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Gene targeting of the KI–KII sequence elements in a model pre-B cell line: effects on germline transcription and rearrangement of the κ locus

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Abstract

To study the role of individual sequence elements in the coordinate regulation of rearrangement and germline transcription of the κ locus, we have developed a gene targeting system with a mouse model pre-B cell line, 38B9. This line can be induced to initiate κ germline transcription and V–J rearrangement. Importantly, the effects of gene disruption in the cell line can be analyzed independent of selective pressures that may mask effects in the developing immune system of the mouse. We focused our study on targeting mutation of the endogenous KI–KII sequence elements to allow a direct comparison with the same gene disruption reported in mouse studies. Mutations were targeted to one allele, and effects on induced transcription and rearrangement were compared to the remaining wild type allele. Our results show that KI–KII mutation has little effect on germline transcription, and reduced the frequency of rearrangement two fold compared to the wild type allele. This report demonstrates that the use of model pre-B cell lines for targeted gene disruption is an attractive alternative to targeting the germline of the mouse.

Keywords: Immunoglobulin; Gene targeting; Rearrangement

1. Introduction

Currently there is a considerable focus on the enzymatic machinery that promotes immunoglobulin (Ig) gene assembly. However, specific sequence recognition motifs have been demonstrated to play an important role in targeting the RAG1/RAG2 mediated cleavage reactions (Grawunder et al., 1998). Moreover, the tissue specific and developmentally regulated accessibility of the various Ig family members appears to depend on transcriptionally mediated accessibility (Chen and Alt, 1993). For example, we (O'Brien et al., 1997) and others (reviewed in Staudt and Lenardo, 1991) have demonstrated the close correlation between sequences and factors that regulate germline transcription of the kappa (κ) locus and Vκ–Jκ recombination. Numerous transfection and transgenic studies with recombination substrates have also correlated factors such as active transcription, open chromatin structure, hypomethylation and transcriptional enhancer elements to recombinational accessibility (Chen and Alt, 1993; Ferrier et al., 1990; Sleckman et al., 1996). Several recent gene-targeted mutational analyses have further supported the importance of the intron and 3′ enhancers in controlling recombinational accessibility of the κ locus (Xu et al., 1996; Takeda et al., 1993). These enhancers are made up of multiple sequence elements that bind a variety of transcription factors and coordinately regulate tissue specific and developmental κ expression (Staudt and Lenardo, 1991).

Although the link between transcription and recombination is established, it is not clear which individual regulatory sequences participate in coordinating the

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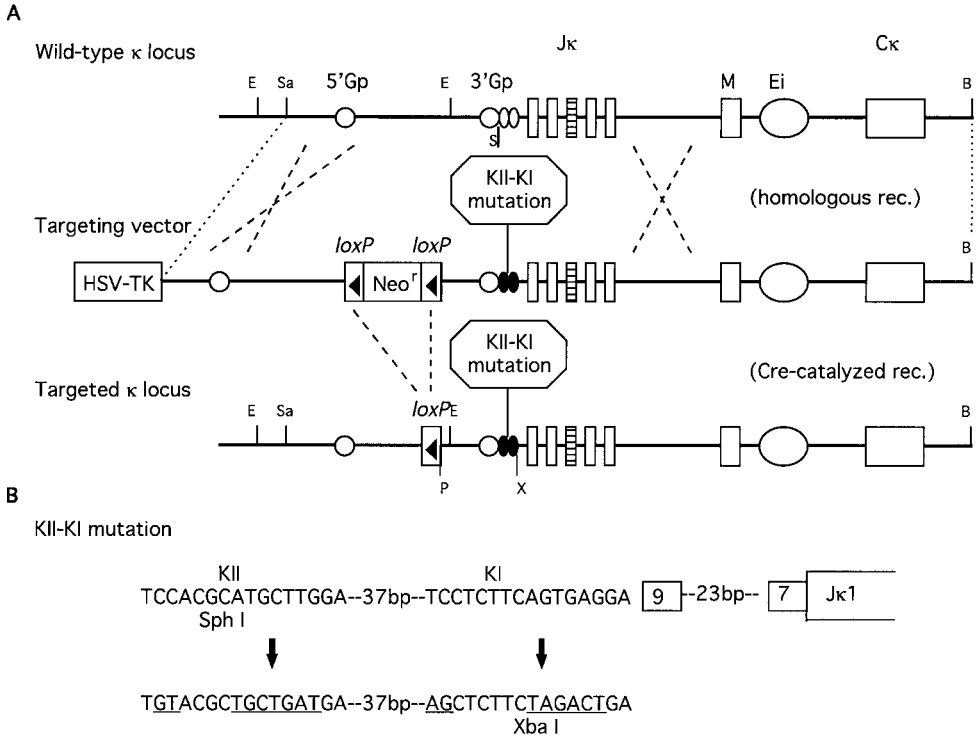

