k chain monoallelic demethylation and the establishment of allelic exclusion

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Allelic exclusion in k light-chain synthesis is thought to result from a feedback mechanism by which the expression of a functional k light chain on the surface of the B cell leads to an intracellular signal that down-regulates the V(D)J recombinase, thus precluding rearrangement of the other allele. Whereas such a feedback mechanism clearly plays a role in the maintenance of allelic exclusion, here we provide evidence suggesting that the initial establishment of allelic exclusion involves differential availability of the two k alleles for rearrangement. Analysis of k+ B-cell populations and of individual k+ B cells that have rearranged only one allele demonstrates that in these cells, critical sites on the rearranged allele are unmethylated, whereas the nonrearranged allele remains methylated. This pattern is apparently generated by demethylation that is initiated at the small pre-B cell stage, on a single allele, in a process that occurs prior to rearrangement and requires the presence in cis of both the intronic and 3' k enhancers. Taken together with data demonstrating that undermethylation is required for rearrangement, these results indicate that demethylation may actually underly the process of allelic exclusion by directing the initial choice of a single k allele for rearrangement.

[Key Words: Methylation; allelic exclusion; immunoglobulin; B lymphocytes]

Received January 29, 1998; revised version accepted April 13, 1998.

The generation of the vast repertoire of antibody molecules during B-cell differentiation is complex and is based on a program of gene-specific rearrangement events (for review, see Lewis 1994; Rajewsky 1996). Ultimately, this program leads to the expression of a single heavy chain (µ) and a single light chain (either k or l) in each mature B cell. All of these rearrangement events are mediated by conserved recombination signal sequences (RSSs) on the DNA and are carried out by a single set of enzymes (RAG-1 and RAG-2) (Schatz et al. 1989; Oettinger et al. 1990; for review, see Gellert 1996). In spite of the sharing of the enzymes and RSSs, recombination takes place in a programmed, sequential manner that involves the ordered selection of the proper gene loci as well as a mechanism for choosing only one allele at a time. This poses an interesting problem in the regulation of the rearrangement process.

Many studies of the regulation of rearrangement have focused on the mechanisms underlying the ordered selection of the proper gene loci. These studies have identified enhancer-mediated changes in chromatin structure as well as changes in the extent of DNA methylation. At various stages of B-cell development, different trans-acting proteins clearly are involved in rendering the µ, k, and l immunoglobulin loci available to the recombination apparatus. Responding to these trans-acting proteins, enhancers and other cis-regulatory sequences mediate local changes in chromatin structure that underlie increased accessibility to the recombinase (Stanhope-Baker et al. 1996). For example, experiments in transgenic mice have clearly shown that enhancer elements can indeed mediate changes in chromatin structure (Jenuwein et al. 1993; Forrester et al. 1994), and in vivo targeted deletions of these sequences strongly repress rearrangement (Chen et al. 1993; Serwe and Sablitzky 1993; Takeda et al. 1993; Xu et al. 1996; Gorman et al. 1996).

DNA methylation plays an important role in regulating transcription and chromatin structure in a number of genes (for review, see Mostoslavsky and Bergman 1997). Methylation has also been correlated with immunoglobulin gene expression; both the µ and k chains have been shown to be heavily modified in most tissues, but unmethylated in mature B cells (Mather and Perry 1983; Storb and Arp 1983; Kelley et al. 1993). Using a transfection assay, we previously demon-
strated that this pattern is brought about by stage-specific demethylation events that are directed by local cis-acting elements. Demethylation of sequences adjacent to the Jk region, for example, can be induced by the presence in cis of a complex DNA signal containing enhancer and matrix attachment region (MAR) sequence elements (Lichtenstein et al. 1994). Additionally, in vitro experiments have demonstrated that methylation inhibits rearrangement (Engler et al. 1991; Hsieh and Lieber 1992). These experiments suggest an important role for demethylation in the regulation of \( \kappa \) gene rearrangement. However, the in vivo role of demethylation in the process of B-cell development has not yet been clarified.

Until now, the random choice of one allele for rearrangement (at a given locus) has not been addressed in studies of chromatin structure or methylation, but rather in experiments that have demonstrated the existence of a feedback mechanism by which the expression of a functional \( \kappa \) light chain on the surface of the B cell leads to an intracellular signal that down-regulates the V(D)J recombinase, thus precluding rearrangement of the other allele. The widely accepted theory is that for a given immunoglobulin gene locus; \( \mu, \kappa, \) or \( \lambda \) inefficiency in the rearrangement process allows one allele to rearrange prior to the other (Coleclough et al. 1981). Then, allelic exclusion results from the feedback down-regulation mechanism discussed above. Whereas such a feedback mechanism clearly plays a role in the maintenance of allelic exclusion, here we provide evidence suggesting that the initial establishment of allelic exclusion involves differential availability of the two \( \kappa \) alleles for rearrangement. We demonstrate that demethylation of the \( \kappa \) gene occurs initially on only one allele in each B cell. We also define two other aspects of the allele-specific demethylation that are critical to its role in allelic exclusion by demonstrating that, in vivo, demethylation takes place prior to and independently of rearrangement. We also demonstrate that the demethylation is driven, in vivo, by the intronic and 3' enhancer sequences that were implicated previously (Lichtenstein et al. 1994; Kirillov et al. 1996) in driving the process in cell lines. These results, when taken together with direct experiments demonstrating that methylation inhibits rearrangement (Engler et al. 1991; Hsieh and Lieber 1992), suggest that demethylation itself may be the rate-limiting step in the rearrangement process. Thus, our results strengthen the model in which changes in DNA methylation play a major role in controlling the program of sequential rearrangement events. Additionally, our data indicating that the demethylation is monoallelic in individual B cells suggest a novel primary basis for allelic exclusion.

