

# Light Chain Replacement: A New Model for Antibody Gene Rearrangement

By Eline Luning Prak and Martin Weigert

From the Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544

## Summary

A functional B cell antigen receptor is thought to regulate antibody gene rearrangement either by stopping further rearrangement (exclusion) or by promoting additional rearrangement (editing). We have developed a new model to study the regulation of antibody gene rearrangement. In this model, we used gene targeting to replace the J $\kappa$  region with a functional V $\kappa$ -J $\kappa$  light chain gene. Two different strains of mice were created; one, V $\kappa$ 4R, has a V $\kappa$ 4-J $\kappa$ 4 rearrangement followed by a downstream J $\kappa$ 5 segment, while the other, V $\kappa$ 8R, has a V $\kappa$ 8-J $\kappa$ 5 light chain. Here, we analyze the influence of these functional light chains on light chain rearrangement. We show that some V $\kappa$ 4R and V $\kappa$ 8R B cells only have the V $\kappa$ R light chain rearrangement, whereas others undergo additional rearrangements. Additional rearrangement can occur not only at the other  $\kappa$  allele or isotype ( $\lambda$ ), but also at the targeted locus in both V $\kappa$ 4R and V $\kappa$ 8R. Rearrangement to the downstream J $\kappa$ 5 segment is observed in V $\kappa$ 4R, as is deletion of the targeted locus in both V $\kappa$ 4R and V $\kappa$ 8R. The V $\kappa$ R models illustrate that a productively rearranged light chain can either terminate further rearrangement or allow further rearrangement. We attribute the latter to editing of autoantibodies and to corrections of dysfunctional receptors.

Allelic and isotypic exclusion lead to the expression of only one kind of antigen receptor per B cell. Exclusion is ensured by mechanisms that shut off antibody gene rearrangement (H/L-STOP), an idea based on the finding that at least 50% of plasmacytoma lines and approximately two thirds of murine splenic B cells have only one productively rearranged kappa locus,  $\kappa^+$ , whereas the other kappa locus in these cells is unrearranged,  $\kappa^0$  (1). This  $\kappa^+/\kappa^0$  genotype is not expected if rearrangements were to continue indefinitely. Exclusion is thought to be governed by the products of productively rearranged heavy (H) and light (L) chain genes. This hypothesis was tested in transgenic mice; it was demonstrated that a functional, transgene-encoded  $\kappa$  L chain and H chain (contributed by endogenous rearrangement) prevented additional antibody gene rearrangements (2).

The products of a productive H and L chain rearrangement may not always shut down further rearrangement. Ongoing  $\kappa$  rearrangement has been inferred from the nature of circular excision products. These episomes, generated by deletion  $\kappa$  recombination (3), sometimes contain V $\kappa$ -J $\kappa$  rearrangements, including rearrangements with productive junctions (4). Evidence consistent with ongoing rearrangement has also been obtained in autoantibody transgenic animals (5-7). It has been proposed that ongoing rearrangements allow autoreactive B cells to edit their receptors, thereby escaping tolerance induction (5-7).

Editing defines a genetic precursor-product relationship, for example that a preexisting V $\kappa$ -J $\kappa$  rearrangement has been displaced by the rearrangement of an upstream V $\kappa$  gene to

a downstream J $\kappa$  gene. So far, a direct demonstration of such a relationship is lacking. It cannot be established in the analysis of circular excision products because the cellular source of these products is not known. Even in studies analyzing multiple rearrangements in individual cell lines (6, 8, 9), the timing of the rearrangements cannot be ascertained. For example, an upstream V $\kappa$ -J $\kappa$  rearrangement may have occurred after the rearrangement to the downstream J $\kappa$  segment. Light chain transgenes are not appropriate for this study because they lack the necessary upstream and downstream recombination signals. Moreover, transgenes are problematic because they differ from normal loci in copy number (usually greater than one) and downstream sequences. For example, truncation of downstream sequences influences the degree to which a given transgene excludes endogenous rearrangements (10).

Here we describe a new mouse model which, unlike the  $\kappa$  transgenics, recreates a functional rearranged  $\kappa$  locus. By homologous recombination, we have replaced the unrearranged J $\kappa$  region with a rearranged V $\kappa$ -J $\kappa$  gene (see Fig. 1). This L chain replacement (V $\kappa$ R) has a single, rearranged L chain gene in the proper genomic context. The V $\kappa$ R model thereby simulates the genotype of a normal B cell with a functional V $\kappa$ -J $\kappa$  L chain gene on one allele ( $\kappa^+/\kappa^0$ ). We have used these animals to reexamine how a productively rearranged L chain gene influences L chain rearrangement.

## Materials and Methods

*Cloning of Targeting Vectors.* Replacement-type targeting vectors (11) were assembled from genomic BALB/c  $\kappa$  DNA (12), pPGK-

Neo (13), pMC101-tk (the thymidine kinase gene [tk]<sup>1</sup> was only used in the V $\kappa$ 8RTV [14]), and V $\kappa$ 4-J $\kappa$ 4 (7) or V $\kappa$ 8-J $\kappa$ 5 (15) DNA as follows: a 1.6-kb genomic EcoR1 fragment located upstream of the BALB/c J $\kappa$  region was subcloned into pBlueScript (Stratagene, La Jolla, CA). The unique EcoRV site in 1.6R1 was converted to a Xho1 site by linker ligation (unphosphorylated Xho1 linkers from New England Biolabs, Beverly, MA). The pPGK-Neo gene was excised from its plasmid as a Xho1 fragment and inserted into the engineered Xho1 site in 1.6R1, in the reverse transcriptional orientation. Digestion of the resultant 3.2R1 insert with EcoR1 yields a 2.8-kb fragment containing the 1.2-kb short homologous recombination arm and PGK-Neo. This 2.8-kb EcoR1 fragment was blunted with T4 polymerase (New England Biolabs) and inserted ~4 kb upstream of the V $\kappa$ 8 and the V $\kappa$ 4 genomic clones in the proper orientation (see Fig. 1). To insert the tk gene in the V $\kappa$ 8 replacement targeting vector, tk was liberated from its plasmid by double digestion with Sal1 and Xho1 and band purified from low melting temperature agarose. The tk Sal1-Xho1 fragment was introduced into the unique Sal1 site of the V $\kappa$ 8R targeting construct, recreating the unique Sal1 site in the final product.

