B cell development in mice that lack one or both immunoglobulin κ light chain genes

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We have generated mice that lack the ability to produce immunoglobulin (Ig) κ light chains by targeted deletion of Jκ and Cκ gene segments and the intervening sequences in mouse embryonic stem cells. In wild type mice, ~95% of B cells express κ light chains and only ~5% express λ light chains. Mice heterozygous for the JκCκ deletion have approximately 2-fold more λ^{+} B cells than wild-type littermates. Compared with normal mice, homozygous mutants for the JκCκ deletion have about half the number of B cells in both the newly generated and the peripheral B cell compartments, and all of these B cells express λ light chains in their Ig. Therefore, homozygous mutant mice appear to produce λ-expressing cells at nearly 10 times the rate observed in normal mice. These findings demonstrate that κ gene assembly and/or expression is not a prerequisite for λ gene assembly and expression. Furthermore, there is no detectable rearrangement of 3' κ RS sequences in λ^{+} B cells of the homozygous mutant mice, thus rearrangements of these sequences, per se, is not required for λ light chain gene assembly. We discuss these findings in the context of their implications for the control of Ig light chain gene rearrangement and potential applications of the mutant animals.
Key words: B cell development/gene rearrangement/gene targeting/κ-deficient mice/RS rearrangement

Introduction

Antigen-independent B cell differentiation involves a series of gene rearrangements by which functional immunoglobulin (Ig) variable region genes are assembled from germline V, (D) and J gene segments. The process generally is initiated at the Ig heavy (H) chain locus, first by rearrangement of a D_H to a J_H segment, followed by joining of a V_H gene segment to the $D_H J_H$ complex (reviewed by Blackwell and Alt, 1989). A variety of studies indicate that a productive H chain gene rearrangement signals the onset of Ig light (L) chain gene assembly (reviewed by Blackwell and Alt, 1989; Rolink and Melchers, 1991). There are two Ig L chain

isotypes encoded by separate gene families, kappa (κ) and lambda (λ). However, individual B cell progenitors functionally rearrange and express only one L chain gene (Alt et al., 1980) to generate mature B cells which are either κ[+] or λ[+] (isotype exclusion, Bernier and Cebra, 1964). In mice, the κ L chain gene family is substantially more complex than the λ gene family (Zachau, 1989; Selsing et al., 1989). Correspondingly, there is a considerable bias towards the expression of the κ isotype in mice, such that ~95% of B cells are κ[+], and only ~5% are λ[+] (Hood et al., 1969; McIntire and Rouse, 1970; McGuire and Vitetta, 1981).

The mechanism by which L chain gene assembly is regulated so as to generate the observed differences in κ versus λ chain gene utilization remains to be elucidated (Blackwell and Alt, 1988). The finding that κ L chain genes are often non-productively rearranged in λ producers but not vice versa suggested that κ genes generally are rearranged before λ genes either due to a preferred order in which the two loci are rearranged or due to a higher probability of κ versus λ rearrangement (Alt et al., 1980). Over the years, various data have been interpreted to support either the 'ordered' or 'stochastic' models (Coleclough et al., 1981; Hieter et al., 1981; Korsmeyer et al., 1982; Lewis et al., 1982; Berg et al., 1990; Nadel et al., 1990; Felsher et al., 1991; Ramsden and Wu, 1991); however, this issue has not been unequivocally resolved.

Both murine and human λ-producing B cell lines and hybridomas frequently have the κ loci deleted by rearrangement of either a Vκ gene segment or sequences adjacent to a partial VDJ recombination recognition sequence (heptamer) within the Jκ−Cκ intron, to a recognition sequence (RS) located ~25 kb 3' of the Cκ exon (referred to as the 3' κ RS element or κDE; Durdik et al., 1984; Moore et al., 1985; Siminovitch et al., 1985; Klobeck and Zachau, 1986; Muller et al., 1990). It has been postulated that 3' κ RS rearrangement may regulate λ gene assembly either by activating a gene required for λ gene assembly or by deletion of a gene sequence that inhibits λ gene assembly (Moore et al., 1985; Muller and Reth, 1988). However, the precise relationship and function of the 3' κ RS rearrangements with respect to the initiation of λ gene assembly remains to be determined.

The elucidation of mechanisms that regulate Ig L chain gene assembly and expression have been facilitated through in vivo analyses of L chain transgenics (reviewed by Storb, 1989). The strength of the transgenic system lies in its potential for assessing the impact of defined DNA sequences on the development and function of the immune system; however, these analyses can be complicated considerably by expression of the endogenous L chain genes (Blackwell and Alt, 1989; Storb, 1989). To provide a better in vivo model system for studying the roles of the κ and λ L chains in B cell development and to evaluate the potential relationship between rearrangement and/or expression of Ig κ genes
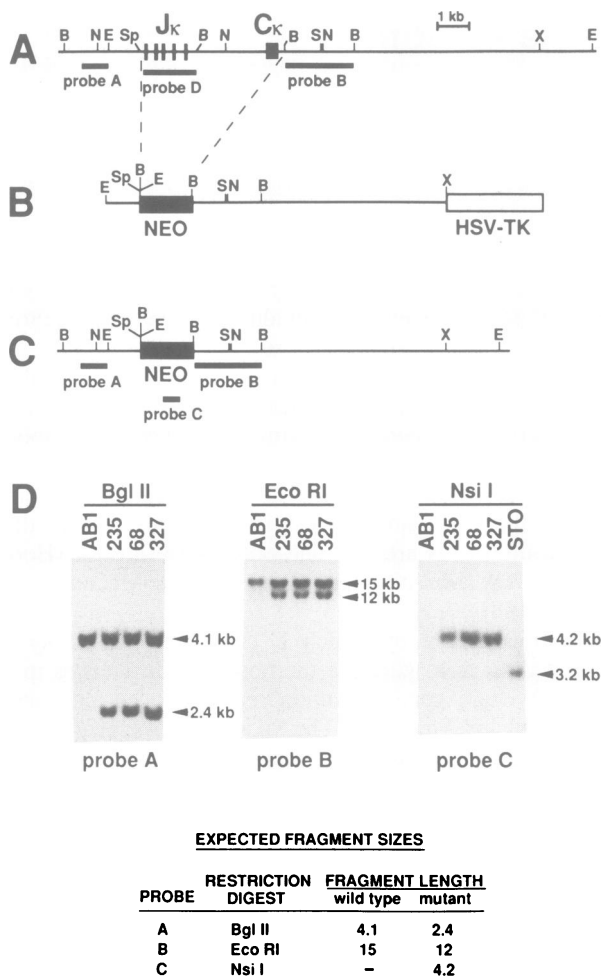
Fig. 1. Targeted inactivation of the x gene by JxCx deletion. (A) Genomic structure and partial restriction map of the mouse Jx and Cx regions. The closed boxes represent exons; the restriction sites are: B, BglII; N, NsiI; E, EcoRI; Sp, SphI; S, SacI; and X, XhoI. Probe A is a 0.8 kb XbaI−EcoRI fragment 5' of Jx, probe D is a 1.7 kb HindIII−XbaI fragment spanning the Jx segments, and probe B is a 2.3 kb BglII fragment 3' of Cx (B) Design of the x targeting construct. x sequences between the SphI site 5' of Jx and the BglII site 3' of Cx were replaced with the PGK-neo expression cassette, and the HSV-tk gene was added to the XhoI site ~8 kb 3' of Cx (see Materials and methods). The construct was excised from plasmid sequences prior to electroporation. (C) The predicted structure of the x locus following homologous recombination with the targeting construct. Probe C is an ~0.6 kb PstI neo gene fragment from pKJ1. (D) Southern blot analysis of targeted ES cell clones. Ten micrograms of DNA from clones 235, 68 and 327 and from control AB1 ES cells was digested with BglII, EcoRI and NsiI and hybridized with probes A, B and C, respectively. DNA from STO feeder cells was also included in the NsiI digest as a control for the faintly hybridizing neo fragment observed in the DNA samples from the targeted clones. The expected sizes of the hybridizing restriction fragments are shown below.

with respect to Ig λ gene rearrangement, we have generated mice in which the x locus has been inactivated by gene targeting in embryonic stem (ES) cells. We describe here the generation of mice heterozygous and homozygous for this mutation and the initial characterization of B cell development in these mutant animals.