**Results**

Allelic differences in Igk gene methylation status in normal B cells

To evaluate Igk methylation patterns in vivo, \( \kappa \)-expressing B cells were isolated from adult mouse bone marrow or spleen and their DNA was subjected to methylation analysis (see map, Fig. 1). DNA was first digested with HindIII, which allows one to visualize both the germ-line (discrete 2.7-kb band) and rearranged alleles. Further digestion with the methyl-sensitive restriction enzyme HhaI revealed that (in a large fraction of DNA molecules) sites located in the junction (J) region upstream of the intronic enhancer are unmethylated, whereas the germ-line band is mostly methylated (Fig. 1a, lanes 1,2). Next, we examined a 1.0-kb BglII–HindIII restriction fragment at the 3' end of the J cluster that is present in both rearranged and germ-line \( \kappa \) alleles. Analysis with HhaI showed clearly that ~50% of the gene copies are indeed unmethylated (Fig. 1a, lanes 3,4).

To further assess the relationship between \( \kappa \) chain gene rearrangement and methylation, \( \kappa^+ \) B-cell DNA was analyzed using several different methyl-sensitive restriction enzymes (see map). The unmethylated germ-line copies (2.7-kb Hind III band) are strikingly resistant to digestion at five individual CpG sites, indicating that most of these molecules are highly methylated (Fig. 1b). In contrast, the rearranged HindIII-treated \( \kappa \) alleles, extracted from the smear region (>2.7 kb) on the original agarose gel (Fig. 1a, lane 1) and further digested with methyl-sensitive restriction enzymes, proved to be completely unmethylated over the entire Jk and intronic region (Fig. 1c). This is most clearly seen in the complete digestion by both SacI and HhaI of the 1.0-kb BglII–HindIII fragment (Fig. 1c, lanes 1–3). Methyl sensitivity is also observed in the HhaI site near J4 where most of the 0.6-kb Avall band disappears upon HhaI digestion (Fig. 1c, lanes 4,5). These results demonstrate the presence of rearranged unmethylated alleles and germ-line methylated alleles in \( \kappa^+ \) B cells. Since each cell expresses \( \kappa \) light-chain protein on its surface, each must have at least one rearranged \( \kappa \) allele. Thus, these experiments demonstrate clearly that in a majority of \( \kappa^+ \) B cells, a fully methylated germ-line copy coexists with an undermethylated, productively rearranged \( \kappa \) allele.

Single-cell analysis of allelic differences in Igk gene methylation

We have developed an assay to directly determine the methylation status of the two \( \kappa \) alleles present in individual \( \kappa^+ \) B cells. In order to accomplish this we first isolated the Mus spretus \( \kappa \) gene and identified polymorphisms with respect to the Mus musculus allele (C57BL/6). Thus, in F1 progeny from a cross between M. spretus and M. musculus, it is possible to distinguish the two alleles. Spleen cells were harvested from F1 progeny and \( \kappa^+ \) B lymphocytes were sorted as single cells directly into PCR tubes using a fluorescence-activated cell sorter (FACS). Genomic DNA from these single cells was subjected to digestion by the methyl-sensitive restriction enzyme Avall to analyze the methylation status of the Avall site in J4 (Fig. 2a). After Avall digestion, oligonucleotide primers flanking the Avall site were used for nested PCR (primers 1–4; see Fig. 2a). Because these primers will not amplify DNA that has been Avall di-
suggested, only DNA molecules that are methylated at the Aval site will amplify. We will refer to this methyl-sensitive restriction enzyme-dependent PCR assay as MSRE–PCR.

Using MSRE–PCR, we obtained PCR products from 80% of sorted κ⁺ B cells. To determine which alleles have been PCR amplified, we took advantage of a polymorphism between the M. spretus and M. musculus κ genes that leads to the presence of an HhaI site in the M. musculus allele and absence of this site in the M. spretus allele. Whereas HhaI is itself a methyl-sensitive restriction enzyme, here it is used after PCR amplification to distinguish the two alleles. PCR products from 86% (89/103) of the κ⁺ B cells revealed only one allele in this assay (Table 1). Representative analyzed PCR products are shown in Figure 2b. Half the alleles are maternally (M. musculus) derived and half the alleles are paternally (M. spretus) derived. The small number of biallelic PCR products likely derive from instances in which the Aval enzyme has failed to cut an unmethylated allele.