**Generation of Targeted ES Cell Lines.** Targeting vectors were linearized outside of the region of homology by Sal1 digestion and transfected by electroporation into E14-1 (16) and C57Bl6-III (17) ES cells. ES cell colonies were expanded on primary embryonic fibroblasts in the presence of 200  $\mu$ g/ml G418 (active drug, GIBCO BRL, Gaithersburg, MD) or 200  $\mu$ g/ml G418 and 2  $\mu$ M of freshly prepared gancyclovir (Cytovene; Syntex Laboratories, Palo Alto, CA). Boiled cell lysates were prepared from neomycin-resistant or double-resistant (neomycin and gancyclovir) ES cell colonies and were screened for homologous recombination events by PCR, using primers situated in the neomycin resistance gene (5'-GGCTCT-ATGGCTTCTGAGG-3') and in the  $\kappa$  locus upstream of the 5' end of the targeting vector (5'-TGCCCTTGGTGAGGGTGAAG-3'). Reactions consisted of 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.1 mg/ml gelatin, 0.31  $\mu$ M of each dNTP, 0.8  $\mu$ M of each primer, and a proteinase K lysate from 50–100 ES cells. Amplifications were carried out as follows: 2 min at 94°C (primary denaturation) followed by 45 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 1 min, and a final extension step at 72°C for 10 min.

**Southern Blotting.** Genomic DNA was purified from ES cell lines and tail samples as described previously (9, 15) and digested overnight with Pst1. Digested DNA was run on 0.8% agarose gels in Tris/acetate/EDTA buffer and transferred to Zeta Probe nylon membranes (Bio Rad Laboratories, Richmond, CA) in 0.4 N NaOH (18). The filter was probed with PKP6, a 0.8-kb genomic fragment upstream of the J $\kappa$  region (3).

**Hybridomas.** B cell hybridomas were prepared from the spleen fragments of hemisplenectomized 3–6-wk-old mice with the following  $\kappa$  genotypes: V $\kappa$ 4R/wt (two animals) and V $\kappa$ 8R/wt (one animal). To prepare the hybridomas, splenocytes were stimulated in vitro with 20  $\mu$ g/ml *Escherichia coli* LPS (Sigma Chemical Co., St. Louis, MO) for 2–3 d before fusion with Sp2/0-Ag14 (19). Hybridomas were selected using azaserine-hypoxanthine (Sigma Chemical Co.). Supernatants were tested for  $\mu$  $\kappa$  and  $\mu$  $\lambda$  antibody production as described previously (9).

**PCR Assays on Hybridoma DNA.** Genomic DNA was prepared from hybridomas grown to high density in 24-well plates as described previously (9). For PCR, 100–150 ng genomic DNA was used per reaction. For the J $\kappa$  typing PCR assays, Vs (20) or L3-L7

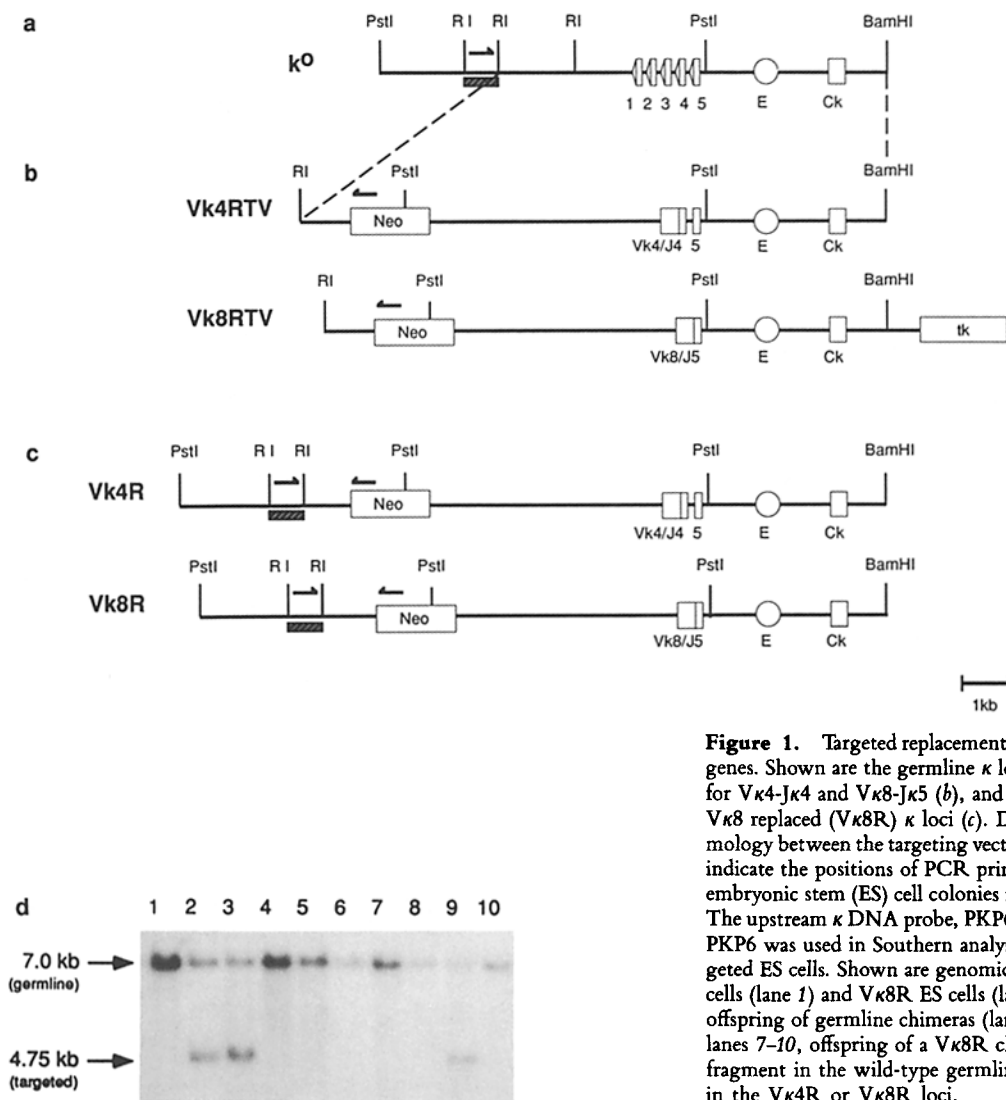
(21) forward V $\kappa$  primers were used with J $\kappa$ 2 (22), J $\kappa$ 4 (9), or J $\kappa$ 5 (22) reverse-J $\kappa$  primers. Primer positions are shown in Fig. 2 a. Reaction conditions and cycling programs are described by Luning Prak et al. (9). PCR assays that specifically amplify V $\kappa$ 4 or V $\kappa$ 8 have been described, (7 and 15, respectively). An assay using the V $\kappa$ 4-specific V $\kappa$  primer (7) and the J $\kappa$ 5 reverse primer (22) used the same reaction mixture and cycling program as the J $\kappa$  PCR (described in reference 9). PCR assays used to identify  $\lambda$ 1,  $\lambda$ 2, and  $\lambda$ X rearrangements were performed as before (9).

