A Targeted Deletion of a Region Upstream from the Jk Cluster Impairs \( \kappa \) Chain Rearrangement In Cis in Mice and in the 103/bcl2 Cell Line

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Summary

We have shown previously that a mutation of the KI-KII site immediately 5' to \( J_{k1} \) on the mouse immunoglobulin light chain \( \kappa \) locus reduces the rearrangement level in cis, although it does not affect transcription. Here we deleted by homologous recombination in mouse embryonic stem cells a 4-kb DNA fragment, located immediately upstream of the KI-KII element, which contains the promoter of the long germline transcript. Analysis of gene-targeted heterozygous mouse splenic B cells showed a strong decrease in rearrangement for the allele bearing the deletion. When both the KI-KII mutation and the 4-kb deletion were present on the same allele, the overall reduction in rearrangement was stronger than with the 4-kb deletion alone underlying the role of these two elements in the regulation of rearrangement. The same deletion was performed by homologous recombination on one allele of the rearrangement-inducible mouse 103/bcl2-hygro\( ^{R} \) pre-B cell line, and resulted in a similar reduction in the induction of rearrangement of the mutated allele. This result validates this cell line as an in vitro model for studying the incidence of gene-targeted modifications of the \( \kappa \) locus on the regulation of rearrangement.

Key words: regulation of rearrangement • mouse immunoglobulin genes • allelic exclusion • germline promoter • positive regulatory element

The cell stage- and locus-specific control of rearrangement remains a critical issue in understanding how T and B cell receptor (TCR and BCR) \( ^{2} \) loci are differentially assembled during lymphoid differentiation (for review see reference 1).

It has been proposed that transcription factors coupled with germline transcription of the different elements before their assembly could be the prerequisite signal permitting access to the recombinase. Supporting this proposition, it has been shown that mutations of transcriptional control elements of TCR and BCR genes significantly impaired their rearrangement efficiency (for review see references 2, 3). Along this line, a region upstream of the TCR-\( \alpha \) locus containing the promoter of the predominant TCR-\( \kappa \) germline transcript was shown to control accessibility of the most 5'Js (4). On the other hand, it has also appeared that transcription factors and germline transcription may not be the sole elements controlling rearrangement, as some experimental settings show a dissociation between the two events (5–8). We have previously shown that the KI-KII motif (9), located immediately upstream of the \( J_{k1} \) segment in the mouse V-Jk intervening sequence, was an enhancer of rearrangement, although it did not seem to play a role on germline transcription initiated immediately upstream of \( J_{k1} \) (10).

To explore whether there are other regulatory elements in the mouse V-Jk intervening sequence, we constructed mice carrying a 4-kb deletion upstream of KI-KII, the deleted fragment encompassing the promoter of the long J-Ck germline transcript (11, 12). It is shown here that this deletion strongly inhibits rearrangement of the \( \kappa \) locus. This result is further confirmed when the same deletion is performed by homologous recombination of the light chain locus in the rearrangement-inducible pre-B cell line 103/bcl2-hygro\( ^{R} \) (13), thus validating the use of this cell line for further gene targeting experiments.

Materials and Methods

Genetic Constructs. The pSPIg8 plasmid, which has a 12.7-kb BamHI-I genomic insert containing the New Zealand black mouse Jk cluster, was provided by B. Van Ness (University of Minnesota,
Minnapolis, MN). The following modifications were introduced in defined subclones, and used thereafter to reconstitute the complete BamHI fragment: the loxP-flanked neo<sup>+</sup> gene, obtained from the pLZ-neo<sup>+</sup> plasmid (provided by H. Gu, NIAID, National Institutes of Health, Bethesda, MD), was blunt-end inserted in the most 5' HindIII site. A XhoI site was introduced 127-bp upstream of j<sub>4</sub>, by site-directed mutagenesis (In Vitro Mutagenesis kit; Bio-Rad) and was used thereafter to insert a XhoI-SalI site-directed mutagenesis (In Vitro Mutagenesis kit; Bio-Rad) and were immediately resuspended in 50 ml RPMI, 10% FCS, 0.05 mM Hepes, pH 7.4 (300 V, 900 mfd). The final constructs thus contained three loxP segments in the same orientation (verified by sequencing) allowing for the Cre-mediated deletion of either the neo<sup>+</sup> gene alone (control mice or clones) or of both the neo<sup>+</sup> gene and the 4-kb fragment (see Fig. 1). For constructions used in the Abelson virus-transformed 103/4 cell line (13), a diagnostic EcoRI site was created instead of the natural StyI site between J<sub>4</sub> and J<sub>2</sub>. Establishment of the 103/bcl2-hygro Cell Line. Since the 103/4-bcl2 cell line was derived by transfection of a bcl2 construct carrying the neo<sup>+</sup> gene that is present in our gene targeting constructs, we established a new 103/bcl2-hygro<sup>+</sup> derivative by transfection of the original 103/4 cell line with the pSFFV bcl2 expression vector (provided by S.J. Korsmeyer, The Rockefeller University Press, New York) in which the neo<sup>+</sup> gene was replaced by a hygro<sup>+</sup> gene (15) driven by the CMV promoter.