We next performed a variety of control experiments to validate our results and show that this assay is efficient enough to detect both alleles in each cell. When Aval digestion is omitted, for example, only 33% (4/12) of κ⁺ cells yield a monoallelic PCR product (Table 1). These monoallelic products reflect either inefficiency in the PCR or the small fraction of κ⁺ B cells that have used J4 or J5 for rearrangement. An additional control involved

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Figure 1. In vivo methylation pattern of the κ chain gene. A map of the κ chain gene locus shows the J segments, the intronic MAR (M) and enhancer (E) sequences, the relevant restriction sites, fragment sizes, and the probes used in these experiments. (a) DNA from bone marrow (lanes 1, 2) or spleen (lanes 3, 4) κ⁺ cells derived from wild-type mice was digested with HindIII (lanes 1–2) or HindIII–BglII (lanes 3–4) and then tested for methylation by additional restriction with HhaI (+). These samples were then subjected to gel electrophoresis and analyzed by blot hybridization using probe 1. It should be noted that equal amounts of DNA were loaded in both lanes 1 and 2 as indicated by ethidium bromide staining (data not shown). When cut with HindIII the bone marrow DNA shows a germ-line band (2.7 kb) as well as numerous rearranged bands (seen as a smear). Full undermethylation results in 1046-, 827-, and 864-bp fragments, but partial digests can also be seen on the gel (1.6 and 1.9 kb). It should be noted that the appearance of a 1.9-kb band indicates that a small fraction of the germ-line allele is also unmethylated at some sites. Similar results were seen when DNA from κ⁺ spleen cells was analyzed in the same manner (data not shown). Digestion with HindIII–BglII concentrates all κ gene copies into a single band (1.0 kb), which if unmethylated is reduced to 0.8 kb when cleaved with HhaI. This site is ~50% methylated in normal κ⁺ cells (lane 4). In this gel, the 1.0-kb band has a lower intensity than the 1.7-kb band, mainly because it hybridizes to a smaller part of the probe and because gel transfer is not as efficient at this molecular weight (as shown by analysis of HindIII–BglII digests of non-B-cell DNA). The upper (1.7-kb) band seen in this gel represents the 5' HindIII–BglII fragment derived from germ-line copies of the gene. (b) DNAs from κ⁺ bone marrow cells were treated with HindIII and further digested with the methyl-sensitive restriction enzymes, HhaI, Aval, or SacI and analyzed as in a. (c) Because the degree of undermethylation on the rearranged κ alleles could not be determined quantitatively from the data shown in Figure 1a, DNA was extracted (Quiagen Kit) from the smear region (>2.7 kb; a, lane 1), further digested with either BglII (lanes 1–3) or Aval (lanes 4, 5) plus methyl-sensitive restriction enzymes SacI or HhaI (indicated by a + or − at the top), and analyzed using either probe 2 or probe 3, as indicated. The resulting BglII–HindIII fragment (region covered by probe 2) yielded a 0.8- or 0.6-kb band when the respective HhaI and SacI sites are unmethylated. The Aval fragment (region covered by probe 3) was cleaved by HhaI to yield a 0.3-kb band if unmethylated.
M. spretus, or F1 genomic DNAs were PCR amplified (Lanes 1–3) Control cells were analyzed. (c) Both alleles were amplified from this cell and in individual cells with PCR products from both regions were analyzed.

allele) has an unrearranged M. spretus allele. All together, 15 individual cells with PCR products from both regions were analyzed.

**Figure 2.** M SRE-PCR analysis of the \( \kappa \) gene in single cells. (a) A map of the \( j_k \) region showing the location of primers used for M SRE-PCR analysis and relevant restriction enzyme sites. Aval was used to detect DNA methylation, whereas the polymorphic Hhal and BsmAI were employed to distinguish between the two \( \kappa \) alleles. (b) (Lanes 1–3) As a control, genomic DNAs from M. musculus, M. spretus, or F1 mice were PCR amplified with primers P3 and P4, digested with Hhal (used here because of a polymorphism, not because of its methyl-sensitivity), and analyzed on a 4% agarose gel. The M. musculus gene is digested, whereas the polymorphic Hhal site in the region amplified by the primers is not cut, and the F1 DNA yields a product that shows 50% digestion. (Lanes 4–7) DNA from two single \( \kappa^+ \) B cells was digested with Aval and PCR amplified. The secondary PCR primers were P1 and P2. Uncut PCR products are in lanes 4 and 6; Hhal digested PCR products are in lanes 5 and 7. Cells 1 and 2 are monoallelic (M. musculus and M. spretus allele, respectively). Most \( \kappa^+ \) B cells analyzed were monoallelic. (Lanes 8, 9) The same analysis of a single midbrain cell (cell 3). Both alleles were amplified from this cell and in most of the control cells analyzed. (c) (Lanes 1–3) Control M. musculus, M. spretus, or F1 genomic DNAs were PCR amplified by primers P7 and P9, digested with BsmAI, and analyzed on a 4% agarose gel. (Lanes 4–7) Tertiary PCR products derived from cells 1 and 2 (from b) using primers P7 and P9. The tertiary amplification signal is dependent on the germ-line-specific primary PCR (using primers P5 and P6). Uncut PCR products are in lanes 4 and 6, and corresponding BsmAI-digested PCR products are in lanes 5 and 7. Cell 1 (shown previously to have a methylated M. musculus allele) has an unrearranged M. musculus allele; cell 2 (shown previously to have a methylated M. spretus allele) has an unrearranged M. spretus allele. All together, 15 individual cells with PCR products from both regions were analyzed.