## Results

**Homologous Recombination of V $\kappa$ -J $\kappa$  and the J $\kappa$  Locus.** The V $\kappa$ 4-J $\kappa$ 4 and V $\kappa$ 8-J $\kappa$ 5 genes were cloned from antibody-secreting hybridomas (7, 15) and introduced into replacement-type targeting vectors (Fig. 1 b). Targeting vectors were linearized outside of the homology region and transfected into E14.1 (16) and C57Bl6-III (17) ES cell lines. Neomycin-resistant ES cell colonies were screened for homologous recombination events by PCR, using primers situated in the neomycin resistance gene and in the  $\kappa$  locus, upstream of the 5' boundary of the targeting vector (Fig. 1). The frequency of homologous recombinants obtained after selection in neomycin was ~1/100 for both L chain replacement constructs.

**Production of ES Cell Chimeras and Germline Transmission of V $\kappa$ R.** Targeted ES cells were injected into blastocysts to produce chimeras. ES cell-derived B cells from these chimeras were studied to verify that the V $\kappa$ 4 and V $\kappa$ 8R constructs were functional. Chimeric animals were identified by coat color, and the presence of the replaced locus was confirmed by PCR analysis of tail DNA using V $\kappa$ 4- or V $\kappa$ 8-specific PCR assays (primers 4R and 8R in Fig. 2 a, data not shown). Splenocytes from PCR-positive chimeras were used to make hybridomas. Hybridoma DNA samples were screened by PCR for V $\kappa$ 4 or V $\kappa$ 8 genes (data not shown). RNA from V $\kappa$ 4- and V $\kappa$ 8-positive clones was amplified by reverse transcriptase PCR (RT-PCR), using V $\kappa$ 4 or V $\kappa$ 8 sequence-specific V $\kappa$  primers for reverse transcription and amplification and a reverse primer in C $\kappa$  for amplification (23). The presence of an appropriately sized RT-PCR product for both V $\kappa$ 4R and V $\kappa$ 8R indicated that both replaced L chain genes were transcribed (data not shown). V $\kappa$ 4R- and V $\kappa$ 8R-positive clones secreted  $\mu$  $\kappa$  antibodies, confirming that the replaced L chain was functional (data not shown). Moreover, the average amount and range of secreted  $\mu$  $\kappa$  in examples that produce exclusively V $\kappa$ 4R or V $\kappa$ 8R (class 1 see below,  $n = 8$ ) are the same as the examples that may express endogenous  $\kappa$  chains (classes 2 and 3,  $n = 8$ ). Furthermore, evidence that the expression level of the replaced allele is normal comes from the phenotype of bone marrow B cells of V $\kappa$ R mice. For this analysis, we used the progeny of V $\kappa$ R mice crossed to  $\kappa$ -deficient mice (9). V $\kappa$ R/ $\kappa$ -deficient heterozygous mice can only express  $\kappa$  chain from the V $\kappa$ R allele and, in the case of V $\kappa$ 8R/ $\kappa$ -deficient mice, even secondary V $\kappa$  rearrangements are precluded. The IgM density at the pre-B/immature B stages (B220<sup>+</sup>CD43<sup>-</sup>(S7)<sup>-</sup>HSA<sup>3+</sup>) of V $\kappa$ 8R/ $\kappa$ -deficient mice is indistinguishable from that of  $\kappa$ -deficient hemizygous littermates and wild-

<sup>1</sup> Abbreviation used in this paper: tk, thymidine kinase gene.