Results

Targeting of the Igx locus in ES cells

Inactivation of the Ig x locus was accomplished by gene targeting with a replacement vector (Thomas and Capecchi,

1987) designed to delete all five Jx segments, the x intron enhancer and the Cx exon, following homologous recombination with the genomic locus. The targeting construct contains 9.2 kb of genomic x DNA flanking a 4.5 kb deletion spanning the SphI site ~100 bp 5' of Jx1 and the BglII site ~200 bp 3' of Cx (Figure 1A and B). The deleted x sequences have been replaced by the neo gene under the transcriptional control of the phosphoglycerate kinase-1 (PGK-1) promoter. The herpes simplex virus thymidine kinase (HSV-tk) gene, also driven by the PGK-1 promoter, was added to the 3' end of the targeting vector to allow selection against random integration events (Mansour et al., 1988).

The linearized vector was electroporated into AB-1 ES cells (McMahon and Bradley, 1990) and colonies were selected with G418 and FIAU. From 3.4 × 10⁷ electroporated cells a total of 435 G418 and FIAU resistant colonies were generated, representing an ~6-fold reduction in the number of colonies seen with G418 selection alone. Genomic DNA was prepared from 358 colonies and screened for homologous recombinants by Southern blot hybridization of BglII-digested DNA with a probe flanking the 5' junction of recombination (probe A in Figure 1A and C). Four colonies showing the predicted 2.4 kb targeted BglII fragment, in addition to the 4.1 kb wild type BglII fragment, were identified (three of the positive clones are shown in Figure 1D), indicating a homologous recombination frequency of ~1/90 G418^r and FIAU^r clones. Three of these clones, numbered 68, 235 and 327, were analyzed in detail to verify homologous recombination at the x locus. Hybridization of EcoRI-digested DNA with a probe 3' of Cx (probe B in Figure 1A and C) generated the predicted 15 kb wild type and 12 kb targeted fragments in all three clones; no additional fragments were seen (Figure 1D). Hybridization of NsiI digested DNA with a neo probe (probe C in Figure 1C) showed the predicted 4.2 kb fragment (Figure 1D) diagnostic of a targeting event. The additional faintly hybridizing fragment of ~3.2 kb in the targeted clones results from hybridization of probe C to the neo gene in the STO feeder cells on which the ES cells were cultured (Figure 1D). These results demonstrate the presence of a single homologously integrated copy of the x targeting vector in all three clones.

Generation of mice bearing the JxCx deletion

Clones 68, 235 and 327 were injected into C57BL/6J blastocysts to generate chimeras. Of a total of 63 pups born, ~50% were chimeric as judged by the extent of agouti coat coloration; and, of this number, the majority (24/30) were males showing significant agouti contribution (~50% or greater coat chimerism). Male chimeras were bred to C57BL/6J females to derive germline transmission of the targeted x locus. The majority of chimeras from clones 68 and 235 fathered agouti offspring, whereas only one of seven chimeras from clone 327 yielded a single agouti pup. Southern blot analysis of tail DNA from the agouti offspring identified ~50% of the animals as heterozygous for the JxCx deletion. Heterozygotes derived from clone 235 were bred together to generate homozygous mutants and the offspring of these matings were genotyped by Southern blot hybridization of BglII digested tail DNA with probe A. Homozygous mutants, heterozygotes and wild type offspring were generated with the expected frequency of 1:2:1 (Figure

A

probe A

B
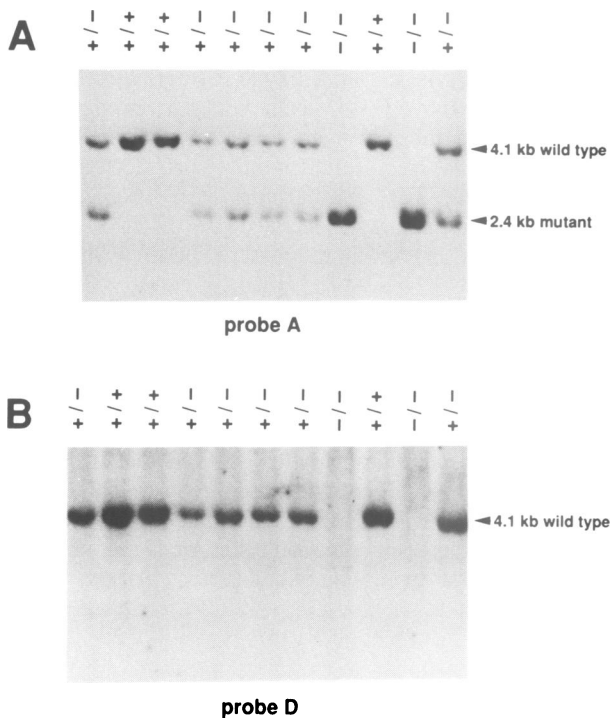
probe D

Fig. 2. Southern blot analysis of tail DNA. Tail DNA was isolated from a litter of pups derived from the mating of parents heterozygous for the JκCκ deletion. (A) Ten micrograms of DNA was digested with BglII and hybridized with probe A which flanks the 5' end of the targeting construct (Figure 1). The 4.1 kb band represents the wild type κ locus and the 2.4 kb band the targeted locus. Two of the 11 mice in the litter show only the 2.4 kb mutant band, indicating that they are homozyygous for the JκCκ deletion. (B) The filter was stripped and hybridized with probe D which spans the Jκ gene segments (Figure 1). The absence of hybridizing sequences in DNA from homozygous mutant mice verifies the deletion of these sequences by the targeting event.

2A; a representative litter is shown). To verify the deletion of κ sequences from the targeted locus, we stripped the probe A hybridized blot and reprobed it with a Jκ probe (probe D in Figure 1A). No hybridization of the Jκ probe was detected to DNA from homozygous mutant mice whereas the predicted 4.1 kb wild type fragment was observed in both heterozygous and wild type littermates (Figure 2B). In addition, male chimeras derived from clone 235 that gave germline transmission were also bred to 129/Sv females to generate a 129 congenic κ-deficient strain.