Fig. 1. The strategy for generating the KI–KII mutation. (A) Targeted distribution of the KI–KII sequences at the κ locus. The strategy is adapted from the use of this vector in targeting ES cells (Ferradini et al., 1996). The targeting vector contains a 11.5 kb Sal I-Bam HI κ gene fragment, which includes the KI–KII mutation, the joining segments 1–5, the Jκ–Cκ cell intervening sequence and the Cκ region. The *neo*^{r} gene flanked by loxP sites was inserted 2 kb upstream of the Jκ1, and the HSV-tk gene was positioned at the 5′ end of the κ gene fragment. The vector was first introduced into 38B9 cells by electroporation. In the second step, the *neo*^{r} gene was excised from the targeted locus by transient transfection of the targeted clones with a CRE recombinase expression vector, leaving behind an intact loxP sequence at the targeted locus. E, B, S, X, P, Sa, M, Ei and Gp represent sites for Eco RV, Bam HI, Sph I, Xba I, Pst I, Sal I, the matrix associated region, the intron enhancer and the germline promoters, respectively. (B) The mutations were introduced by site-directed mutagenesis and confirmed by sequencing. Modified nucleotides are underlined. The original KII and KI sequences (upper) were replaced by mutant sequences (lower) simultaneously. To facilitate analyzing the mutations, a Sph I restriction site in the wild-type KII sequence was destroyed and a Xba I site was introduced in the mutant KI sequence. The heptameter and nonamer sequences of the recombination signal sequence are represented by closed boxes and the Jκ1 segment by an open box.

two processes. Moreover, other potential sequences have been proposed to impact on recombination events, independent of their role in transcriptional regulation (Lewis, 1995; Weaver and Baltimore, 1987; Ferradini et al., 1996). In chicken, in addition to the enhancer and promoters, sequences located in the V–J intervening sequence regulate rearrangement of the lambda light-chain (Lauster et al., 1993). Silencers and anti-silencers were proposed to control rearrangements of constructs transfected into mouse pre-B cells. One of the chicken anti-silencer elements is homologous in sequence and location to palindromic motifs (called KI and KII) in both mouse and human (Weaver and Baltimore, 1987). In mouse, these sites lie immediately upstream of the nonamer-heptamer recombination signal sequences of the Jκ1 segment and are separated by 37 bp from each other. A protein (κ locus protein, KLP) from pre-B and mature B cell lines has been identified by gel mobility shift assay to specifically recognize these sequences (Weaver and Baltimore, 1987). Based on the conservation and position of KI–KII and the tissue-specific expression of KLP, the interaction between KLP and KI–KII has been proposed to be involved in the regulation of κ rearrangement (Lauster et al., 1993; Ferradini et al., 1996). A recent knockout study in mice suggests that the KI–KII sequences are only involved in the regulation of rearrangement but not germline transcription (Ferradini et al., 1996). However, the exact mechanism by which the KI–KII sequences regulate rearrangement is still unknown.

Targeting sequences in the mouse germline has limitations in the study of the developing immune system. Significant effects can be masked by the selective pressures to expand limited numbers of B cells in the animal. Indeed, KI–KII mutations in mice appeared on one hand to reduce rearrangement events, yet mice that were heterozygous and homozygous for the mutation were found to have normal B cell numbers, with typical κ/λ ratios (Ferradini et al., 1996).

To study the role of individual κ sequence elements in the coordinate regulation of rearrangement and germline transcription, we have developed a gene targeting system with a model mouse pre-B cell line, 38B9 (O'Brien et al., 1997). The advantage of this approach is (1) this cell line has both κ alleles in the germline configuration; (2) the line can be induced with lipopolysaccharide (LPS) or cytokines to undergo germline transcription and rearrangement; (3) unlike the limitation in the mouse, there are no selective pressures to expand individual subpopulations that would mask the functional consequences of gene alterations. By targeting one allele in the cell line, we could directly compare the effects of the genetic alteration on one allele with the other unaltered allele in the same cells. Moreover, we could follow gene rearrangements in individual cells. Our first approach, reported here, was to target the KI–KII sequence elements and to compare and contrast our results with the same gene targeting in the mouse (Ferradini et al., 1996).

2. Materials and methods

2.1. Plasmid construction and mutagenesis

The KI–KII targeting vector was kindly provided by Dr L. Ferradini. The targeting vector contains a 11.5 kb Sal I-Bam HI κ gene genomic fragment, which includes the KI–KII mutation, the joining segments 1–5, the Jκ–Cκ intervening sequence and the Cκ region. The KI–KII mutations were introduced by site-directed mutagenesis and confirmed by sequencing. To facilitate analyzing the mutations, a Sph I restriction site in the wild-type KII sequence was destroyed and an Xba I site was introduced in the mutant Ki sequence. The *neo*^{r} gene flanked by loxP sites was inserted 2 kb upstream of the Jκ1 segment, and the HSV-tk gene was positioned at the 5′ end of the κ gene fragment (Fig. 1). A unique Bam HI restriction site at the 3′ end of the κ gene fragment was used to linearize the vector prior to transfection. A Cre recombinase expression vector pBS185 was purchased from GIBCO.