**Figure 1.** Targeted replacement of the  $J\kappa$  region with functional  $V\kappa$ - $J\kappa$  genes. Shown are the germline  $\kappa$  locus (a), replacement targeting vectors for  $V\kappa 4$ - $J\kappa 4$  and  $V\kappa 8$ - $J\kappa 5$  (b), and the resultant  $V\kappa 4$  replaced ( $V\kappa 4R$ ) or  $V\kappa 8$  replaced ( $V\kappa 8R$ )  $\kappa$  loci (c). Dashed lines denote the borders of homology between the targeting vector and the germline locus. Arrowheads indicate the positions of PCR primers used to screen neomycin-resistant embryonic stem (ES) cell colonies for homologous recombination events. The upstream  $\kappa$  DNA probe, PKP6 (3), is denoted by a cross-hatched bar. PKP6 was used in Southern analysis (d) to confirm the genotype of targeted ES cells. Shown are genomic DNA samples from untransfected ES cells (lane 1) and  $V\kappa 8R$  ES cells (lane 2) and tail DNA samples from the offspring of germline chimeras (lanes 3–6, offspring of a  $V\kappa 4R$  chimera; lanes 7–10, offspring of a  $V\kappa 8R$  chimera).  $PstI$  digestion yields a 7.0-kb fragment in the wild-type germline  $\kappa$  locus (a) and a 4.75-kb fragment in the  $V\kappa 4R$  or  $V\kappa 8R$  loci.

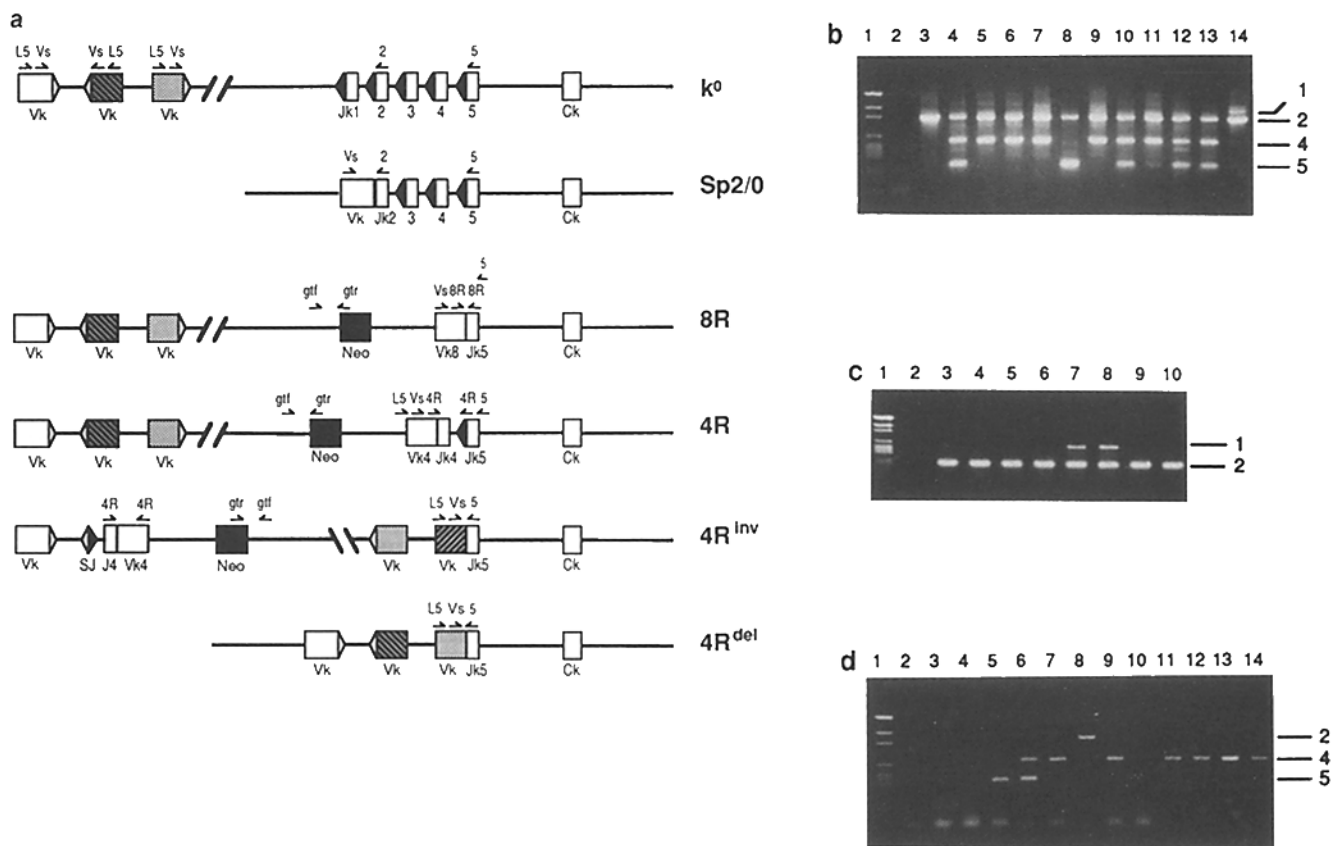
type mice (Luning Prak, E., R. R. Hardy, and M. Weigert, manuscript in preparation).

Germline transmission was achieved in a C57Bl6 (ES cell)/ICR chimera for  $V\kappa 4R$  and in an E14.1 (ES cell)/C57Bl6 chimera for  $V\kappa 8R$ . Offspring in which the replaced  $\kappa$  locus was present were identified by  $V\kappa 4$ - or  $V\kappa 8$ -specific PCR assays of tail DNA (data not shown). Transmission of  $V\kappa R$  was confirmed by Southern analysis (Fig. 1 d).  $PstI$  digestion of genomic DNA yields a 7.0-kb fragment in the wild-type germline  $\kappa$  locus and a 4.75-kb fragment in the  $V\kappa 4$  or  $V\kappa 8$  L chain replaced locus.