B cells in homozygous mutant mice are exclusively λ^+

The effect of the JκCκ deletion on the development and phenotype of B cells was analyzed by flow cytometry, using cells from wild type (+/+) and heterozygous (+/−) littermates as controls for homozygous mutant (−/−) mice. The majority of the splenic B cells from wild type and heterozygous mice expressed κ light chains (Figure 3A); cells expressing λ light chains comprised only a minority of the B cell population (Figure 3B). In contrast, no κ$^+$ B cells were detected in the spleens of mice homozygous for the JκCκ deletion (Figure 3A); instead all of the splenic B cells expressed λ light chains (Figure 3B). Similar results were obtained upon analysis of B cells from lymph node, bone marrow, peripheral blood and peritoneum in a total of 12 homozygous mutant mice ranging in age from 11 days to

4 months (data not shown). Furthermore, the serum of homozygous mutants lacked anti-κ reactivity but was reactive to anti-λ by ELISA assay (data not shown).

The B cells in the homozygous mutant mice appeared to be normal in their surface phenotype and tissue distribution. The staining profiles for IgM, IgD, CD45R (the B lineage-specific B220 surface marker) and CD23 (Figure 3C and E, and data not shown) were idential to that of control littermates; and, as was the case in wild type littermates, most of the λ$^+$ B cells in homozygous mutants were of the λ_1 isotype (data not shown). Staining of cells for the CD5 (Ly-1) surface antigen, the expression of which is characteristic of a distinct subset of B cells found primarily in the peritoneum, showed an identical tissue distribution of CD5$^+$ B cells in κ-deficient and control mice. Almost all of the B cells from the spleen, lymph node and peripheral blood of κ-deficient mice were conventional (CD5$^-$) B cells (data not shown), while CD5$^+$ B cells resided predominantly in the peritoneum (Figure 3D). In addition, the CD5$^+$ B cells in κ-deficient mice had the typical Ig^{bright}, IgD^{dull} and B220^{dull} phenotype characteristic of this B cell subset (data not shown; reviewed by Hardy, 1992). Approximately 10% of B cells in the spleens of homozygous mutants were very bright for both IgM and λ staining compared with splenic B cells from wild type and heterozygous mice (Figure 3B). These B cells were also duller for CD23-staining and were larger than other B cells (data not shown), suggesting that they were activated (Waldschmidt et al., 1988); however, the levels of serum Ig were comparable in homozygous mutants and control littermates (data not shown).

T cell development and surface phenotype were not grossly affected by the JκCκ deletion. Flow cytometry analysis of thymocytes and T cells from spleen and lymph nodes of homozygous mutant mice revealed that the distribution and expression patterns of CD3, CD4, CD8 and CD5 surface markers were idential to those of wild type and heterozygous littermates (data not shown).

Mice heterozygous for the JκCκ deletion have more λ^+ B cells

Mice heterozygous for the JκCκ deletion had approximately twice as many λ$^+$ B cells in spleens and bone marrow as did their wild type littermates (Figure 3B and data not shown). This increase was observed in all 14 heterozygous mice analyzed, ranging in age from 11 days to 6 months (Figure 4). To rule out the possibility that the increased number of λ$^+$ B cells was simply a consequence of the C57BL/6J × 129/Sv cross (chimeras derived from ES cells of 129/Sv origin were mated with C57BL/6J females to derive germline transmission of the targeted κ locus), the 129 strain congenic heterozygotes were also analyzed and similarly found to have about twice as many λ$^+$ B cells as their wild type littermates (data not shown). In addition, CD5$^+$ B cells as well as conventional B cells in the peritoneum of heterozygous mice also showed an approximate doubling in λ L chain usage compared with wild type littermates (data not shown). Since both wild type and heterozygous mice had similar total numbers of B cells in their respective lymphoid organs (Figure 5 and data not shown), this increase represents an increase in the absolute numbers of λ$^+$ B cells, indicating an apparent gene dosage effect of the JκCκ deletion on λ chain expression.

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## Alternations in pre-B and B cell numbers in x-deficient mice

Although the B cells of the homozygous mutant mice were phenotypically normal, they generally had about half as many (on average) B cells in the various lymphoid tissues (Figure 3C–E); independent of the age of the mice (Figure 5, and data not shown). Analysis of peritoneum washout cells showed that while there were fewer conventional B cells in x-deficient mice, the numbers of CD5+ B cells appeared unaffected (Figure 3D and data not shown). Therefore, the deletion of JxCx appears to reduce preferentially the number of conventional B cells without affecting the CD5+ B cell population.

During B cell differentiation Ig L chain gene rearrangement generally is initiated after a functional H chain gene rearrangement has taken place. The effect of the JxCx deletion on pre-B cell differentiation was assessed by flow cytometry analysis of bone marrow cells. Mutant and control mice had approximately the same number of B-lineage (B220+) cells in the bone marrow; however, there tended

to be a slight (~20% on average) increase in the number of B cell precursors (B220+IgM−) in homozygous mutants relative to wild type and heterozygous littermates (Figure 3E and data not shown). Consistent with our findings in the periphery, the number of mature (B220+IgM+) B cells in the bone marrow of x-deficient mice also decreased by ~2-fold (Figure 3E). Furthermore, this decrease was observed both in the newly generated (B220^{dull}IgM+) B cell fraction (Figure 3E) and in the mature (B220^{bright}IgM+) B cell fraction.