2.2. Cell culture

The 38B9 mouse pre-B cell line was obtained from Dr Eugene Oltz (Vanderbilt University). The culture conditions, transfections, and induction of κ germline transcription and rearrangement are as previously described (O'Brien et al., 1997). Double selection for cells with homologous recombination events was carried out in RPMI 1640 media containing 800 μg/ml G418 (Geneticin, GIBCO) and 50 ng/ml ganciclovir (Roche) for 2–3 weeks. Single cell clones were isolated by limited dilution of selected cell populations in 96 well plates.

2.3. Screening of single cell clones by PCR and Southern blot analysis

To screen homologous recombination events in single cell clones after G418 and ganciclovir selection, long-range PCR assays were performed with two oligonucleotides. One oligonucleotide is complementary to an endogenous κ region (upstream of a Sal I site, Fig. 1) 5′ of the κ gene fragment included in the targeting vector, and the other is complementary to the *neo*^{r} gene in the targeting vector. The sequences of these oligos are as follows: 5′ primer (upstream of the Sal I site), 5′-GTTGTGAGGCCATGGTGAGATCCC-3′; 3′ primer (within the *neo*^{r} gene), 5′-CAGCCAAGCTAGCTTGGCTGGACG-3′. The PCR was carried out for 40 cycles at 94°C for 45 s, 66°C for 45 s and 72°C for 4 min, and it produced a product of approximately 4 kb. The enzyme used in the PCR assay was KlentaqLA, purchased from Dr W. Barnes at the Washington University (St Louis).

Southern blot analysis of homologous recombination events in single cell clones was performed as described by Sambrook et al. (1989). Genomic DNA samples were digested with Eco RV before electrophoresis and blotting. An Eco RV fragment of the κ gene was labeled with ^{32}P-dCTP by DNA polymerase I and used to detect both wild-type allele and altered allele carrying the *neo*^{r} gene insertion. The probe detects an approximately 3.9 kb fragment from the wild-type allele and an approximately 5.2 kb fragment from the altered allele.

To determine if a double homologous recombination event actually carried the Ki–KII mutation, PCR amplification of the region containing the KI–KII sequences was performed. The PCR product was then digested with diagnostic enzyme Xba I to confirm the presence of the KI–KII mutation.

The excision of the *neo*^{r} gene insertion after transient expression of Cre recombinase was verified by appropriate PCR assay or Southern blot analysis, which showed shortened product or fragment due to the loss of the 1.3 kb *neo*^{r} gene insertion.

2.4. Analysis of germline transcription by reverse transcriptase (RT)–PCR assays

Total RNA, 1 μg, was reversed transcribed with SuperScript II reverse transcriptase (GIBCO) at 42°C for 1 h with 300 ng oligo(dT) (Pharmacia) in a 20 μl reaction. The cDNA was amplified by PCR using 24–26 cycles at 94°C for 15 s, 70°C for 25 s and 72°C for 30 s. Sequences of two oligonucleotides designed to amplify germline transcripts initiating within the KII

sequence for both wild-type and mutated alleles are as follows: 5′ primer (between the KII and KI sites), 5′-GAG-GGGGTTAAGCTTTCGCCTACCCAC-3′; 3′ primer (with the Ck region), 5′-GTCGTTCACTG-CCATCAATCTTCCACT-3′. Sequences of oligonucleotides for detecting germline transcripts from the wild-type allele are as follows: 5′ primer (complementary only to the wild-type allele), 5′-CTACCCAC-TGCTCTGTTCCTCTTCAGT-3′; 3′ primer (as before). Sequences of oligonucleotides for detecting germline transcripts from the KI–KII mutated allele are as follows: 5′ primer (complementory only to the KI–KII mutated allele), 5′-CCCACTGCTCTGTAG-CTCTTCTAGACT-3′; 3′ primer (as before). Sequences of oligonucleotides for detecting transcripts from already rearranged substrates are as follows: 5′ primer, 5′-GTCCCTGCCAGGTTYAGTGGCAGT-GGRTCWRGGAC-3′ (where Y = C or T, R = A or G, and W = T or A); 3′ primer (as before).

2.5. Analysis of recombination by PCR assays

To detect DNA remaining in the germline configuration, genomic DNA was isolated and subjected to PCR with 24–26 cycles at 94°C for 30 s, 66°C for 30 s and 72°C for 2.5 min. Sequences of oligonucleotides used to detect unrearranged DNA of the wild-type allele are as follows: 5′ primer, 5′-CTACCCACT-GC-TCTGTTCCTCTTCAGT-3′; 3′ primer, 5′-TTTGATCTGCGCTGTTTCATCCTCTGGGTCAT-TC-3′. Sequences of oligonucleotides used to detect unrearranged DNA of KII–KI mutated allele are: 5′ primer, 5′-CCCACTGCTCTGTAGCTCTTCTA-GACT-3′; 3′ primer (as before). A pair of oligonucleotides complementing to the Cκ region was used as an internal control for template loading and amplification efficiency. Their sequences are: 5′ primer, 5′-CCACGGACGAGTATGAACGACATAACAGCTA-TAC-3′; 3′ primer, 5′-GTGTAATCTCACGGTATA-GAGGTCTCTTGAAG-3′. To detect already rearranged substrates, PCR was cycled as follows: 4 cycles of 97°C for 45 s, 65°C for 1 min and 72°C for 2.5 min; and 21 cycles of 94°C for 45 s, 65°C for 1 min and 72°C for 2.5 min (87). Sequences of oligonucleotides used in this assay are: 5′ primer, 5′-GTCCCTGCCAGGTTYAGTGGCAGTGGRTCWR-GGAC-3′ (where Y = C or T, R = A or G, and W = T or A); 3′ primer, 5′ TTAGTGGCTCTGTTCCT-ATCACTGTGTCCTCAGG-3′. The same control was also used in this assay. Results presented are representative of several targeted clones.