**Analysis of L Chain Genotypes in  $V\kappa R$  Hybridomas.** To study the effect of  $V\kappa 4R$  and  $V\kappa 8R$  on the rearrangement of other L chain genes, LPS hybridomas were prepared from  $\kappa$  hemizygous  $V\kappa 4R/\kappa^0$  and  $V\kappa 8R/\kappa^0$  mice. The rearrangement status of  $\kappa$  and  $\lambda$  genes in individual IgM-secreting lines was tested using a series of PCR assays. First, each clone was tested for the presence of  $V\kappa 4R$  or  $V\kappa 8R$  DNA by PCR. Next, additional  $\kappa$  rearrangements on the targeted allele and on the

wild-type  $\kappa$  allele (when they occurred) were identified using a series of PCR amplifications with forward  $V\kappa$  primers and reverse  $J\kappa$  primers (primer positions are shown in Fig. 2 a). The size of the amplification product in these assays is diagnostic of the  $J\kappa$  segment used in the rearrangement (Fig. 2 b–d). For example, using  $V_s$  and  $J\kappa 5$  primers (Fig. 2 b), rearrangement to  $J\kappa 1$  gives a 1.6-kb product, whereas  $J\kappa 2$  rearrangements are 1.2 kb,  $J\kappa 4$  are 600 bp, and  $J\kappa 5$  are 270 bp. Because  $J\kappa 1$  rearrangements are not always discernible by  $V_s + J\kappa 5$  PCR,  $V_s$  and  $J\kappa 2$  primers were used to verify  $J\kappa 1$  rearrangements (Fig. 2 c). The  $L5 + J\kappa 5$  PCR (Fig. 2 d) was used to type  $J\kappa 2$  rearrangements on the untargeted  $\kappa$  allele. (The  $V_s + J\kappa 5$  PCR cannot be used for this purpose because the  $J\kappa 2$  rearrangement of the fusion partner is amplified in all of the hybridomas).

The pattern of  $J\kappa$  segment usage revealed by these assays will in nearly all cases reveal the rearrangement status at each  $\kappa$  allele, yielding a  $\kappa$  genotype for each hybridoma ( $\kappa$  genotypes are shown in Fig. 2 a and all observed genotypes are



**Figure 2.** (a)  $\kappa$  locus genotypes and the positions of PCR primers.  $\kappa^0$  (germline configuration), Sp2/0 (the hybridoma fusion partner harbors a nonproductive V $\kappa$ -J $\kappa$ 2 rearrangement), 8R (V $\kappa$ 8 replacement), 4R (V $\kappa$ 4 replacement), 4R<sup>inv</sup> (rearrangement of a V $\kappa$  gene to J $\kappa$ 5, inverting V $\kappa$ 4R), and 4R<sup>del</sup> (rearrangement of a V $\kappa$  gene to J $\kappa$ 5, deleting V $\kappa$ 4R). The forward V $\kappa$  primers are Vs (Schlüssel degenerate V $\kappa$  primer, binds 80–90% of V $\kappa$  genes, reference 20), L5 (Huse V $\kappa$  primer, binds 50–60% of V $\kappa$  genes; reference 21 and Luning Prak, E., and M. Weigert, unpublished observations), 8R (V $\kappa$ 8R specific, reference 15), and 4R (V $\kappa$ 4R specific, reference 7). Not shown are additional V $\kappa$  primers (L3, L4, L6, and L7, see reference 21) used to confirm the R/0 genotype. The reverse J $\kappa$  primers are 5 (J $\kappa$ 5, reference 22), 2 (J $\kappa$ 2, reference 22), 4R (specific for V $\kappa$ 4R junction, reference 7), and 8R (spans the V $\kappa$ 8R CDR3-J $\kappa$ 5 junction, reference 15). Gene targeting primers (*gtf* and *gtr*) are described in Fig. 1. Not drawn to scale. (b) J $\kappa$  typing with Vs + J $\kappa$ 5 PCR primers. The size of the amplified product corresponds to the J $\kappa$  segment used in the rearrangement (see Materials and Methods). Rearrangement to J $\kappa$ 1 gives a 1.6-kb product, whereas J $\kappa$ 2 products are 1.2 kb, J $\kappa$ 4 products are 600 bp, and J $\kappa$ 5 products are 270 bp. The J $\kappa$ 2 rearrangement in the fusion partner is amplified in all of the hybridomas. Lane 1, pGEM molecular weight standards; lane 2, water control; lane 3, Sp2/0 DNA; and lanes 4–14, genomic DNA samples from V $\kappa$ 4R hybridomas. (c) Typing J $\kappa$ 1 rearrangements with Vs and J $\kappa$ 2 primers. Because J $\kappa$ 1 rearrangements are not always discernible by V $\kappa$  + J $\kappa$ 5 PCR (see Fig. 2 b), Vs and J $\kappa$ 2 primers were used to verify J $\kappa$ 1 rearrangements. Lane 1, pGEM molecular weight standards; lane 2, water control; lanes 3–10, V $\kappa$ 4R hybridoma DNA samples (these do not correspond to the V $\kappa$ 4R hybridoma samples in b). All hybridomas have a J $\kappa$ 2 band (190 bp) because of the Sp2/0 J $\kappa$ 2 rearrangement. Two clones (lanes 7 and 8) also have a J $\kappa$ 1 rearrangement (540 bp). (d) J $\kappa$  typing with L5 and J $\kappa$ 5 primers. The L5 + J $\kappa$ 5 PCR was used to type J $\kappa$ 2 rearrangements on the untargeted B cell  $\kappa$  allele. This assay relies on the fact that the L5 primer does not amplify the J $\kappa$ 2 rearrangement of Sp2/0. Lane contents: 1, pGEM molecular weight markers; 2, water control; lanes 3–15, DNA samples from V $\kappa$ 4R hybridomas (these do not correspond to the V $\kappa$ 4R samples shown in Fig. 2, b or c). Sizes of amplicons are 1.78 kb (J $\kappa$ 1), 1.38 kb (J $\kappa$ 2), 780 bp (J $\kappa$ 4), and 450 bp (J $\kappa$ 5).