The kinetics of \( \kappa \) chain demethylation in vivo

We have analyzed populations of B cells at various stages of B cell development to determine the timing of \( \kappa \) demethylation relative to rearrangement. The \( \kappa \) locus is methylated in RAG-1−/− mouse B220+ bone marrow cells (Fig. 3b, lanes 1,2), which are known to be arrested at the pro-B cell stage of differentiation (fraction C, Fig. 3a). We also examined IL-7 dependent cultured bone-marrow-derived cells (Rolink et al. 1991), which represent one of the earliest stages in B-cell development (fraction B, Fig. 3a), and observed full demethylation of the \( \kappa \) locus.

Table 1. Allelic methylation of the \( \kappa \) gene in single cells

| Allelic product | Cells with monoallelic PCR product | Nonlymphoid cells |
|-----------------|-----------------------------------|-------------------|
|                 | \( \kappa^+ \) B cells | \( \kappa^- \) B cells | (\( \kappa^- \) Val) |
| 89/103 (86%)    | 4/12 (33%)                   | 6/39 (15%)        |

Single-cell PCR using primer sets P1–P4 was carried out as described in Materials and Methods with (+) or without (−) pre-treatment with Aval, and the products were digested with Hhal to determine the contribution of the M. spretus and M. musculus alleles (examples shown in Fig. 2b). It should be noted that the monoallelic products (15%) seen in the nonlymphoid control cells are probably due to inefficient PCR reactions. In the absence of Aval, B-cell DNA yields a high background level (33%) of monoallelic products primarily because of rearrangements to J4 or J5, which remove the primer sequences.
Role of \(\kappa\) demethylation in B-cell development

![Diagram of murine B-cell development](image)

Figure 3. \(\kappa\) chain demethylation in B-cell development. (a) Schematic diagram of murine B-cell development (adapted from Nutt et al. 1997). The different developmental stages of B lymphopoiesis are shown together with their characteristic cell surface markers, which are used for classification according to Rolink et al. (1994) (top) or Hardy et al. (1993) (bottom). As the correlation between the two classification systems is not straightforward in all aspects, the reader is referred to the original literature for details. (b) DNAs from bone marrow B220\(^+\) cells at different stages of B-cell development (see the diagram) were tested for methylation by digestion with HhaI. The different developmental stages of B lymphopoiesis are shown together with their characteristic cell surface markers, or after rearrangement, we studied the effect of demethylation in B-cell development. (data not shown). In contrast, analysis of bone marrow cells derived from Rag-1\(^{-/-}\) mice carrying a prerearranged \(\mu\) transgene (Rag-1\(^{-/-}\)/\(\mu\)^+; Spanopoulou et al. 1994), demonstrated clear-cut \(\kappa\) gene undermethylation (Fig. 3b, lanes 3,4), suggesting that demethylation occurs for the first time in these pre-B cells (fraction D, Fig. 3a). This was, in fact, confirmed by the observation of \(\mu\) light chain expression (Fig. 3b, lanes 5,6). The low level of demethylation observed is expected as fraction D is made up of a mixed population containing both large and small pre-B cells and \(\kappa\) rearrangement is restricted primarily to the smaller cells (ten Boekel et al. 1995). These kinetic studies demonstrate that demethylation of the \(\kappa\) region occurs at about the same developmental stage in which the first \(\kappa\) rearrangement events take place (Hardy et al. 1991; Ehlich et al. 1993; ten Boekel et al. 1995).

\(\kappa\) chain gene allelic demethylation is independent of rearrangement

To determine whether demethylation takes place before or after rearrangement, we studied \(\kappa\) gene methylation patterns in cells derived from mice in which rearrangement of the endogenous \(\kappa\) locus is prevented. In IExT\(^{-/-}\) mice, where both copies of the \(\kappa\) intronic enhancer are replaced by the neomycin resistance (neo) gene, rearrangement of the \(\kappa\) locus is strongly inhibited and, as a result, immunoglobulin-producing cells express the \(\lambda\) light chain exclusively (Takeda et al. 1993). It is likely that this dominant effect is mainly caused by the presence of the foreign neo gene, since this bacterial sequence has been shown to inhibit rearrangement even when placed at other positions in this gene (Takeda et al. 1993). We have isolated B220\(^+\) splenic B cells from these animals and analyzed their methylation pattern at the endogenous \(\kappa\) locus. Despite the fact that both alleles remain in the germ-line configuration in these cells, ~50% of the \(\kappa\) gene copies undergo demethylation at HhaI sites in the J region (Fig. 4a, lanes 3,4). In addition, alleles that are unmethylated at the SacI site are also completely unmethylated at both HhaI sites (Fig. 4b, lane 3). These multiple demethylation events take place although ~50% of the \(\kappa\) molecules remain highly modified, indicating that in vivo demethylation mainly behaves cooperatively and occurs on one allele at a time.