2.6. Quantification of PCR products by PhosphorImager and statistical analysis

All images were acquired with a model 445SI PhosphorImager (Molecular Dynamics). Visualization and Quantification of images were performed with IPLab Gel software, 1.5 (Signal Analytics, Vienna, VA). Typically, three RT–PCR or PCR assays were performed for each sample set, and average values were plotted with standard error of mean.

3. Results

3.1. The strategy for generating the KI–KII mutation at the endogenous κ locus in the 38B9 mouse pre-B cell line

To study the role of the KI–KII sequences in the regulation of κ rearrangement and germline transcription in a model pre-B cell line, we took an approach that combined homologous recombination and the Cre-loxP system to mutate Ki–KII sequences on one allele at the endogenous κ locus in 38B9 pre-B cells. The 38B9 cells are Abelson virus-transformed pre-B cells that express endogenous RAG genes and can be induced to undergo k gene rearrangements by lipopolysaccharide (LPS). As we have previously shown, this correlates with induction of germline transcription (O'Brien et al., 1997). These cells show no background rearrangement in the absence of inducers; thus, controlled staging and expansion of cell populations before and after induced rearrangements can be done. We directly compared the effects of the Ki–KII alteration on rearrangement and germline transcription to that of the wild-type allele. The vector used to generate the KI–KII mutation was kindly provided by Dr L. Ferradini (Ferradini et al., 1996). It contains a mouse κ genomic fragment in which the KII and KI sequences were simultaneously mutated by site-directed mutagenesis, and replaced by 9 and 8 bp substitution mutations, respectively (Fig. 1). The mutations effectively destroy any specific factor binding activities of both sequences in B cells (Ferradini, personal communication). The targeting vector also had a thymidine kinase (TK) gene promoter-driving neomycin resistance (*neo*^{r}) gene flanked by loxP sites at each end of the *neo*^{r} gene and a herpes simplex virus (HSV)-tk gene at the 5′ end of the κ gene fragment. Both the *neo*^{r} and HSV-tk genes were used in drug selections for the correct homologous recombination events that deliver the KI–KII mutation to the endogenous locus. To eliminate the potential effect of a transcriptionally active *neo*^{r} gene on rearrangement and germline transcription, the Cre-loxP system of bacteriophage P1 was used to delete the *neo*^{r} gene from the targeted locus (see Fig. 1).

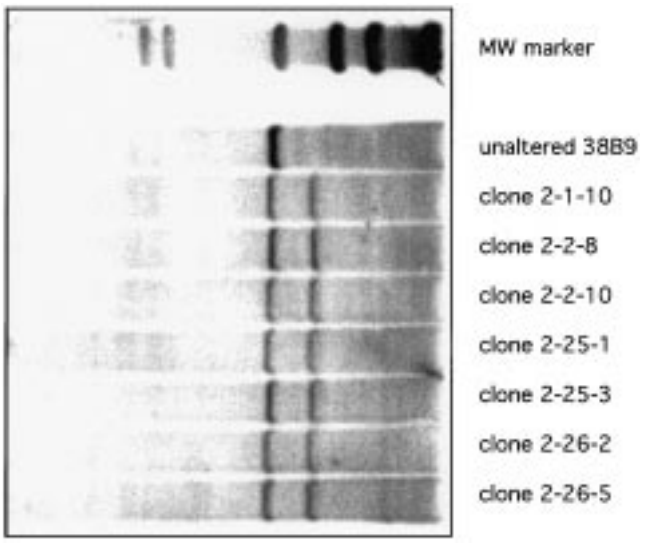

Fig. 2. Southern blot analysis of homologous recombination events in single cell clones. Positive single cell clones from the PCR screening were expanded and their genomic DNA was extracted and subjected to Southern blot analysis. An Eco RV fragment was radiolabeled and used to detect both the wild-type allele and the altered allele carrying the *neo*^{r} gene insertion (see Fig. 1). The genomic DNA samples were digested with Eco RV enzyme before blotting. The probe detects an approximately 3.9 kb fragment from the wild-type allele and a 5.2 kb fragment from the altered allele.