listed and defined in Table 1). For example, V $\kappa$ 4R hybridomas with J $\kappa$ 2-, J $\kappa$ 4-, and J $\kappa$ 5-sized bands (Fig. 2 b, lanes 4, 10, and 13) have a J $\kappa$ 2 band from the fusion partner, a rearrangement to J $\kappa$ 4 on one allele, and a rearrangement to J $\kappa$ 5 on the other. These three clones type positive for V $\kappa$ 4R in the V $\kappa$ 4-specific PCR assay (data not shown). As the primers for the V $\kappa$ 4 assay amplify V $\kappa$ 4-J $\kappa$ 4 DNA (see Fig. 2 a), it is not certain that V $\kappa$ 4R is still in proximity to J $\kappa$ 5 and C $\kappa$ ; the genotype of these clones is therefore either R/5 (having V $\kappa$ 4R on one allele and a rearrangement to J $\kappa$ 5 on the other) or 4R<sup>inv</sup>/4 (inverting V $\kappa$ 4R by rearrangement to J $\kappa$ 5 on the

targeted allele and rearranging to J $\kappa$ 4 on the other  $\kappa$  allele). To distinguish R/5 from 4R<sup>inv</sup>/4 genotypes, amplifications were carried out with 4R and J $\kappa$ 5 primers (not shown). In all 18 V $\kappa$ 4R clones with J $\kappa$ 4 and J $\kappa$ 5 rearrangements, the genotype was R/5 (Table 1).

**Classes of Light Chain Gene Rearrangements.** Four classes of L chain genotypes were observed. The first class expresses only the V $\kappa$ R L chain (R) and has no additional  $\kappa$  rearrangements (R/0, Table 1). The R/0 genotype corresponds to the  $\kappa^+/\kappa^0$  genotype of a normal B cell. The 10 V $\kappa$ 4R clones (one of the 11 V $\kappa$ 4R R/0 clones is excluded because it has

**Table 1.**  $\kappa$  Rearrangements in  $V\kappa4R$  and  $V\kappa8R$  B Cells

J $\kappa$ genotype	$V\kappa4R$ -1	$V\kappa4R$ -2	$V\kappa4$ -total	$V\kappa8R$
R/0	4	7	11 (19)	16 (42)
$4R^{inv}/0$	6	0	6 (10)	NA
$4R^{inv}/2$	1	0	1 (2)	NA
$4R^{del}/0$	0	2	2 (3)	NA
$4R^{del}/4$	1	0	1 (2)	NA
del/0	0	0	0	1 (3)
del/1	0	2	2 (3)	0
del/2	0	1	1 (2)	0
del/4	1	0	1 (2)	0
del/5	0	0	0	1 (3)
Subtotal	9	5	14 (24)	2 (6)
R/1	5	3	8 (14)	9 (24)
R/2	3	4	7 (12)	5 (13)
R/4	*	*	*	4 (10)
R/5	9	9	18 (31)	2 (5)
Subtotal	17	16	33 (57)	20 (52)
Total	30	28	58 (100)	38 (100)

The numbers of  $V\kappa4R$  and  $V\kappa8R$  hybridomas with a particular  $\kappa$  genotype are listed. Results are also expressed as percentages (given in parentheses) of the total number of hybridomas in the combined  $V\kappa4R$  panels or in the  $V\kappa8R$  panel. Two hybridoma panels from different hemizygous  $V\kappa4R$  mice ( $V\kappa4R$ -1 and  $V\kappa4R$ -2) and one hybridoma panel from an hemizygous  $V\kappa8R$  mouse are shown. The genotypes refer to the J $\kappa$  segment used at each  $\kappa$  allele. The replaced allele is to the left of the slash mark. R/0 means that the  $V\kappa4$ -J $\kappa4$  or  $V\kappa8$ -J $\kappa5$  replacement is in proximity to C $\kappa$  on the targeted allele and that the other  $\kappa$  allele is in the germline configuration.  $4R^{inv}$  indicates that  $V\kappa4R$  has been inverted by the rearrangement of an upstream  $V\kappa$  segment to J $\kappa5$  (these clones are positive by  $V\kappa4$ -J $\kappa4$  and gene targeting PCR, but yield no J $\kappa4$ -sized bands with Vs and J $\kappa5$  primers, see Fig. 2).  $4R^{del}$  means a rearrangement to J $\kappa5$  has deleted the  $V\kappa4R$  (these clones type negative by  $V\kappa4$ -J $\kappa4$  and gene targeting PCR). Clones with the del/1, del/4, or del/5 have deleted the replaced  $\kappa$  locus and undergone rearrangements to J $\kappa1$ , J $\kappa4$ , or J $\kappa5$ , respectively, on the other allele. One clone, del/0, lacks  $V\kappa8R$  DNA and expresses a  $\kappa$  antibody, but the  $V\kappa$  gene is not amplified by any of the  $V\kappa$  primers (Vs, L5, L3, L4, L6, and L7). R/1 indicates that the replacement is in proximity to C $\kappa$  and that a  $V\kappa$ -J $\kappa1$  rearrangement is present on the untargeted  $\kappa$  allele. Only observed genotypes are listed. \* In the case of  $V\kappa4R$ , it was not possible to distinguish clones with an R/0 genotype from those with an R/4 genotype (see text). NA, not applicable.

a  $\lambda$  rearrangement, see Table 2) and the 16  $V\kappa8R$  clones represent an upper limit of the number of R/0 cells. Since the PCR assays do not distinguish the R/0 genotype from R/4 in  $V\kappa4R$ , some R/0 cells may be mistyped. However, rearrangements to J $\kappa4$  are ordinarily rare among splenic B cells (9, 24). In  $V\kappa8R$ , some R/5 cells may be mistyped as R/0 because de novo rearrangements to J $\kappa5$  can only be distinguished from  $V\kappa8$  to J $\kappa5$  with one  $V\kappa$  primer, L5 (see Fig. 2 a). However, as the L5 primer amplifies at least 50% of

murine  $V\kappa$  genes (Luning Prak, E., and M. Weigert, unpublished observations), mistyped R/5 cells are unlikely to account for more than  $\sim 4$  of the 16  $V\kappa8R$  R/0 cells (Table 1). The R/0 genotype could also be falsely assigned to hybridomas that had lost a rearranged L chain locus (other than  $V\kappa R$ ). However, loss of chromosome 6 in these hybridomas is infrequent because only one clone of 100 tested failed to secrete antibody (data not shown).