The B cell precursor population defined by the B220+IgM− phenotype consists of large cells representing pro-B and early pre-B cells, as well as small, resting pre-B cells which are the direct precursors of IgM+ B cells (Coffman and Weissman, 1983). A size analysis of the B220+IgM− precursor population showed that the slight increase in pre-B cell number observed in many mice appeared to represent cells of the small size fraction (Figure 3F). To define this cell population better, bone marrow cells were analyzed for expression of cell surface markers CD43
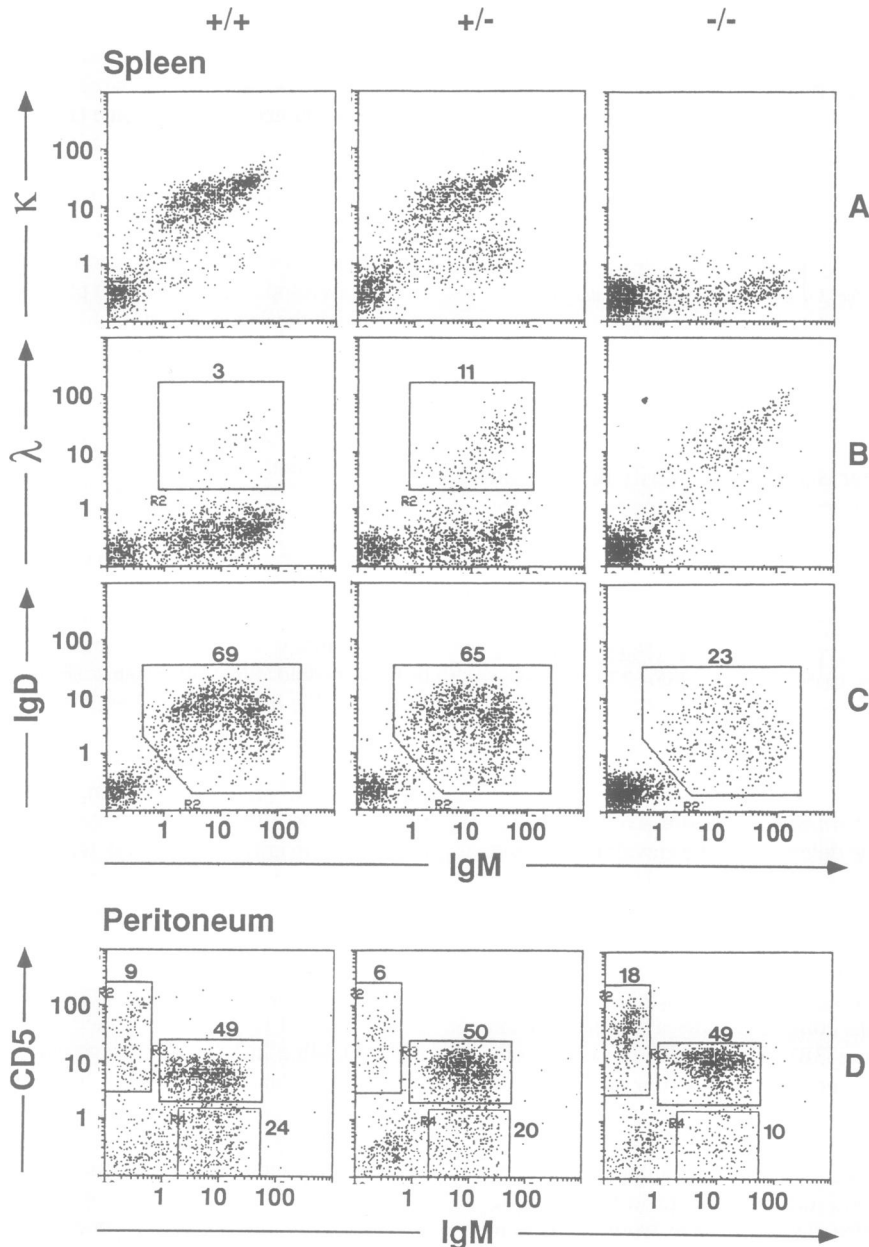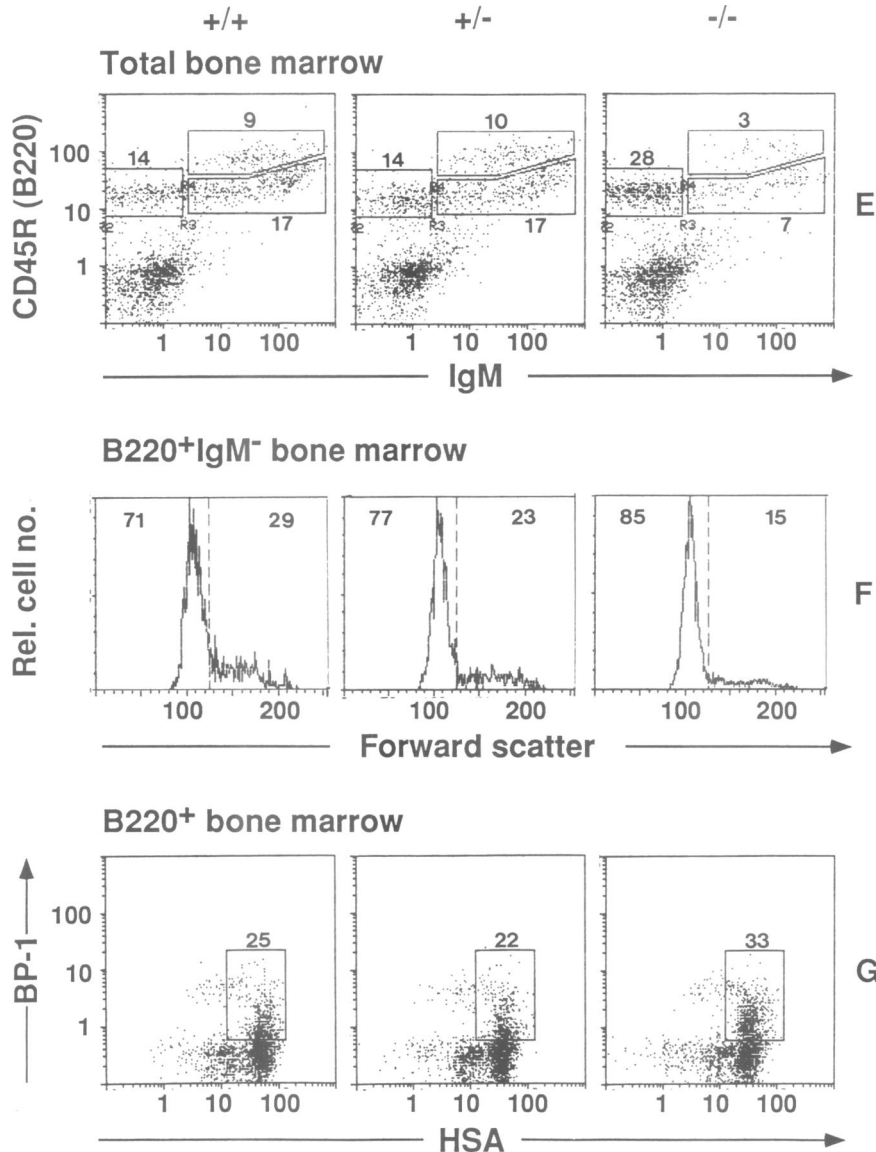
Fig. 3. Flow cytometry analysis of cells from spleen, peritoneum washout and bone marrow of homozygous mutants ($-/-$), heterozygous mutants ($+/-$), and wild type ($+/+$) littermates. Spleen cells (**A, B** and **C**) from 31-day-old mice were stained with the following combinations: ^{fl}anti-x, ^{PE}anti-λ and ^{bi}anti-IgM; or ^{fl}anti-IgD, ^{PE}anti-λ and ^{bi}anti-IgM. Peritoneum washout cells (**D**) from 31 day-old mice were stained with ^{fl}anti-IgM and ^{PE}anti-CD5. Bone marrow cells from 27 day-old mice (**E, F** and **G**) were stained with following combinations: ^{fl}anti-IgM and ^{PE}anti-CD45R (B220); ^{fl}anti-BP-1, ^{PE}anti-CD45R (B220) and ^{bi}anti-IgM. or ^{fl}anti-BP-1, ^{PE}anti-CD45R (B220) and ^{bi}anti-HSA (heat stable antigen). Cells in the lymphocyte gate, as defined by light scatter (Forster *et al.*, 1989), were analyzed and results are presented as a two-dimensional dot plot in which each dot represents an individual cell. The numbers indicate the percentage of cells staining for a particular phenotype in the various boxed regions. Cell populations reanalyzed for cell size by forward scatter are displayed as histograms (**F**) in an arbitrary scale of channel numbers. At least eight mice of each genotype were analyzed for each staining; the plots shown each represent staining of a single mouse. The IgM$^+$, x^{dull} stained cell population in heterozygous spleen (panel A) are λ$^+$ B cells, as suggested by a x versus λ two-dimensional plot (data not shown). These λ$^+$ B cells are stained by anti-x antibody probably because they have absorbed serum Ig(x) molecules through Fc receptors *in vivo*; note that λ$^+$ B cells in x-deficient mice, which do not make any Ig(x) molecules, do not stain for x.