3.2. Selection of single cell clones with the KI–KII mutation and neo^r gene insertion

The targeting vector was linearized with Bam HI, and introduced into 38B9 cells by electroporation. The transfected cells were then subjected to selection with G418 and ganciclovir at optimized concentrations, and surviving cells were subcloned for single cell clones. After expansion, the cell clones were first screened by long-range genomic DNA PCR assays using two primers (one primer complementary to an endogenous κ region 5′ of the κ gene fragment contained in the targeting vector, and the other complementary to the *neo*^{r} gene in the targeting vector). Positive clones from the PCR screening were further analyzed by Southern blot analysis to verify the structure of the targeted locus and presence of the KI–KII mutation. As shown in Fig. 2, the hybridization probe detects an additional band at 5.2 kb as a result of the alteration. The homologous recombination events that introduced both the *neo*^{r} insertion and KI–KII mutation were detected at a frequency of 2% of screened cell clones.

3.3. Isolation of single cell clones with excision of the neo^r gen via Cre recombinase-catalyzed, loxP site-specific recombination

Because previous reports have shown that a transcriptionally active *neo*^{r} gene can affect expression of its neighboring genes (Xu et al., 1996), we carried out the additional step to eliminate the inserted *neo*^{r} gene from the targeted locus in the single cell clones by transiently introducing a Cre recombinase expression vector into the targeted single cell clones. The Cre recombinase is capable of catalyzing loxP site-specific recombination events that will result in the excision of the *neo*^{r} gene from the targeted locus, and leaves an intact loxP sequence at the targeted locus. A copy of the intact loxP sequence has been shown to have no effect on expression of its neighboring genes (Ferradini et al., 1996). Transiently transfected cells were further subcloned. Each single cell clone was expanded and divided in two cultures. One culture was maintained in normal non-selective media while the other was maintained in G418 selective media. The G418 sensitive clones were likely to be the clones that lost *neo*^{r} gene, and *neo*^{r} loss was confirmed by appropriate restriction digestion, PCR assays and Southern blot analysis (data not shown). We found that 15–30% of selected cell clones had lost *neo*^{r} gene. Thus, the final clones have one wild-type allele and one KI–KII mutated allele.

3.4. Mutation of KI–KII sequences does not affect germline transcription at the κ locus

To assess the effect of the KI–KII mutation on germline transcription in 38B9 cells, a quantitative, allele-specific RT–PCR assay was developed to specifically measure germline transcripts produced from both κ alleles. Two germline transcripts have been previously reported at the κ locus (Martin and Van Ness, 1990). One transcript initiates approximately 3.5 kb upstream of the Jκ1 segment from a 5′ germline promoter, the other transcript initiates within 0.1 kb upstream of the Jκ1 segment from a 3′ germline promoter. Because the 3′ germline promoter closely adjoins the KI–KII sequences, the transcript initiating from the 3′ germline promoter was analyzed by the RT–PCR assay in KI–KII mutated 38B9 pre-B cells (Fig. 3(A)). The Xba I site included in the KI mutation was used to distinguish transcripts originating from the mutant allele. RT–PCR products from both alleles were digested with Xba I, resulting in two fragments of different sizes, representing transcripts from either the wild-type (274 bp) or mutant (233 bp) allele. As shown in Fig. 3(B) and (C), transcripts at a comparable level were detected on both wild-type and KI–KII mutated alleles, suggesting that the KI–KII sequences are not required for germline transcription in these cells.