Cells in the second genotype class have rearranged the  $V\kappa R$  locus in two ways. One way deletes the  $V\kappa R$  locus (the del genotype in Table 1). This mechanism presumably involves rearrangement of upstream  $V\kappa$  genes to recombination signal sequences located downstream of C $\kappa$ . Rearrangements to these RS sequences account for the deletion of the  $\kappa$  locus frequently observed in  $\lambda$  B cells from mice and humans (1, 25–27). The other way in which class 2 cells rearrange involves the downstream J $\kappa5$  segment in  $V\kappa4R$ . Rearrangement to J $\kappa5$  results either in the loss of  $V\kappa4$ -J $\kappa4$  ( $4R^{del}$ , Fig. 2 a) or in its dissociation from J $\kappa5$  and C $\kappa$  ( $4R^{inv}$ , Fig. 2 a). These results are consistent with the described mechanisms of deletional and inversional recombination at the  $\kappa$  locus (3, 28).

Ongoing L chain rearrangement in  $V\kappa R$  B cells also yields the third class of B cells (having the R/1, R/2, R/4, or R/5 genotypes; Table 1). As in class 2, class-3 genotypes result from continued rearrangement in  $\kappa^+/\kappa^0$ . It is possible that the replaced L chain has undergone deleterious mutations and that the resultant  $\kappa^-/\kappa^0$  genotype was converted to a  $\kappa^-/\kappa^+$  genotype by further rearrangement. However, this would require a high mutation rate that is not ordinarily observed in LPS/IgM hybridomas. Sequence analysis of  $V\kappa4$ -J $\kappa4$  in an R/1 hybridoma reveals no mutations (data not shown). Assuming therefore that the  $V\kappa R$  locus remains functional at this stage of B cell development, additional rearrangements of the untargeted  $\kappa$  allele will result in a  $\kappa^+/\kappa^+$  or  $\kappa^+/\kappa^-$  genotype.

The fourth class of cells have  $\lambda$  rearrangements (Table 2).  $\lambda$  L chain rearrangements occurred in 4 of 58 ( $\sim 7\%$ )  $V\kappa4R$  hybridomas). Two  $V\kappa4R$  clones with  $\lambda$  rearrangements secrete  $\mu\lambda$  antibodies, consistent with productive  $\lambda$  rearrangements. The other two  $V\kappa4R$   $\lambda$  clones only appear to produce  $\mu\kappa$  antibodies, suggesting that their  $\lambda$  rearrangements were nonproductive (Table 2). Three of four clones with  $\lambda$  rearrangements also had evidence of additional  $\kappa$  rearrangements (Table 2). In contrast, no clone of 39  $V\kappa8R$  hybridomas had a  $\lambda1$ ,  $\lambda2$ , or  $\lambda X$  L chain rearrangement (data not shown). Furthermore, not one  $V\kappa8R$  hybridoma (of 51 surveyed) secreted  $\lambda$  L chains, suggesting that the frequency of  $\lambda$  B cells is reduced in  $V\kappa8R$ . However,  $V\kappa8R$  (and  $V\kappa4R$ ) animals do have  $\lambda$  antibodies in the serum (data not shown).

## Discussion

We interpret the four genotype classes as follows. The first class, R/0, is found in multiple clones in both  $V\kappa4R$  and  $V\kappa8R$  hybridomas. The presence of several clones with this genotype indicates that the L chain replacement constructs are expressible as normal L chains and are capable of inhibiting

**Table 2.** *VκR Hybridomas with Lambda Rearrangements*

Source	Productive L chain	Jκ genotype	Lambda genotype λ1/λ2/λX
Vκ4R-1	κ	4R <sup>inv</sup> /0	- /0/0
Vκ4R-1	λ	4R <sup>inv</sup> /0	0/0+
Vκ4R-1	κ	R/0	- /0/0
Vκ4R-2	λ	R/5	± / ± /0

Individual clones having λ DNA rearrangements are shown. The animal source of the clone corresponds to the panels shown in Table 1; 58 Vκ4R hybridomas and 51 Vκ8R hybridomas were screened for IgM κ and IgM λ expression by solid-phase enzyme-linked immunosorbent assay (described in ref. 9). Of the 58 IgM secreting Vκ4R hybridomas shown in Table 1, 56 produced only κ L chains, while two expressed only λ L chains. All 51 Vκ8R hybridomas (of which the 38 shown in Table 1 were chosen at random) secreted only κ L chains. Shown are the κ L chain genotypes (see Table 1 for nomenclature) and the λ genotypes (+, productive; -, nonproductive; ±, rearranged (could be productive or nonproductive); and 0, germline [unrearranged]). λ rearrangements were typed by λ1, λ2, and λX-specific PCR assays (see Materials and Methods).

further κ rearrangement. These results reaffirm the H/L-STOP model, recapitulating the observations in earlier studies of κ transgenics (2). Similarly, the low frequency of λ hybridomas in Vκ8R points to a role for the replaced L chain in shutting off λ rearrangement (Table 2). In the absence of an H/L-STOP signal, the VκR genotype would resemble that of a hemizygous κ-deficient B cell (kdel/wt). In kdel/wt, ~10% of B cells produce λ L chains (16, 29, and Luning Prak, E., and M. Weigert, unpublished observations). Therefore, even among Vκ4R B cells, the frequency of λ-expressing clones, 3%, is lower than would be expected if the replaced allele exerted no effect on further rearrangement.

Not predicted by the H/L-STOP model is the majority of cells that are found in the second and third genotype classes. These cells represent a complete departure from the H/L-STOP model and indicate that the H/L-STOP signal is often delayed or never activated. Class 2 VκR cells provide the first direct demonstration of secondary rearrangement at a productively rearranged locus within individual B cells. The VκR model also establishes that, given downstream RS sequences, a productively rearranged κ locus can serve as a substrate for locus deletion.