(S7), HSA (heat stable antigen) and BP-1, which can be used to correlate pre-B cell surface phenotypes with the status of Ig gene rearrangements (Hardy *et al.*, 1991). In four separate homozygous mutant mice, we observed a significant ($\sim40\%$) increase in the percentage of pre-B cells of the CD43$^-$HSA$^+$BP-1$^+$ fraction (Figure 3G; and data not shown); these cells represent pre-B cells that have undergone a functional H chain gene rearrangement and are in the process of L chain gene assembly (Hardy *et al.*, 1991). A size analysis of this precursors subset again showed that the increase in cell number generally represented small cells: 60% of the HSA$^+$BP-1$^+$ cells in homozygous mutants were

small, whereas only 40% were small in control littermates (data not shown). Therefore, mice homozygous for the JxCx deletion tend to have slightly more pre-B cells at the L chain gene rearrangement stage.

3′ x RS rearrangement in λ$^+$ B cells from x-deficient mice

Murine λ-producing B cell lines and hybridomas have frequently deleted the x loci by rearrangement of the 3′ x RS sequence (Durdik *et al.*, 1984; Moore *et al.*, 1985). At the time of this study, the status of 3′ x RS sequences in normal B cells was unknown. The x-deficient mice provide
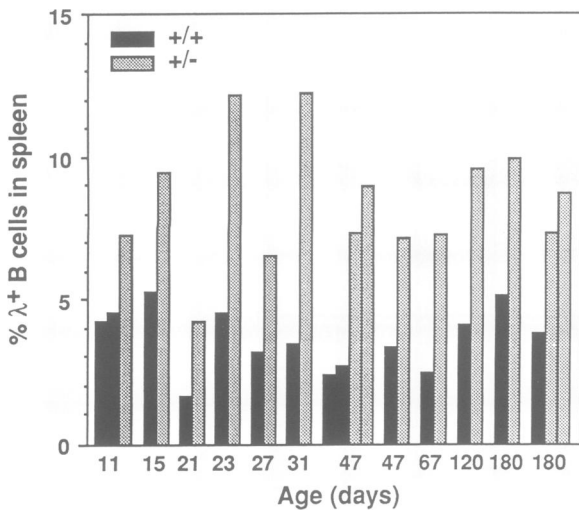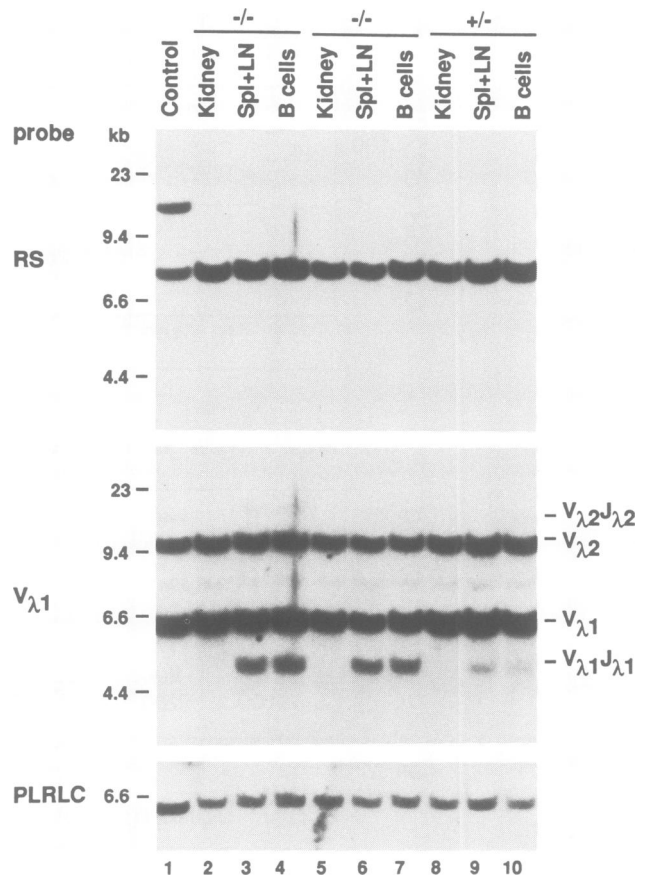
Fig. 4. Percentage of λ^+ B cells in the spleen of heterozygous mutant (+/−) and wild type (+/+) mice at various stages of ontogeny. Spleen cells were analyzed as in Figure 3. The percentage of B cells defined as λ^+, IgM^+ as in Figure 3B were compared in groups. Each group of mice represents littermates which were analyzed in the same experiment. Since wild type and heterozygous mice from the same litters have very similar numbers of B cells in the spleen, as shown in Figure 5, the approximate doubling in λ^+ B cells in heterozygous mice represents an increase in the absolute number of λ^+ B cells.
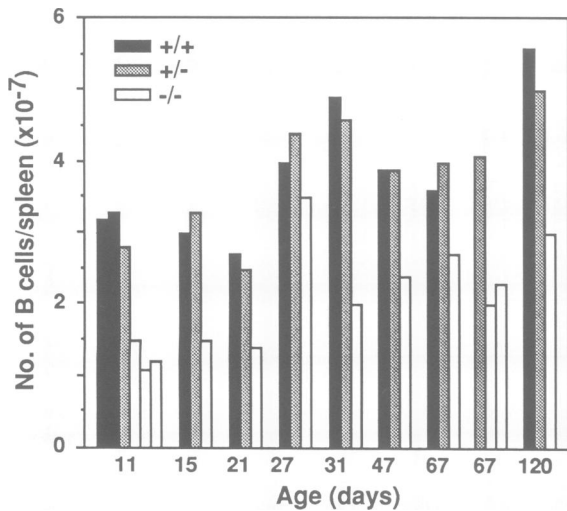
Fig. 5. Number of B cells in the spleen of homozygous mutant (−/−), heterozygous mutant (+/−), and wild type (+/+) mice at various stages of ontogeny. Spleen cells were stained for [fl]anti-IgD, [PE]anti-λ and [bi]anti-IgM. Each group of mice represents littermates which were analyzed in the same experiment. The total number of Ig^+ cells (or $B220^+$ cells, data not shown) in the spleen was obtained by multiplying the percentage of B cells by the number of nucleated cells.

Fig. 6. Southern blot analysis of 3′ $\varkappa$ RS rearrangement in DNA isolated from an enriched λ^+ B cell population from $\varkappa$-deficient mice. B cells from the spleen and lymph nodes of homozygous mutant mice were enriched to 75% purity as described in Materials and methods and DNA was isolated from the enriched λ^+ B cells, spleen and lymph node cell pools, and kidney. **(Top)** 10 μg of DNA was digested with *Bam*HI and hybridized with an RS probe, the 0.8 kb *Sau*3AI fragment 3′ of RS sequences (Durdik *et al.*, 1984), to assay for RS rearrangement. **(Middle)** The blot was stripped and rehybridized with the 0.9 kb *Xba*I−*Hin*dIII Vλ1 probe (Blackwell *et al.*, 1986) to assay for λ rearrangements. This Vλ1 probe hybridizes to both germline Vλ1 and Vλ2 as well as their rearrangement products (Hagman *et al.*, 1990; Nadel *et al.*, 1990). **(Bottom)** The filter was stripped again and hybridized with the PRLC-A cDNA probe (precursor lymphocyte-specific regulatory L chain, Oltz *et al.*, 1992) for standardizing the amount of DNA loaded in each lane. Lane 1, DNA isolated from pre-B cell line BM18-4.20.2.14 (kindly provided by Dr Eric Selsing, Tufts University) which has an RS rearrangement at one $\varkappa$ locus (Persiani *et al.*, 1987). Lanes 2, 5 and 8, DNA isolated from kidney. Lanes 3, 6 and 9, DNA isolated from spleen and lymph node cell pools. Lanes 4, 7 and 10, DNA isolated from the enriched λ^+ B cell population. −/− and +/− represent homozygous mutant and heterozygous mutant mice, respectively. Migration of the *Hin*dIII-digested λ DNA marker is indicated.