Transfection and Homologous Recombination in Embryonic Stem and 103/4 Cell Lines. E14.1 embryonic stem (ES) cells were cultured, transfected, and selected as previously described (10). The analysis of recombinant clones before and after Cre-mediated deletion of the neo<sup>+</sup> gene and/or of the 4-kb DNA fragment was performed by Southern blot analysis similarly for ES and 103-derived cells. The transfection protocol of the 103/4 cell line was as follows: 10<sup>7</sup> cells were electroporated with 30 μg linearized DNA in 800 μl RPMI, 10 mM Hepes, pH 7.4 (300 V, 900 μfd). The cells were immediately resuspended in 50 ml RPMI, 10% FCS, 0.05 mM β-ME, and subcloned in 96-well plates at 20,000 cells/well. Selection with 1.5 mg/ml hygromycin B (Boehringer Mannheim) or G418 (GIBCO BR) was applied 48 h later. The resistant clones were then subcloned at 0.5 cells/well, grown in culture for 3 wk, and analyzed by Southern blot after expansion. Approximately 10% of each of the transfecants were targeted to the κ locus, but only in a fraction of them did the recombination extend over the 10 kb containing the three loxP sites and the EcoRI restriction site. In a typical experiment, 66 clones were obtained from 10<sup>7</sup> cells, of which 6 were targeted to the κ locus and 1 contained the three loxP modifications.

Generation of Chimeric Mice. Chimeric mice were obtained after aggregation of gene-targeted ES cells (see below) with CD-1 morulas. Analysis of ES-derived lymphoid cells took advantage of a polymorphism that frequently occurs between the CD-1 and 129 cells at the Ly5 locus.

Spleen Cell Sorting. The anti-mouse Ly5.2-PE and Ly5.1-PE antibodies were provided by B. Rocha (INSERM, Faculté de Médecine Necker, Paris, France). Anti-mouse CD19-FITC was purchased from PharMingen. Spleen cells from chimeric mice were prepared and labeled as previously described (10), and Ly5.2<sup>+</sup>, CD19<sup>+</sup>, and CD19<sup>-</sup> cells (~50% chimerism in the spleen) were purified using a FACSVantage cell sorter (Becton Dickinson).

Germline Retention Analysis of DNA in Mouse Spleen Cells and in 103/bcl2-hygro Cells. Genomic DNA preparation was performed as described (10). Alkaline Southern blots after digestion of genomic DNA with BamHI and hybridization with a 1.8-kb SplI-PstI random-labeled DNA fragment (probe C) encompassing the mouse j<sub>4</sub> cluster were performed as previously described (10). Germline retention in control mice was carried out by PCR with the primers used in the reverse transcription (RT)-PCR detection of the long germ line transcript from the wild-type allele (see below).

Quantitative PCR Analysis of Rearrangement in 103/bcl2-hygro Cells. Total RNA was extracted with Trizol (GIBCO BR) from 10<sup>6</sup> cells cultured at the permissive (34°C) and the nonpermissive (40°C) temperatures for 6 and 12 h. After reverse transcription with an equimolar mixture of oligo-dT and random primers (Stratagene), cDNA was resuspended in 50 μl of water. For each sample, four different amounts of CDNA (2, 1, 0.5, and 0.25 μl) were amplified in separate reactions for each primer described below to avoid plateau effects. For the long germ line transcript, the 3' primer was 5'-AGCAATTCCTCTCCTAAA-3', the 5' primer was 5'-GCTGTAAGAGGAGTTACGTCCAGC-3' (hereafter referred to as loxP<sup>+</sup>-primer) for the mutant allele containing a loxP site (1 min 94°C; 1 min 66°C; 1.5 min 72°C; 30 cycles) and 5'-CTATGGAAAGAGCAGCGAGTGCC-3' (hereafter referred to as wt-<sup>+</sup>-primer) for the wild-type allele (same PCR parameters, but 27 cycles). Normalization was performed with respect to β-actin (mouse β-actin amplifier set; Clontech). The PCR products were blotted onto nylon membranes and hybridized with the oligonucleotide DAR25 (17) for the long transcript, and with AACTGGCATTTGACCT for β-actin.

Quantitation. Southern blots and PCR hybridizations were exposed to phosphor screens, scanned with a Storm 480 machine (Molecular Dynamics), and analyzed with the public domain NIH Image program (developed at NIH and available at http://rsb.info.nih.gov/nih-image).