An outcome of secondary rearrangement at a productively rearranged locus is revision of the antigen receptor. This process of receptor editing may allow autoreactive cells to escape clonal elimination (5–7). Receptor editing has been inferred from the increased frequencies of distal Jκ rearrangements in autoantibody transgenics (6, 9, 30). Whereas most splenic B cells in normal animals have rearrangements to Jκ1 or to Jκ2 (31, 32), splenic B cells from mice with an anti-DNA-H chain transgene (3H9) exhibit a skewing toward Jκ5 rearrangement (6). Secondary rearrangements to Jκ5 were also demonstrated in 3H9 transgenics that were hemizygous for

κ L chain deficiency ( $H^+/\kappa^{\text{del}}/\kappa^0$ ), but again, the precursor-product relationship remained unverified (9).

Receptor editing has also been described in anti-MHC class 1 transgenics (5). Anti-H-2K<sup>k,b</sup> B cells are deleted in mice expressing the H-2K<sup>k</sup> or K<sup>b</sup> allele (5, 33). However, autoreactive B cells are present in the bone marrow, where they express elevated levels of recombinase gene products and actively rearrange λ (5). Edited B cells in the periphery have lost the anti-H-2K<sup>k,b</sup> specificity, suggesting that the editing process results not only in λ rearrangement, but also in the deletion of κ (reference 5; and Nemazee, D., personal communication). Editing by successive κ rearrangements or by κ deletion is illustrated in class 2 VκR B cells. Here, for the first time, the precursor-product relationship is known because all cells start out with a productive Vκ-Jκ rearrangement. Editing disables VκR and replaces it with a new L chain.

Ongoing L chain rearrangement in VκR B cells also yields the third class of B cells which have a  $\kappa^+/\kappa^+$  or  $\kappa^+/\kappa^-$  genotype. Regardless of which genotype occurs in class-3 cells, the H/L-STOP signal appears to have been switched off or modified. The H/L-STOP signal could be canceled if the VκR L chain and the H chain gave rise to an autoreactive receptor. However, in contrast to class 2 cells, editing in class 3 cells does not disable the autoreactive L chain. For a  $\kappa^+/\kappa^+$  cell to escape deletion, it has been proposed that the nonautoreactive L chain successfully completes against the autoreactive L chain for pairing with the H chain (7). Alternatively, the H/L-STOP signal may be modified because the VκR L chain pairs poorly with the H chain (7). According to this model, poor pairing between H and L chains results in the production of too few receptors to effect an H/L-STOP signal. Therefore, rearrangement continues until an adequate level of H/L pairs is reached or until a new L chain that can efficiently generate the H/L-STOP signal is formed. Here, the quality of an H/L pair, rather than its specificity for self or nonself antigens, is what drives further gene rearrangement. Such a corrective process may be mechanistically distinct from receptor editing.

The preponderance of class 2 and class 3 genotypes is surprising, given that these classes are not as common in splenic B cells from normal animals (1). This difference may result from a failure to recruit R/0 cells into the peripheral B cell pool in VκR mice. Protective selection of B cells may favor classes 2 and 3, because these cells comprise a more diverse set of antigen receptors than do R/0 cells. In contrast, the  $\kappa^+/\kappa^0$  genotype in normal animals does not impart a substantially different diversity than the other genotypes. A second possibility is that the prevalence of class 2 and class 3 cells is the consequence of having a functional L chain rearrangement present at the inception of κ rearrangement. For example, prematurely rearranged L chain may confer transcriptional competence (and perhaps availability to recombinase) to the locus. If L chain rearrangements initially proceed in the absence of an H/L-STOP signal, then rearrangements during this early period would take place without regard for the functional status of VκR. Eventually the H/L-STOP signal is activated and further L chain rearrangements (such as the later rearrangements to λ in class 4 cells) are inhibited.

Yet, it seems improbable that all class 2 and class 3 cells represent the outcome of "uncensored" rearrangement or the failure to positively select R/0 cells because the frequency of R/0 cells is different in V $\kappa$ 4R (19%) and V $\kappa$ 8R (42%). Also, neither explanation can account for the frequent distal J $\kappa$  rearrangements in V $\kappa$ 4R class 3 cells (Table 1). Rather, these differences in V $\kappa$ 4R and V $\kappa$ 8R may reflect an intrinsic difference in the nature of the L chains and to the degree they are subjected to editing. In this regard, mutated forms of V $\kappa$ 4 are frequent among autoantibodies from autoimmune mice (34), and here V $\kappa$ 4 appears often to be edited by genetic replacement or the operational equivalent, phenotypic replacement by L chain competition (7). Consistent with editing in V $\kappa$ 4R, 4 of 16 R/5 clones harbor J $\kappa$ 1 or J $\kappa$ 2 rearrangements, indicating that multiple rearrangements took place on the untargeted allele (data not shown). V $\kappa$ 8, on the other

hand, is unmutated and is used in the response of normal mice to influenza (35). Most V $\kappa$ 8R class 3 cells have proximal J $\kappa$  rearrangements on the untargeted  $\kappa$  allele (see Table 1).

The V $\kappa$ R models illustrate two vital aspects of the immune system. Mechanisms such as H/L-STOP have evolved to fix important specificities. However, these mechanisms appear to be reversible, reflecting the dynamic nature of the immune response. Somatic mutation during clonal expansion (and in the same sense, embellishment of the inherited antibody repertoire by junctional diversity) is an ongoing source of diversity (36, 37). Such variety inevitably includes dysfunctional antibodies such as autoantibodies or nonfunctional antibodies. In these cases, H/L-STOP is reversed or modified, allowing revision of deleterious or nonfunctional mutants.

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Address correspondence to Dr. Martin Weigert, Department of Molecular Biology, Princeton University, Princeton, NJ 08544.

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