a rich source of λ^+ B cells allowing us to assay readily for the occurrence and frequency of 3′ $\varkappa$ RS rearrangement in this cell population. λ^+ B cells were enriched from spleen and lymph node of homozygous mutants by complement-mediated lysis of T cells. Southern analysis of DNA from a cell population in which 75% of the cells were λ^+ B cells failed to detect any apparent RS rearrangement, while hybridization of the same filter with a V$_{\lambda1}$ probe readily detected V$_{\lambda1}$−J$_{\lambda1}$ and some V$_{\lambda2}$−J$_{\lambda2}$ rearrangements (Figure 6). Since the J$\varkappa$C$\varkappa$ deletion also deleted the J$\varkappa$−C$\varkappa$ intron heptamer used in RS rearrangement, any RS rearrangement in the $\varkappa$-deficient mice would have to occur

between the V$\varkappa$ and RS sequences. RS rearrangement from multiple V$\varkappa$ gene segments would generate heterogeneous RS hybridizing DNA fragments which would probably be below the level of detection by Southern blot hybridization. However, such rearrangements would diminish the intensity of the germline RS hybridizing fragment to a degree commensurate with the extent of RS rearrangement. Quantification of the intensity of these bands in DNA samples from enriched λ^+ B cells failed to detect any diminution of intensity (data not shown). In addition, a PCR assay using a degenerate V$\varkappa$ oligonucleotide primer (Schlissel and

Baltimore, 1989) and a primer 3′ of the RS sequence could readily detect RS rearrangement in DNA from spleen of wild type mice, but not in DNA from the enriched λ^+ B cells of x-deficient mice (data not shown).

Discussion

Phenotype of JxCx mutant mice

In mice, ~95% of B cells express Ig x light chains, while only ~5% express λ L chains. We now describe the generation and initial characterization of mice that cannot produce Ig x chains as a result of germline deletion of the Jx and Cx gene segments and the intervening sequences. Mice homozygous for this deletion display a complete absence of x^+ B cells and the presence of a B cell compartment that exclusively expresses λ L chains. The absolute numbers of λ^+ B cells is approximately double in heterozygous mutants and ~10-fold in homozygous mutants over that of wild type mice. In addition to the λ^+ B cell phenotype, mice homozygous for the JxCx deletion have about half as many Ig^+ B cells in the bone marrow and periphery and a small (variable) increase in the number of B cell precursors in the bone marrow. All of these phenotypic changes are maintained throughout the life of the animals. In addition, an essentially identical phenotype was observed in two independent gene-targeted mutations that inactivated expression of the x locus (Zou et al., 1993); therefore, the observed changes reflect neither the precise mutation generated (see below) nor the mouse strain used.

The effects of JxCx deletion are quite distinct from those of other recently reported mutations that affect B cell development. For example, inactivation of the recombinase activation genes RAG 1 or 2 fully eliminates mature B cells due to an inability of B cell precursors to initiate Ig variable region gene assembly (Shinkai et al., 1992; Mombaerts et al., 1992); disruption of the membrane exons of the μ heavy chain completely blocks B cell development at the large pre-B cell stage (Kitamura et al., 1991); and inactivation of the λ_5 gene reduces the rate of B cell production due to a leaky block in differentiation at the large pre-B cell stage, such that B cells slowly accumulate in the periphery over time (Kitamura et al., 1992).

Reduction of B cell number in x-deficient mice

Newly generated B cells in the bone marrow are stringently selected for their Ig variable region gene expression. Self-reactive B cells are either deleted by cell death or inactivated through anergy (Goodnow et al., 1989; Hartley et al., 1991; Russell et al., 1991). B cells that survive migrate from the bone marrow to peripheral lymphoid organs such as spleen and lymph node. In the absence of antigen stimulation, these peripheral B cells do not divide, and undergo programmed cell death. Thus the total number of B cells in a normal mouse is maintained at a steady state determined by the rate of production and the rate of turnover (Osmond, 1991; Rolink and Melchers, 1991). Mice heterozygous for the JxCx deletion have twice as many λ^+ B cells although these animals should retain the ability to express a complete repertoire of x variable region genes from the intact allele. Furthermore, homozygous mutants produce ~10-fold more λ^+ B cells than wild type mice. These findings are consistent with the possibility that loss of a functional x allele leads to an increased generation of λ-producing cells in these animals (see below) and that there is no major selection for

or against the increased numbers of B cells that express λ chains in heterozygous mutants.

Mice homozygous for the JxCx deletion have a dramatic increase in λ-producing B cells but have about half as many total B cells in the bone marrow and periphery than do wild type mice. Such a decrease could be due to unknown factors that affect the selection or life-span of λ^+ B cells in the x-deficient background. However, given the apparent decrease in the number of newly generated B cells in the bone marrow of homozygous mutants, it seems likely that a significant factor contributing to the reduction in B cell number in x-deficient mice is a decreased overall rate of B cell production. In any case, there appear to be no mechanisms operating to restore the sizes of B cell pools in the mutants to wild type levels since the reduced numbers are maintained throughout life.

Control of Ig L chain gene rearrangement

Only one L chain isotype is expressed on the surface of a mature B cell (isotype exclusion, Bernier and Cebra, 1964); in the mouse the number of x^+ B cells exceeds that of λ^+ B cells by ~20-fold (i.e. 95% x^+ B cells and 5% λ^+ B cells; Hood et al., 1969; McIntire and Rouse, 1970; McGuire and Vitetta, 1981). In x-producing cells, λ genes are usually in the unrearranged germline configuration, whereas in λ-producing cells, x genes tend to be non-productively rearranged or deleted (Alt et al., 1980; Coleclough et al., 1981; Hieter et al., 1981; Durdik et al., 1984; Siminovitch et al., 1985). These observations suggested that non-functional x gene rearrangement generally precedes λ gene assembly during the differentiation of a λ-producing B cell. Two alternative general models were proposed to explain these findings: either there is a sequential mechanism that acts first at x and then at λ genes; or x and λ rearrangement are initiated simultaneously but there is a much greater probability of x than λ gene rearrangement which yields an apparent order (Alt et al., 1980). Importantly, both of these models rely on cessation of L chain gene rearrangement following the assembly and expression of a functional light chain gene to ensure allelic and isotype exclusion (Alt et al., 1980; Coleclough et al., 1981; Hieter, et al., 1981). Although our findings do not unequivocally prove or eliminate either of these models, they help to define further some of the outstanding questions.