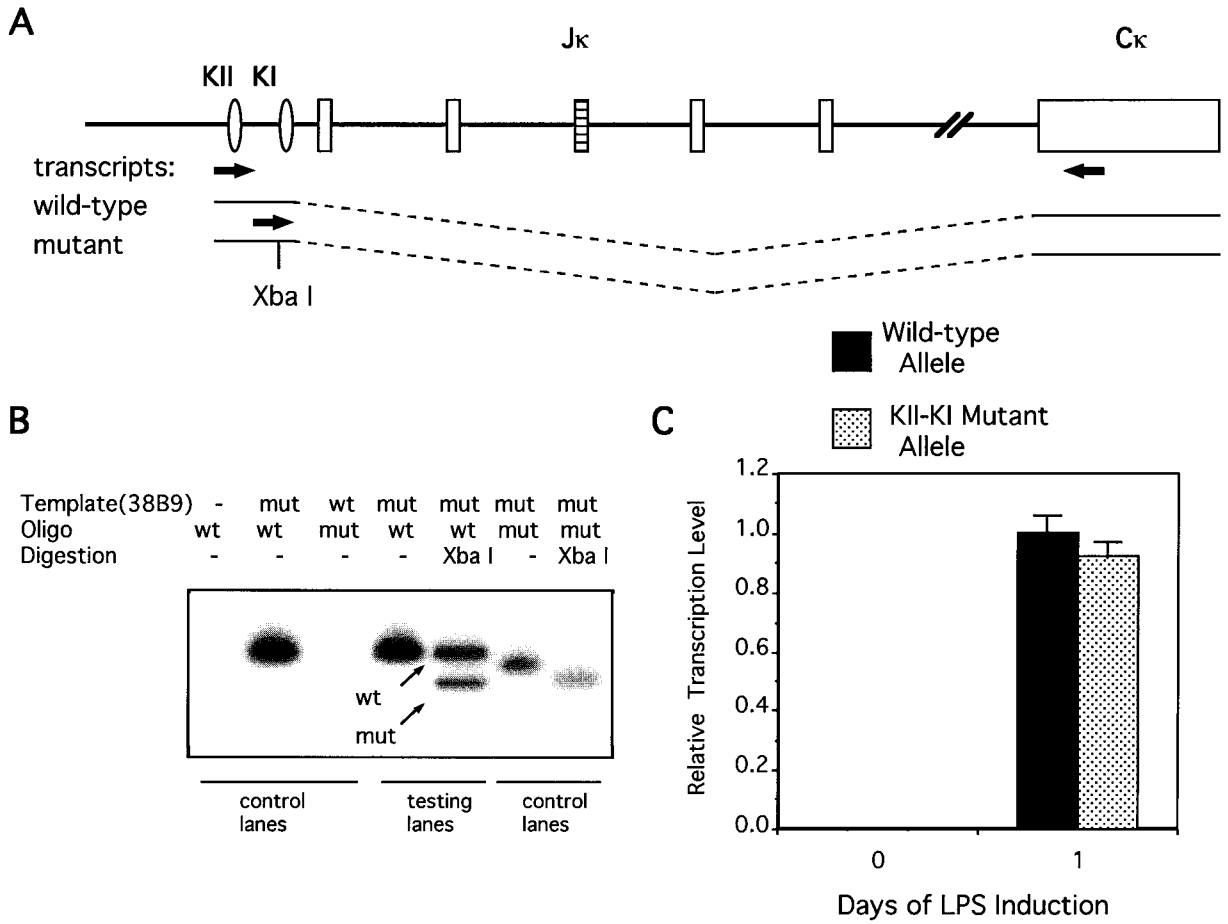

Fig. 3. Comparison of germline transcription levels between the wild-type and KI–KII mutated alleles by a quantitative RT–PCR assay. (A) Germline transcripts initiating within the KII sequence from the wild-type and mutated alleles are indicated. Primers designed to amplify transcripts from both alleles or mutated allele are shown above the transcripts. Wild-type primer is complementary to both alleles, and mutant primer is only complementary to the mutated allele. The Xba I site included in the KI mutation was used to distinguish transcripts originating from the mutant allele. (B) Analysis of RT–PCR products. Total RNA samples were isolated from 1–2 day LPS-induced cell pools. RT–PCR assay was performed according to a standard procedure (see Section 2 for details). The Cκ primer was radiolabeled before RT–PCR assay. RT–PCR products were digested with Xba I to completion and resolved on a non-denaturing acrylamide gel. The completion of Xba I digestion was monitored by a parallel Xba I digestion of the RT–PCR product amplified with the Cκ primer and a mutant primer that is specific for the mutant transcript. The image was acquired through a PhosphorImager. (C) The intensities of germline transcripts from the wild-type and mutated alleles were quantified and normalized to the controls.

3.5. Mutation of the KI–KII sequences reduces the frequency of rearrangement at the κ locus

Despite the lack of activity in regulating germline transcription, given their close proximity to the Jκ1 recombination signal sequences and their conservation among human and mouse κ genes, the KI–KII sequences might still be important for rearrangement. We tested whether the KI–KII sequences were important for rearrangement of the κ locus by performing several different assays designed to measure the frequency of rearrangement events on both the wild-type and mutated alleles. The first assay was a quantitative, allele-specific genomic DNA PCR assay using KI-KII mutated cell pools induced with LPS for 2 weeks. Because the KI and KII sequences are lost upon rearrangement, mutant vs wild-type alleles can only be distinguished when the κ alleles have not rearranged, namely, in the germline configuration. Therefore, the proportion of DNA (both wild-type and mutant alleles) remaining in the germline configuration was used to estimate their relative rearrangement frequencies. The PCR assay was designed to measure the amount of DNA remaining in germline configuration on both alleles (see Fig. 4(A) and Section 2 for details). After carrying out the assay, the intensities of radiolabeled products were quantified and the relative values were plotted (Fig. 4(B) and (C)). Based on PCR assays and random single cell analysis, no rearrangement events were detected in the starting population. Therefore, assuming that 100% DNA was unrearranged at the starting point, after LPS induction the

