-, PE-, PE-TxR-, Cyc (Tricolor)-, and biotin-conjugated (bi) antibodies and analyzed using an Epics XL-MCL (Beckman Coulter) or MoFlo (DAKO). The sources of individual antibodies and detailed staining procedures are available upon request from the authors. For most FACS plots, ≥100,000 events were collected; dead cells were excluded by propidium iodide (SIGMA) staining or by forward and side scatter gating. Data were analyzed with FlowJo (Tree Star, Inc.) software. For sorting pro- and pre-B cells, total BM cells were first depleted of IgM^+ cells using rat-antimouse IgM microbeads on AutoMACS column (Miltenyi Biotec) according to manufacturer's instructions. To prepare splenocytes expressing human Igκ ($h\kappa^+$), mouse Igκ ($m\kappa^+$) or CD19, cells were first positively selected using PE-conjugated antibody followed by anti-PE-microbeads and AutoMACS separation. Cell sorting was performed on an Epics Elite (Beckman Coulter) or MoFlo (DAKO). The purity of sorted population was verified by post sort analysis (≥2,000 cells analyzed) and found to exceed 99%.

RT-PCR Analysis

Total RNA was purified from 25,000-sorted cells using Trizol (Invitrogen) and reversed transcribed. cDNA samples were normalized by TaqMan real-time PCR (Applied Biosystems) using primers specific for mouse HPRT and semiquantitative PCR analysis for MHC class I (H2) gene, respectively. PCR reactions were performed as follows: 5 min at 95°C, 23–25 cycles of 94°C for 30s, 60°C for 30s, and 72°C for 1 min, and a final extension step of 7 min at 72°C. Primer sequences used in RT-PCR are described in Supplemental Data (available on *Cell* website). PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining or subjected to Southern blotting with specific oligonucleotide probes.

PCR, LMPCR, and MSRE-PCR Assays

Sorted cells were enumerated by hemocytometer, embedded in agarose plugs, or lysed directly for genomic DNA extraction (Schlissel, 1998). Multiplex PCR was performed using Advantage 2 Polymerase (Clontech) under conditions recommended by the manufacturer. LMPCR assays to detect coding end breaks derived from Jκ gene segments were performed essentially as described (Schlissel, 1998). For MSRE-PCR assay, genomic DNA from 50,000-sorted cells was first subjected to digestion with 300 units of HhaI, SmaI, or XmaI for 24 hr followed by phenol/$CHCl_3$ extractions. Restricted genomic DNA was recovered and quantified using the Fluorescent DNA Quantitation Kit and VersaFluor Fluorometer (BioRad) prior to PCR analysis. The sequence of oligonucleotides used to analyze these restricted DNA samples are described in Supplemental Data (available on *Cell* website).

Short-Term Culture and Differentiation of Pre-B Cells

We modified a previously reported primary cell culture system in which the differentiation of B cell precursors is promoted by homotypic interactions (Stoddart et al., 2001). FACS-sorted pre-B cells ($IgM^-CD43^-B220^+$) from the h/G mice were further separated into GFP^- and GFP^+ pre-B cells. Both populations were enumerated, and seeded into round-bottom 96-well plates (Nunc) at a density of 20,000 cells/100 μl/well. Cells were cultured in complete media (RPMI-1640, 10% FCS) supplemented with recombinant mouse BAFF (~50 ng/ml) from the culture supernatant of 293T cell transiently transfected with a mBAFF expression vector. After 18 hr of culture in 5% CO_2 at 37°C, cells were pooled from individual wells, counted, and stained with antibodies against hκ and mκ light chains to monitor the progress of B cell maturation by flow cytometry.

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