The finding that most A-MuLV transformed pre-B cells tend to exclusively rearrange x genes without rearranging λ genes has provided the strongest support for a sequential mechanism of x versus λ rearrangement (Lewis et al., 1982; Muller and Reth, 1988). There are at least two distinct mechanisms that would lead to ordered L chain gene assembly. One is a program in which, following expression of H chain, the resultant pre-B cell can exclusively rearrange x genes during a given developmental window after which, in cells that fail to rearrange and express x genes, λ rearrangement is initiated ('strict ordered model'). In the context of this model, aberrant rearrangements (deletions) of x genes would play a major role in determining the proportion of cells that reach the λ rearranging stage. A second version of an ordered model argues that the probability of x rearrangement is high and that of λ is low (or non-existent) in newly generated pre-B cells; but if x rearrangements are unsuccessful, the probability of λ rearrangement increases ('relative ordered model'). In the context of this model, the rate of λ^+ B cell generation would also be determined by

the frequency of cells that harbor non-productive (or deleted) x loci from which elements that interfere with or inhibit λ rearrangement have been deleted (Moore *et al.*, 1985; Muller and Reth, 1988; see below).

The occasional rearrangement of λ in a x producer or the occasional presence of germline x loci in λ-producing cell lines (Coleclough *et al.*, 1981; Berg *et al.*, 1990; Nadel *et al.*, 1990; Felsher *et al.*, 1991) or splenic λ^+ B cells (Zou *et al.*, 1993) is consistent with a probabilicist (stochastic) model of x versus λ rearrangement. This model argues that the x and λ loci become accessible for rearrangement simultaneously in developing pre-B cells and that rearrangements at each locus occur independently of each other (i.e. the probability of making λ rearrangements should not increase in cells that have deleted both x loci). Therefore, the relative generation rate of x^+ versus λ^+ B cells depends solely on the probability of making productive rearrangements at the two L chain loci. Possible mechanisms for preferential x rearrangement in a stochastic model might be the limited numbers of substrate $V\lambda$ gene segments relative to Vx gene segments (Alt *et al.*, 1980; Coleclough *et al.*, 1981) and/or the possibility that λ RS sequences are not as effective as some x RS sequences (Ramsden and Wu, 1991).

In normal mice, the number of pre-B cells available for L chain gene rearrangement is maintained at a steady-state level (Rolink and Melchers, 1991; Osmond, 1991). The number of division cycles cells spend at the L chain gene rearrangement stage has been estimated to range from one to several (Crippen and Jones, 1989; Forster *et al.*, 1989; Park and Osmond, 1989; Rocha *et al.*, 1990). Mice heterozygous for the $JxCx$ deletion have a pre-B pool size similar to that of wild type despite an apparent 2-fold increase in production of λ^+ B cells. Mice homozygous for the $JxCx$ deletion have an only slightly expanded pre-B pool size despite a 10-fold increase in λ^+ B cell production (assuming the steady-state level of newly generated B cells reflects the B cell production rate). Thus, mutant mice appear to generate many more λ^+ B cells from a pre-B pool similar in size to that of wild type. This finding can be explained by the relative ordered model which predicts that the $JxCx$ deletion should increase the probability of cells undergoing λ rearrangement. The finding could also be explained by a strict ordered model if the $JxCx$ deletion increased the number of pre-B cells available for λ gene rearrangement. However, to explain the conservation of pre-B pool size in mutant mice in the context of this model, one would have to argue further that the transition from x to λ rearrangement normally occurs with little or no cell division. A purely stochastic model argues that the $JxCx$ deletion should not influence the rate of λ^+ B cell generation from precursor cells; according to this model, increased λ^+ B cell production should require a corresponding increase in λ-progenitor numbers. Therefore, this model, at least in its simplest form, is not easily accommodated by our finding of increased λ^+ B cell production from a conserved pre-B pool size in mutant animals.

Role of 3' x RS rearrangement

Murine λ-producing B cell lines and hybridomas frequently have the x loci deleted by 3' RS rearrangement (Durdik *et al.*, 1984; Moore *et al.*, 1985). Likewise, most but not all normal λ-producing B cells have undergone 3' x RS

rearrangement (Zou *et al.*, 1993). We do not detect 3' x RS rearrangements in the peripheral λ^+ B cells from mice homozygous for the $JxCx$ deletion. This finding clearly demonstrates that rearrangement of the 3' x RS sequence *per se* is not a prerequisite for efficient λ gene assembly. Moreover, reduced levels of 3' RS rearrangement were found in λ^+ B cells from mice in which only the Cx-encoding sequences were deleted, and no rearrangements of the 3' x RS sequences were found in λ^+ B cells from mice in which the intronic x enhancer was deleted (Zou *et al.*, 1993). A possible interpretation of these results is that there is no relationship between 3' x RS rearrangement and λ variable region gene assembly. However, it seems more likely that the various targeted germline deletions somehow serve the same function as normal 3' x RS rearrangements in facilitating λ gene assembly.

Rearrangements to the 3' x RS usually involve either a Vx gene segment or a sequence adjacent to a heptamer within the $Jx-Cx$ intron (Durdik *et al.*, 1984; Siminovitch *et al.*, 1985). The common DNA region deleted in such rearrangements extends from the intronic hepatmer to the 3' x RS sequence 25 kb downstream of Cx, and includes intronic Ex, Cx and 3' Ex (Muller *et al.*, 1990). In the context of either ordered or stochastic models for L chain gene rearrangement, the various germline x deletions could simply serve to increase the number of pre-B cells available for λ rearrangement. Likewise, it is possible that germline deletion of the $JxCx$ region removes or inactivates putative DNA sequences that interfere with efficient assembly of λ variable region genes. Such potential negative elements might include sequences that exclude λ loci from entry into a rearrangement site or perhaps even the many Vx RS sequences that compete for VDJ recombinase. It is notable that the intronic x enhancer deletion appears to generate a λ-only phenotype identical to that observed for the $JxCx$ deletion (Zou *et al.*, 1993; this study). Furthermore, λ^+ B cells in Ex deleted mice had neither Vx-Jx rearrangements nor 3' x RS rearrangements (Zou *et al.*, 1993). Therefore, deletion of the intronic Ex element apparently blocks activation of the x locus for VDJ recombinase-mediated rearrangements. Similarly, deletion of this element may also block the activation of putative negative elements within the locus and thereby serve the same function as larger deletions involving the 3' x RS sequences.

The effect of $JxCx$ deletion on $CD5^+$ B cells

Unlike conventional B cells, $CD5^+$ B cells are mostly generated in fetal liver during ontogeny and are capable of self-renewal (Hayakawa *et al.*, 1985; Herzenberg *et al.*, 1986; Hardy, 1992). In contrast to the 50% reduction in conventional B cells in homozygous $JxCx$ deleted mice, the number of $CD5^+$ B cells is apparently not reduced. In this context, λ_5-deficient mice have a severe reduction in the number of conventional B cells and a modest reduction in the number of $CD5^+$ B cells at 2 weeks of age with normal numbers of $CD5^+$ cells by 5 weeks of age, presumably due to the expansion of the cell population by self-renewal (Kitamura *et al.*, 1992). Therefore, the $JxCx$ deletion is much more 'leaky' than the λ_5 mutation with respect to generation of conventional B cells. Likewise, the self-renewal capacity of $CD5^+$ B cells probably completely masks any effect of the homozygous $JxCx$ deletion on that population early in life.