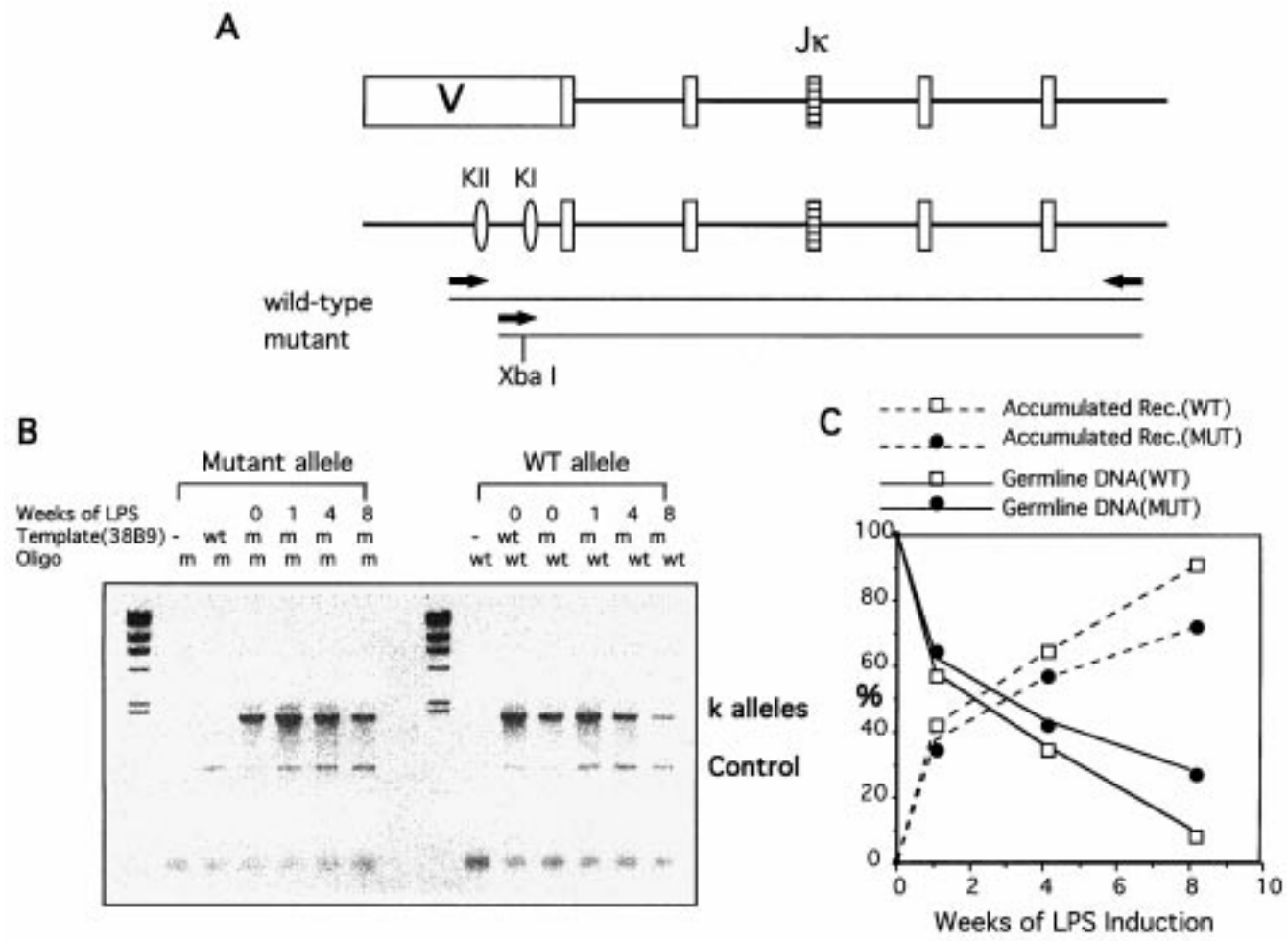

Fig. 4. Comparison of frequencies of accumulated rearrangement events between the wild-type and KI–KII mutated alleles by a quantitative PCR assay. (A) The strategy for detection of DNA remaining in the germline configuration at both κ alleles by a quantitative genomic DNA PCR assay. Allele-specific primers are represented by arrowheads. The wild-type primer is specific only for the wild-type allele and the mutant primer is specific for the mutated allele. (B) Genomic DNA samples were isolated from LPS-treated KI–KII mutated cell pools after indicated periods of LPS induction, and subjected to the quantitative PCR assay. A loading control containing two primers complementary to the Cκ region was also included in the assay (see Section 2 for details). (C) The intensities of the PCR products were quantified and normalized to the loading control, and plotted (vs weeks of LPS induction). The converted values reflecting the accumulated rearrangement frequencies were also plotted.

accumulated rearrangement frequency is equal to the time zero values minus the percentage of DNA remaining in the germline configuration measured by the PCR assay. Based on this calculation, we present the results in two different forms: one is in a form of DNA remaining in the germline configuration vs weeks of LPS induction; the other is the accumulated rearrangement frequency vs weeks of LPS induction (Fig. 4(C)). We conclude from this analysis that KI–KII sequences are not required for κ rearrangement, although at later time points there is a significant reduction in the rearrangement frequency of KI–KII mutated alleles.