Results

Deletion of the 4-kb Fragment on One κ Allele in Mice (B Cells) Results in a Strong Decrease in Rearrangement when Compared with the Wild-type λ Allele. Gene-targeted Ly5.2<sup>+</sup> B and non-B cells were sorted from spleens of chimeric mice obtained by aggregation of mutated ES cells with Ly5.1<sup>+</sup> morulas. In a first series of mice (D5, D6, D7) the sorted cells carried the 4-kb deletion on one κ allele (construct depicted in Fig. 1 e). Analysis by Southern blot (Fig. 2 and Table I) of the percentage of wild-type over mutated allele germline retention in B cells of three different mice (14/86, 15/85, and 23/77) versus non-B cells (47/53, 44/56, and 49/51) showed a strong decrease in rearrangement levels for the mutated allele, indicating that a positive regulatory element of rearrangement had been inactivated upon deletion. Germline retention analysis of B cells from a chimera bearing only the two loxP sites (control mice) indicated that these two elements had no effect on rearrangement (Fig. 2 d).

A Positive Regulatory Element in the Mouse κ V-J Intervening Region
The 4-kb deletion and the KI-KII mutation have cumulative effects. A similar analysis was carried out in a second series of mice (DM1 and DM4), which had the KI-KII mutation on the same allele as the 4-kb deletion (Fig. 1 e, with mutated KI-KII motif). We previously showed that the KI-KII mutation results in a very strong reduction of rearrangement in cis (10). The analysis showed that rearrangement of the mutated allele was further reduced when compared with the wild-type allele when both the deletion and the KI-KII mutation were present in cis (7/93 and 3/97 percentage wild-type over mutated allele germline retention in B cells versus 47/53 in non-B cells) (Fig. 2 and Table I). This stronger reduction in rearrangement in the presence of both mutations was confirmed by PCR analysis (data not shown), indicating that deletion of the 4-kb DNA fragment and mutation of the KI-KII motif have cumulative effects.

Deletion of the 4-kb Fragment Reduces Rearrangement of the Mutated Allele in the 103/bcl2-hygroR Pre-B Cell Line. The 103/bcl2-hygroR cell line, transformed with a temperature-sensitive mutant Abelson virus, rearranges the light chain locus when shifted to the nonpermissive temperature...
It was shown that this rearrangement process follows a kinetics where 20–30% of \( \kappa \) loci are rearranged in 48 h; to a lesser degree, the \( \lambda \) loci of these cells also rearrange during this time course. After 4 d most of the cells have rearranged both \( \kappa \) alleles (13). Upon subcloning after 24 h of induction, we have observed that among 17 clones, 6 had rearranged one \( \kappa \) allele, 2 had rearranged both alleles, and 9 had their \( \kappa \) alleles in germline configuration (data not shown).

Rearrangement of the wild-type over mutated alleles was assessed in the 103/bcl2-hygroR–derived clones carrying the following mutations, introduced by homologous recombination on one \( \kappa \) allele: the 4-kb deletion alone, the KI-KII mutation alone with the two \( \text{loxP} \) sites, the two mutations together, or the control \( \text{loxP} \) sites only (Fig. 3 and Table II). We have chosen clones where the rearrangement status of the \( \kappa \) loci was close to germline at the permissive temperature, since it has been reported previously that a small percentage of these cells can rearrange spontaneously (13).

Rearrangement levels were quantified by PCR at several time points after induction (Table II). In the cell line carrying the 4-kb deletion on one \( \kappa \) allele (clone 7.1.18.3), there was a strong decrease of the rearrangement level of the mutated allele when compared to the wild-type allele (24:76 at 48 h). This indicates that the deletion gives similar results in the cell line and in mice. The control \( \text{loxP} \) clone (7.1.18.9) showed no difference between the two alleles, confirming the neutrality of the two \( \text{loxP} \) inserted in the \( \kappa \) locus on the induction of rearrangement.

The KI-KII mutation alone in clone 19.3.9.10 showed no significant effect on rearrangement of the modified allele 48–96 h after induction. A small difference favoring the KI-KII mutation–bearing allele was seen in this clone 12–24 h after induction (see Table II), but these differences did not stay consistent at later points of the assay. Accordingly, in the cell line carrying both the deletion and the KI-KII mutation, the reduction in rearrangement was identical to the one obtained with the sole 4-kb deletion (clone 19.3.9.14, 24:76 at 48 h). Altogether, these results suggest that in this particular in vitro model the 4-kb deletion mimics the effect obtained with the corresponding mutated mice. On the other hand, the regulatory mechanism involving the KI-KII motif could be nonoperative in these conditions.