Endogenous \(\kappa\) gene rearrangement is also prevented in mice carrying a prerearranged \(\kappa\) transgene (Betz et al. 1994). This effect presumably occurs because the readily synthesized transgene product constitutively turns off RAG1 and RAG2 expression in the B-cell lineage. We analyzed the methylation patterns of the endogenous alleles present in these mice and found that ~50% of these \(\kappa\) alleles underwent demethylation (Fig. 4a, lanes 5,6). Whereas these results indicate that demethylation can take place prior to rearrangement, we still wanted to rule out the possibility that the rearranged state itself can trigger demethylation. To this end, we examined heterozygous mice carrying a single \(\kappa\) prearranged gene (3–83kI) permanently targeted (via homologous recombination) into the normal endogenous \(\kappa\) locus (Fig. 5a) (Pelanda et al. 1996). It should be noted that in these animals, the rearranged construct is actually under the control of one of the endogenous cis-acting DNA elements that normally regulate \(\kappa\) chain expression. As shown in figure 5b, the allele that underwent homologous reco-
Both κ enhancers are needed for demethylation in vivo.

A recent series of mouse knockout experiments has demonstrated that both the intronic and 3' enhancers play a role in κ rearrangement, since a single deletion of either element (Gorman et al. 1996; Xu et al. 1996) partially inhibits this process. In parallel, we have shown by transfection experiments that both the intronic and 3' enhancer sequences can individually induce κ demethylation in B-cell lines (Lichtenstein et al. 1994; Kirillov et al. 1996). To assess the precise role of these enhancers in the demethylation process in vivo, we analyzed a series of transgenic mice carrying constructs with a prerearranged κ light-chain gene (Sharpe et al. 1991; Betz et al. 1994). In all of these transgenes, the VkOx1 sequence is present in the rearranged configuration adjacent to κ5 which is linked to a 3' region, which is either wild-type (Lx), lacking the 3' enhancer (Δ3'E), or lacking the intronic enhancer (ΔEiκ) (Fig. 6). To study tissue-specific demethylation, B220+ spleen cells were isolated from these three mouse lines and the exogenous sequences then analyzed for their methylation patterns. It should be noted that transgenes in general undergo de novo methylation at the stage of implantation in the same manner as their endogenous counterparts (Frank et al. 1991). Although some B-cell-specific demethylation is
enhancers are present on the same molecule (Fig. 6, lanes 1, 2) or constructs individually lacking either the 3′ enhancer [Δ3′Ex] (lanes 3, 4) or the intronic enhancer [ΔEx] (lanes 5, 6) (Betz et al. 1994) was digested by HindIII–KpnI with (+) or without (−) HhaI. The 1.3-kb HindIII–KpnI fragment and its 0.6-kb unmethylated digestion product are derived exclusively from the exogenous transgene; the 0.8-kb band is a common product of both the endogenous and transgenic copies. The accompanying map shows the various transgenes used in this experiment (Betz et al. 1994).

Figure 6. Enhancers required for κ gene demethylation in vivo. B220+ spleen cell (>95% purity by FACS analysis) DNA from mice carrying a complete Vακ rearranged transgene [Lκ] (lanes 1, 2) or constructs individually lacking either the 3′ enhancer [Δ3′Ex] (lanes 3, 4) or the intronic enhancer [ΔEx] (lanes 5, 6) (Betz et al. 1994) was digested by HindIII–KpnI with (+) or without (−) HhaI. The 1.3-kb HindIII–KpnI fragment and its 0.6-kb unmethylated digestion product are derived exclusively from the exogenous transgene; the 0.8-kb band is a common product of both the endogenous and transgenic copies. The accompanying map shows the various transgenes used in this experiment (Betz et al. 1994).

Discussion

In B cells functional rearrangement at a given immunoglobulin locus occurs on only one of two identical alleles, a phenomenon termed allelic exclusion. Inefficiency in the rearrangement process itself has been suggested as a potential mechanism underlying this selection (Coleclough et al. 1981). Inefficiency would then require a feedback mechanism to shut off further rearrangement when a functional receptor has been made. Otherwise, even an inefficient process would eventually result in rearrangement on the second allele. The well-documented existence of feedback down-regulation of the V(D)J recombinase maintains the viability of the inefficiency model explaining the establishment of allelic exclusion (Rajewsky 1996). However, another possibility is that when the trans-acting factors establish a cellular environment conducive to rearrangement of a given family, only one of the two alleles is available initially for rearrangement. Indeed, the experiments we present here lead us to suggest that the establishment of κ gene allelic exclusion involves a demethylation event that marks one of the two κ alleles for subsequent rearrangement.