One of the advantages in using this model cell line system is that we can follow the course of rearrangement by isolating single cell clones after LPS induction. Thus, to more quantitatively assess the effect of the KI–KII mutation on rearrangement, we measured the frequencies of rearrangements at both the wild-type and mutated allele in single cell clones. To perform the single cell clonal analysis, cell pools were first treated with LPS for 2 weeks and then single cell clones were expanded in the absence of LPS. Four different types of PCR assays were used to determine rearrangement events in each single cell (see Section 2 for details). The PCR assays include (1) an allele-specific RT–PCR assay, similar to the one in Fig. 3, but also including primers specific only for the wild-type transcript, was used to detect a loss of germline transcript from either allele indicating a rearrangement event, (2) an RT–PCR assay, using a consensus V region oligonucleotide, was performed to detect tran-

Table 1
Frequencies of rearrangement events on both wild-type and KI–KII mutated alleles measured by a combined analysis of single cell clones

Weeks of LPS induction	2
Total single cell clones analyzed	133
Total clones with κ rearrangement(s)	42 (32%)
Clones with κ rearrangement on wt allele	36 (27%)
Clones with κ rearrangement on mutant allele	18 (13%)

scripts from a rearranged locus, (3) an allele-specific PCR assay to detect the existence of either allele in its germline configuration (similar to the one in Fig. 4) and (4) a PCR assay, using a consensus V region oligonucleotide, to detect already rearranged genes from both alleles (O'Brien et al., 1997). By combining these different PCR assays, we were able to determine which allele(s) was rearranged in each single cell clone. The results of this combined analysis are summarized in Table 1. Of 133 single cell clones analyzed, 42 showed a rearrangement event on at least one of the two κ alleles, suggesting that approximately 32% cells had rearrangement events. Since some cells rearranged both alleles, the total allelic frequency of rearrangement was 54/266 (20%). When the rearrangement frequency on either the wild-type or mutant allele was examined, 36 clones (27%) rearranged the wild-type allele and 18 clones (13%) rearranged the mutant allele, indicating that the wild-type allele rearranged at a frequency twice that of the mutant allele. Therefore, by examining multiple single cell clones, we confirmed that KI–KII sequences are not required for rearrangement, but mutation of these sequences reduced the frequency two fold.

4. Discussion

We previously have demonstrated that the 38B9 pre-B cell line can serve as a model of induced rearrangement of the κ locus (O'Brien et al., 1997). There is a very tight regulation, with both germline transcription and rearrangement dependent on the addition of inducers such as LPS, IFN-γ and IL-1 (O'Brien et al., 1997). In this report we have examined whether targeted disruptions of the endogenous locus by homologous recombination could serve as a useful model to identify sequence elements involved in both transcription and recombination of the locus. We initiated the study with KI–KII disruption because it allowed a direct comparison with the same gene disruption strategy reported in the mouse (Ferradini et al., 1996). We have demonstrated successful gene targeting and strategies for analyzing the effects on transcription and rearrangement in both pooled cultures as well as single cell clones. Because the cells were designed to carry one mutant and one wild-type allele, direct allelic comparisons could be made.

Numerous previous studies utilizing cell lines, transgenic and knockout mice suggest that there is a correlation between germline transcription and rearrangement at immunoglobulin loci (reviewed in Chen and Alt, 1993). Indeed, we have reported a very closed correlation between germline transcription and rearrangement of the κ locus in 38B9 pre-B cells (O'Brien et al., 1997). Because both transcription and rearrangement involve multiple sequence elements, targeted disruption of individual sequence elements could shed light on the relationship between germline transcription and rearrangement. Targeted deletions of the intron and 3′ enhancers in mice have shown that these enhancers may be involved in the regulation of both germline transcription and rearrangement (Takeda et al., 1993; Xu et al., 1996). The KI–KII sequences were proposed to regulate rearrangement and recently, their role in contributing to rearrangement has been suggested by an ES knockout study (Ferradini et al., 1996). Notably, the ES knockout study also showed that KI-KII mutation had a measurable but not complete effect. The quantitative impact of the KI–KII mutations was difficult to evaluate given the selective pressures in the mouse to expand B cell populations to normal levels. Our results in 38B9 cells are entirely consistent with the ES targeted approach, suggesting germline transcription is not impacted by KI–KII sequences, but a measurable effect is seen on rearrangement. It is important to note, however, that both studies demonstrate that KI–KII sequences are not absolutely required. The fact that similar conclusions have been reached in both the cell line and mouse studies encourages us to consider this as a valid approach to addressing other sequence requirements. A recent report demonstrated the use of 'hit and run' gene targeting approach to delete the matrix associated region (MAR) in a pre-B cell line 103 (Hale and Garrard, 1998). Interestingly, they were unable to isolate clones that maintained the germline allele in the absence of the MAR, because of an unexpected hyper-recombination of Vκ–Jκ joining in the MAR deleted allele.

We have also used the 38B9 cell as a model to examine receptor editing (manuscript submitted). Indeed, V–J replacements can be observed on rearranged κ alleles. Since the KI–KII elements were deleted by the primary rearrangement, it is clear that secondary rearrangements on the same allele do not require KI–KII. None of these results rule out the importance of transcriptional activation of the locus for targeting the allele for rearrangement. Initial germline transcription correlates with primary rearrangement events, while transcription of a rearranged allele could serve the same purpose for secondary rearrangements.

A targeted disruption of the germline promoter sequences could address this issue.

The use of model pre-B cell lines such as 38B9 to address the role of sequence motifs in the κ locus provides an attractive alternative to targeting the germline of the mouse.

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