Germline Transcription in the 103/bcl2-hygro Pre-B Cell Line Clones. Overall expression levels of long germline transcripts increased upon induction at the nonpermissive temperature at 6 h in the 103/bcl2-hygro pre-B cell line (Fig. 4) and started to decrease at 12 h when rearrangement levels start to increase (see Fig. 3).

The 4-kb deleted fragment contains the promoter of the long germline transcript. Long germline transcripts were expressed at similar levels from the wild-type and \( \text{loxP} \)-bearing alleles in the control clone 19.3.9.10 (Fig. 4). Analysis of transcription initiating upstream from the \( \text{loxP} \) site on the allele carrying the 4-kb deletion in the 19.3.9.14 de-
Table II. Wild-type/Mutated Allele Rearrangement in 103/bcl2-hygroR Clones

| 103/bcl2-hygroR- derived clone | 12 h | 24 h | 48 h | 72 h | 96 h |
|---------------------------------|------|------|------|------|------|
| 7.1.18.3                        | 80/20| 70/30| 74/26| 75/25| 76/24|
| 19.3.9.14                       | 82/18| 75/25| 74/26| 70/30| 73/27|
| 7.1.18.9                        | 53/47| 52/48| 52/48| 53/47| 56/44|
| 19.3.9.10                       | 29/71| 45/55| 53/47| 51/49| 50/50|

Deletion clone showed negligible amounts of RT-PCR product, indicating that the deletion did not force an ectopic initiation of transcription upstream of the 5′ boundary of the deleted fragment. Moreover, the presence of the loxP site near KI-KII had no effect of transcription of the long germline transcript.

Discussion

Here we show that a deletion of 4 kb immediately upstream of the KI-KII sites in the κ V-J intervening sequence results in a dramatic reduction of rearrangement in cis in mouse spleen B lymphocytes heterozygous for the mutation. When added to the 4-kb deletion, mutations of the KI-KII sites, which had been previously characterized as enhancers of rearrangement, further decreased rearrangement, leading to the almost exclusive use of the unmutated allele. These results clearly demonstrate that a second positive regulatory element of rearrangement is located in the 4-kb fragment adjacent to KI-KII and that both elements must act in concert to enhance rearrangement of the locus.

The same mutations were introduced by homologous recombination of one κ allele in the rearrangement-inducible 103/bcl2-hygroR cell line. This pre-B cell line, which has been transformed with a temperature-sensitive Abelson virus mutant, has both heavy chain alleles rearranged non-productively and initiates rearrangement of the light chain loci when put at a nonpermissive temperature. As in normal pre-B cells, rearrangement initiates in the 103 cell line on one κ allele, but in the absence of a feedback inhibition of the recombinase it then proceeds to the other allele and to the λ locus if the cell is maintained at the nonpermissive temperature for 48 h (13). A reduction of rearrangement was obtained for the allele carrying the 4-kb deletion in the 103/bcl2-hygroR cell line, quantitatively similar to the reduction observed in mutant mice. Several results have been obtained that imply germline transcription as a prerequisite event to rearrangement. When tested in the 103/bcl2-hygroR cell line, transcription of the long germline transcript could only be induced from the unmutated allele upon induction of rearrangement. Therefore, it is highly probable that the effect we have observed with the 4-kb mutation is due to the deletion of the promoter of the long germline transcript, but one cannot exclude that this segment may contain additional regulatory elements.
the other hand, the KI-KII mutation did not alter the efficiency of rearrangement in the 103 cell line, contrary to the result obtained in vivo. It has been shown that the KI-KII motifs can bind the transcription factor Pax-5 (18). This factor also binds a locus control region located at the 3' end of the IgH locus (19, 20), and seems to be involved in VH gene accessibility before the VH-DJH rearrangement step. We have found Pax-5 expression in the 103 cell line (data not shown), but specific cofactors (21) necessary for the KI-KII enhancement effect may nevertheless be absent from this cell line.

In the context of the regulation of allelic exclusion that starts by initiating rearrangement on one chromosome, one can envision that a limited balanced set of positive and negative factors present during a short window of development may control accessibility to one allele only. These factors will bind at the promoter and the enhancer regions but also at some specific sites in the V-(D)-J intervening segments (4, 22-24) or upstream of the V regions (25, 26). Alternatively, only one allele per progenitor could be accessible to the recombinase if it is marked by a modification enzyme as recently proposed (27) or by a specific anatomical location in the nucleus. It has been shown recently that in double positive thymic cells the excluded β allele was mostly hypomethylated and transcriptionally active (28). This result would fit better with a repression of the silent allele in the second phase of the allelic exclusion process than with a regulation operating via methylation. The 103/bcl2-hygro<sup>β</sup> cell line may be a valuable tool to address such issues.

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