κ demethylation is monoallelic

We have performed two distinct experiments, each of which on its own demonstrates that in normal κ+B cells, κ demethylation is monoallelic. One analysis involved examining DNA prepared from populations of purified κ+B cells using methyl-sensitive restriction enzymes and Southern blotting. We also developed a novel assay that allowed us to analyze the methylation status of the two κ alleles in individual κ+B cells. These experiments demonstrate that κ+B cells contain a rearranged, unmethylated allele coexisting with a germ-line allele that is methylated, and, to our knowledge, are the first to analyze the methylation status of the two κ alleles in individual cells in vivo. Previous studies using B lineage cell lines had suggested that germ-line alleles are undermethylated to the same extent as rearranged copies (Mather and Perry 1983; Storb and Arp 1983). However, in light of our in vivo results, it is likely that the generally unmethylated pattern they observed is caused by a cell culture or transformation artifact.

Demethylation precedes rearrangement

By analyzing B cells isolated from bone marrow of both normal and mutant mice, we determined that κ demethylation in vivo takes place at the pre-B cell transition stage (see Fig. 3a,b). It was not clear from these studies, however, whether the κ gene undergoes demethylation before or after the rearrangement event. We have used a genetic approach to distinguish between these two possibilities. We examined mice in which the rearrangement of the κ alleles is inhibited in cis (Takeda et al. 1993), and mice in which κ rearrangement is prevented by a trans-acting mechanism (Betz et al. 1994). In both cases, roughly half of the endogenous (germ-line) alleles undergo demethylation in κ+ lymphocytes, a pattern clearly reminiscent of the allele-specific demethylation observed in normal B cells. These results thus indicate that rearrangement is not necessary for the κ allele to undergo demethylation. We have also been able to prove that rearrangement is not sufficient to bring about demethylation. Indeed, our studies show that a prerearranged κ gene inserted into the normal κ locus (Pelanda et al. 1996) only undergoes partial demethylation in B cells. Taken together, these results strongly suggest that...
during normal B-cell development demethylation immediately precedes rearrangement.

Demethylation is necessary for rearrangement

The strong correlation between undermethylation and rearrangement raises the possibility that DNA methylation itself may inhibit the rearrangement process; several published experiments support this model. Using a simple transfection system, for example, it has been shown that plasmids containing the minimal heptamer/nonamer signal sequences readily undergo rearrangement in B cells. However, when these templates are first methylated in vitro and then allowed to undergo replication in the transfected cells, rearrangement is severely inhibited (Hsieh and Lieber 1992). More convincing evidence that methylation represses rearrangement was obtained by examining mice carrying a transgene construct containing basic rearrangement signals. In a C57BL/6 background, this transgene is apparently methylated in every tissue. However, when bred into the DBA/2 strain, this transgene remains unmethylated throughout the organism and only in this unmethylated state does B-cell-specific rearrangement take place (Engler et al. 1991). It should be noted that in this experiment, the methylation pattern is controlled by modifier genes that have nothing to do with the signals normally used in B cells, and for this reason it provides convincing evidence that local DNA methylation itself plays a critical role in inhibiting gene rearrangement.

By employing transgenes containing a prerearranged \( k \) gene configuration (Betz et al. 1994), it was possible to evaluate the effect of the intronic and 3' \( k \) enhancers on demethylation in vivo independent of the rearrangement process. Our results indicate that although each enhancer alone can induce a small amount of B-cell-specific demethylation, full demethylation of the \( k \) locus is only seen when both are present together in cis. Recent mouse knockout experiments have demonstrated that single deletions of either the intronic or 3' enhancer are sufficient to partially inhibit \( k \) gene rearrangements (Takeda et al. 1993; Gorman et al. 1996; Xu et al. 1996). It thus appears that in vivo the same genetic elements are involved in both demethylation and rearrangement. One attractive explanation for these results is that the enhancers work by inducing demethylation which, in turn, allows rearrangement at the same locus.

It is interesting to consider data suggesting a role for methylation in regulating rearrangement at the heavy-chain gene locus in the mouse. The \( \mu \) enhancer together with its accompanying MAR sequences have been shown to induce demethylation on transfected constructs in B-cell cultures and in vivo (Fernex et al. 1995; Kirillov et al. 1996). Furthermore, when this element is deleted from its normal locus in vivo, heavy-chain rearrangement is inhibited (Chen et al. 1993; Serre and Sablitzky 1993). Because unmethylated constructs lacking either the heavy- or light-chain enhancer elements readily undergo rearrangement following transfection into pre-B cells in culture (Hesse et al. 1987; Lieber et al. 1987), it is clear that these cis-acting sequences are not required for carrying out the mechanical aspects of recombination. It is likely that in vivo the absence of the \( \mu \) enhancer prevents this site from undergoing stage-specific demethylation, and it is for this reason that rearrangement is repressed. In a similar manner, DNA methylation may also play a role in the control of rearrangement at the T-cell-receptor locus (Oyashiki et al. 1992; Capone et al. 1993; Okada et al. 1994; Bories et al. 1996).