Applications of the x-deficient genotype

The JxCx deletion should be of considerable utility in the study of B cell differentiation and function. x-Deficient mice provide a novel model system for investigating the ability of a very limited number (two) of well-characterized L chain Vλ regions to interact with the V_H repertoire, respond to different antigens, and undergo somatic mutation. It should be noted that experiments carried out several years ago, in which x L chain expression was suppressed in mice by repeated administration of anti-x antibodies, suggested that a potentially large repertoire of antibodies can be generated using λ L chains (Weiss et al., 1984). In addition, the JxCx deletion should provide a useful system for investigating regulation of x rearrangement and expression in x trans-genics since the analysis of x transgenics can be considerably complicated by expression of the endogenous x repertoire (reviewed by Blackwell and Alt, 1989; Storb, 1989). Furthermore, the JxCx deleted mice can be bred with mice that carry disrupted Ig heavy chain genes (Chen,J., Trounstine,M., Alt,F.W., Young,F., Kurahara,C., Loring,J.F. and Huszar,D., manuscript in preparation) to generate a mouse that can form neither endogenous H chains nor x L chains. Breeding such mice with transgenics that carry human germline Ig H and L chain gene segments should facilitate the generation of fully human antibodies in mice.

Materials and methods

Construction of the JxCx targeting vector

The PGK-neo expression cassette from the plasmid pKJ1, described by Tybulewicz et al. (1991), contains the neo gene under the transcriptional control of the mouse phosphoglycerate kinase (PGK-1) promoter and the PGK-1 poly(A) addition site. pKJ1 was subcloned into EcoRI+HindIII digested pGEM7-Zf(+) to generate pGEM7 (KJ1). The EcoRI−SphI x genomic fragment 5' of Jx1 (Figure 1A) was isolated from a BALB/C λ genomic library and inserted 5' of the neo cassette in pGEM7 (KJ1) following addition of a SphI−XbaI−BglIII−EcoRI adapter to the SphI site of the genomic fragment. An 8 kb BglIII−XhoI genomic fragment, from a C57BL/6J λ genomic library, extending 3' of Cx (Figure 1A) was subcloned 3' of the neo cassette, in the same orientation as the 5' SphI−EcoRI fragment and the neo gene, as a contiguous 1.2 kb BglIII−SacI fragment and a 6.8 kb SacI−XhoI fragment. A PGK-tk expression cassette, comprising HSV-tk coding sequences flanked by PGK regulatory sequences (described by Tybulewicz et al., 1991) was inserted 3' of the genomic sequences at the XhoI site. The targeting vector was excised from plasmid sequences prior to electroporation into ES cells.

Transfection and screening of ES cells

The AB1 ES cell line was cultured on SNL76/7 mitotically inactive feeder cells as described (McMahon and Bradley, 1990). For electroporation, cells were trypsinized and resuspended at a concentration of 1.1×10^7/ml in PBS (Ca^{2+} and Mg^{2+} free; Gibco). An 0.9 ml aliquot (1×10^7 cells) was mixed with 20 μg of DNA and pulsed at 250 V, 500 μF (Bio-Rad Gene Pulser), after which the cells were diluted in culture medium, plated at 2×10^6 per 100 mm plate containing feeder cells, and placed under selection 24 h later in either G418 sulfate (400 μg/ml powder, Gibco) or G418 and FIAU (0.5 μM; 1-[2'-deoxy, 2'-fluoro-β-D-arabinofuranosyl]-5 iodouracil; Oclassen Pharmaceuticals, Inc.) for 9 days. G418 and FIAU double resistant clones were picked, dissociated with trypsin and divided into one well each of a 96-well and a 24-well feeder plate. Upon confluence, ES cells were frozen in the 96-well plates (Ramirez-Solis et al., 1992) and DNA was prepared from the 24-well plates for Southern blot analysis.

Southern blot analysis

Genomic DNA was prepared from ES cells in 24-well plates by the method of Laird et al. (1991) and from tail biopsies, kidney, spleen and lymph node by the method described previously (Huszar et al., 1991). 10 μg of genomic DNA was digested with the indicated restriction enzymes, electrophoresed through a 0.8 or 1% agarose gel, transferred to GeneScreen (DuPont) or

Zetaprobe membrane (BioRad) and hybridized with the ^{32}P-radiolabeled probes described in the text.

Generation of x-deficient mice

Targeted ES clones were recovered from frozen 96-well plates and injected into C57BL/6J blastocysts as described by Bradley (1987) to generate chimeras. Male chimeras were bred with C57BL/6J females and agouti offspring (representing germline transmission of the ES genome) were screened for the presence of the targeted x locus by Southern blot hybridization of BglII-digested tail DNA using probe A (Figure 1). Offspring heterozygous for the mutation were bred together and mice homozygous for the inactivated x locus were identified by Southern blot hybridization of BglII-digested tail DNA with probe A.

Flow cytometry analysis

Single cell suspensions from spleen, bone marrow, lymph node, peripheral blood, thymus and peritoneal washout were prepared from mice of 11 days to 6 months of age by standard methods (Parks et al., 1986). 14 wild type (+/+), 14 heterozygous (+/−) and 12 homozygous (−/−) mice on the F_2 background of an F_1 (129/Sv × C57BL/6J) heterozygote intercross were analyzed. Three wild type and three heterozygous mice on a 129/Sv congenic background were analyzed. At least four individual animals underwent the analysis shown in Figure 3. Prepared cells were stained with fluorescein (fl)-, phycoerythrin (PE)- or biotin (bi)-conjugated monoclonal antibodies and were analyzed by a FACScan (Becton-Dickinson). Biotin conjugates were revealed by Steptavidin Cy-Chrome (PharMingen, San Diego). Cells present in the lymphocyte gate defined by light scatter (Forster et al., 1989) were analyzed.

The following monoclonal antibodies used in the study were generously provided by Dr Alan M.Stall, Columbia University: fl-conjugated JC5 (λ), 187.1 (x); PE-conjugated 11-26 (IgD) and RA3-6B2 (CD45R/B220); and bi-conjugated 11-26 (IgD). The following monoclonal antibodies were obtained from PharMingen, San Diego, CA: fl-conjugated R5-240 (x) R26-46 (λ), R6-60.2 (IgM), AMS9.1 (IgD^a), 217-70 IgD^b), RA3-6B2 (CD45R(/B220), 6C3 (BP-1), S7 (CD43), B3B4 (CD23), RB6-8C5 (granulocytes) and 53-6.7 (CD8a); PE-conjugated 3009-91 (λ), 53-7.3 (CD5/Ly-1), RA3-6B2 (CD45R/B220) and Rm-4-5 (CD4); and bi-conjugated R6-60.2 (IgM), M1/69 (heat stable antigen (HSA) and 500A2 (CD3ε). Fl-conjugated M1/70 (Mac-1) was from Boehringer Mannheim.

T cell depletion by cytotoxic elimination

Single cell suspensions were prepared from spleen and lymph node. Cells from the same mouse were pooled. T cells were eliminated from the pool by staining with purified monoclonal anti-Thy1.2 antibody, followed by incubation with rabbit complement (Hathcock, 1991). B cell enrichment was assessed by flow cytometry analysis for CD45R(B220) and CD5 (Ly-1). DNA was isolated from the enriched B cell population as described above.

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