Role of demethylation in rearrangement and allelic exclusion

On the basis of our results it is now possible to understand how DNA methylation may play a role in regulating \( k \) gene rearrangement. In pro-B cells, at a stage when the heavy-chain locus undergoes multiple rearrangement events, the \( \kappa \) alleles are still fully methylated, and it is likely that this methylation, in the context of chromatin structure, which prevents their rearrangement despite the presence of RAG-1 and RAG-2 activity in these cells (Grawunder et al. 1995). The expression of a productive \( \mu \) chain appears to bring about a number of dramatic changes in the \( \kappa \) locus, including the initiation of germ-line transcription (Spanopoulou et al. 1994), an increase in chromatin accessibility (Stanhope-Baker et al. 1996), and monoallelic demethylation of the sequences adjacent to the \( J_\kappa \) region (Fig. 3b). All of these events probably occur prior to rearrangement, and it is likely that they are mediated through trans-acting factors that are triggered by the presence of a functional \( \mu \) chain. NF-\( \kappa \)B, for example, is already bound to the \( \kappa \) intronic enhancer at this stage (Shaffer et al. 1997), and has been demonstrated to be required for \( k \) demethylation in a transfection system (Demengeot et al. 1995; Kirillov et al. 1996).

Our results clearly suggest that \( k \) gene demethylation takes place preferentially on only one allele in each cell, and it is probably this demethylation that selects the gene copy that will ultimately undergo rearrangement. The synthesis of a functional \( \kappa \) chain completes the generation of the B-cell receptor and thus down-regulates the expression of RAG-1 and RAG-2. This down-regulation probably serves as a backup mechanism for preventing further rearrangement. In the absence of a productive \( \kappa \) chain it is possible that, given enough time, the second allele in the same cell will eventually undergo demethylation and subsequent rearrangement. This is consistent with our findings (Fig. 1) that all rearranged alleles in B cells are unmethylated.

A number of previous observations also support the idea that differences between the two alleles render one available for rearrangement prior to the other. For example, in IgM+ immature B cells, double-stranded DNA breaks were detected at the \( j_\kappa \) site, but not at the initially preferred \( j_\kappa \) site, strongly suggesting that one allele can undergo repeated rearrangement events, whereas the other \( k \) copy in the same cell is maintained in the germ-line configuration (Constantinescu and Schlissel 1997). These results are consistent...
with the observation that in cells carrying a prerearranged autoreactive κ gene, secondary rearrangements that introduce alternate variable regions take place preferentially on this same allele (Chen et al. 1997). This editing procedure presumably serves as a molecular mechanism for rescuing cells from deletion or anergy. It thus appears that complex regulation of κ gene rearrangement timing and allele selection is most likely mediated through changes in accessibility, and our data clearly indicate that demethylation plays an important role in this process. Yet another line of support comes from single-cell analysis of B cells carrying a prerearranged knock-in (Vκ8R or Vκ4R) on one allele (Luning-Prak and Weigert 1995). Our calculations show that in >90% of these cells, either the targeted or the natural allele, but not both, undergo rearrangement events. This suggests that there is indeed a mechanism for selecting one allele at a time which is independent of whether or not a productive κ gene can be synthesized. If such a primary mechanism did not exist, the rearranged construct should have brought about complete closure of the second allele in every cell.

There are several ways to explain how demethylation might initially occur on a single allele in each developing B cell. One possibility is that the demethylation enzyme machinery is present in limiting amounts in the cell and only suffices to remove methyl moieties from one allele at a time. In this model, demethylation itself would represent the rate-limiting step for allele selection. Alternatively, one κ allele in each B cell may already carry a preset cis-acting molecular marker that serves to direct the demethylation process. Although there is no direct evidence for either mechanism, our data show that a multi-copy prerearranged κ transgene, integrated at an ectopic site in the genome, can undergo full demethylation in B cells, clearly suggesting that the demethylation potential is not rate limiting. In contrast to the κ transgene, a prerearranged construct knocked in to the endogenous κ locus itself became only partially unmethylated, probably because it is subject to cis-acting restrictions normally dictated by this gene region. In light of these data, we favor the hypothesis that the two alleles are differentially marked well before the initiation of κ gene rearrangement.

It thus appears that the process of allele selection at the κ locus may actually be analogous to the mechanism that controls olfactory receptor gene expression. In this case, as well, although the choice of receptor gene activation in any given olfactory neuron is carried out stochastically, chromosome structure limits this process to only a single allele (Chess et al. 1994). Thus, for both the immunoglobulin and olfactory receptor loci, structural allelic exclusion appears to be a primary mechanism for insuring the production of a single gene product from a large array.

**Materials and methods**

**Animals**

Wild-type C57BL/6 × BALB/c F1 mice were obtained from our own colony. iEκT−/− and 3-B3k-1 mice were obtained from S. Takeda (Kyoto University, Japan) and K. Rajewsky (University of Cologne, Germany). Vκ8R mice were obtained from M. Weigert, and Lκ, Δ3′Ex and ΔEκ mice from M. Neuberger (MRC, Cambridge, UK). All mice were analyzed at the age of 8–12 weeks.

**Immunofluorescence staining, cell sorting, purification, and analysis**

Cell suspensions from bone marrow were prepared by flushing six to eight femura with PBS and then washing by centrifugation through PBS. Spleen cells were prepared by disruption in PBS, followed by gentle pipetting, and centrifuging through PBS.

Mouse spleen or bone marrow B cells were enriched by means of staining with either a biotinylated anti-κ or anti-B220 antibody (Pharmingen), followed by incubation with streptavidin-coated magnetic beads (Miltenyi Biotech). Cells were sorted by magnetic cell sorting using the MACS system (Miltenyi et al. 1990). Cells bound to the column in the magnetic field were eluted and recovered for subsequent DNA analysis. Purity (>90%) was assessed by flow cytometry (FACS) analysis after re-staining with streptavidin-conjugated phycoerythrin (Jackson Laboratory).

Wild-type fraction D cells were prepared by a dual laser-dye flow counter (FACStar, Becton-Dickinson). Cells were stained with anti-B220-PE, anti-CD43-FITC, and anti-IgM-phycoerythrin (Pharmingen). MSRE-PCR analysis was performed by the method of Bertram et al. (1995) with the addition of a second PCR round except that 2 µl of the first-round amplification product was used as template. This second round was carried out in 50 µl using primers P1 (5′-GGGGATCTTCTATTGATGCAC-3′) and P2 (5′-GTGGGAGAAATGAGAAAGGAACAG-3′) and P3 (5′-GGGGATCTTCTATTGATGCAC-3′) and P4 (5′-GTGGGAGAAATGAGAAAGGAACAG-3′). The genomic DNA was subjected to digestion overnight at 37°C with 20 units of Aval.

PCR was performed with Taq DNA polymerase (Promega) under standard conditions (50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl2, 10 mM Tris-HCl at pH 8.3) in individual PCR tubes. The genomic DNA was liberated using protease K (0.001 mg/ml at 60°C for 2 hr), that was then inactivated with PMSF (0.002 mg/ml, 60°C, for 1 hr). The genomic DNA was eluted and recovered for subsequent DNA analysis. Purity (>90%) was assessed by flow cytometry (FACS) analysis after re-staining with streptavidin-conjugated phycoerythrin (Jackson Laboratory). Wild-type fraction D cells were prepared by a dual laser-dye flow counter (FACStar, Becton-Dickinson). Cells were stained with anti-B220-PE, anti-CD43-FITC, and anti-IgM-phycoerythrin (Pharmingen). MSRE-PCR analysis was performed by the method of Bertram et al. (1995) with the addition of a second PCR round except that 2 µl of the first-round amplification product was used as template. This second round was carried out in 50 µl using primers P1 (5′-GGGGATCTTCTATTGATGCAC-3′) and P2 (5′-GTGGGAGAAATGAGAAAGGAACAG-3′) and P3 (5′-GGGGATCTTCTATTGATGCAC-3′) and P4 (5′-GTGGGAGAAATGAGAAAGGAACAG-3′). The genomic DNA was subjected to digestion overnight at 37°C with 20 units of Aval.
product was subjected to digestion with HHal and analyzed by agarose gel electrophoresis.

MRE-multiplex PCR on single cells was performed to simultaneously amplify the unrearranged κDNA in addition to the region flanking the Aval site (described above). The following primers were used simultaneously: P1, P2, P5 (5'-AGATCCTAGTGAAGACGGACGGAG-3'), and P6 (5'-TTTACCTCCA-CAAAAACCTTCCC-3'). 50 cycles of amplification were performed using the conditions of the first-round PCR described above. In the second-round PCR, the two distinct regions were amplified in separate tubes in reactions whose conditions were identical (except that each time segment was 45 sec) to the previously described second-round. The following primers (nested within the first-round primers) were used: P7 (5'-CCGG-GATCCCGGGTGAGGACGGAG-3') and P8 (5'-GCTCTAGACCTCCACAAAAACCTTCCC-3'). To visualize products from a larger fraction of cells, a third round (40 cycle) PCR was carried out using primers P7 and P9 (5'-CTGATCTGGAGATGATGTGACGACGGAG-3'). This third-round PCR product was subjected to digestion by the restriction enzyme BsmAI and analyzed by agarose gel electrophoresis.

Acknowledgments
We thank K. Rajewsky, S. Takeda, M. Wiegert, and M. Neuberger for providing the trangenic mice used in this study; S. Jung, T. Wirth, and S. Schaal for their collaboration and for providing antibodies; G. Nieman from the Interdepartmental Equipment Unit at the Hebrew University Medical School for technical assistance with the FACS sorting; and G. Paradis of the MIT flow cytometry core facility. We also acknowledge the assistance of A.C. is a Rita Allen Foundation Scholar and R.M. is the German–Israeli Foundation for Scientific Research and Development (Y.B.), the National Institutes of Health (H.C.), the German–Israeli Foundation for Scientific Research and Development (Y.B.), the German–Israeli Foundation for Scientific Research and Development (Y.B.), the National Institutes of Health (H.C.), the Israel Cancer Research Fund (H.C.), the Israel Research Foundation (H.C.), and the Ira W. DeCamp Foundation created under the will of Elizabeth DeCamp-Mcknry (A.C.). A.C. is a Rita Allen Foundation Scholar and R.M. is the recipient of a fellowship from the Israel Ministry of Science and the Arts.

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κ chain monoallelic demethylation and the establishment of allelic exclusion

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*Genes Dev.* 1998, 12:
Access the most recent version at doi:10.1101/gad.12.12.1801

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