The EMBO Journal vol.12 no.6 pp.2329-2336, 1993

Deletion of the immunoglobulin κ chain intron enhancer abolishes κ chain gene rearrangement *in cis* but not λ chain gene rearrangement *in trans*

Shunichi Takeda, Yong-Rui Zou[1], Horst Bluethmann[2], Daisuke Kitamura[1,3], Urs Muller and Klaus Rajewsky[1]

Basel Institute for Immunology, Grenzacherstrasse 487, CH-4005, Basel, Switzerland, [1]Institute for Genetics, University of Cologne, Weyertal 121, D-5000 Cologne 41, Germany, and [2]Central Research Units, F.Hoffmann-La Roche & Co. Ltd, CH-4002 Basel, Switzerland [3]Present address: Medical Institute of Bioregulation, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka, Japan

Communicated by K.Rajewsky

Immunoglobulins (Ig) secreted from a plasma cell contain either κ or λ light chains, but not both. This phenomenon is termed isotypic κ−λ exclusion. While κ-producing cells have their λ chain genes in germline configuration, in most λ-producing cells the κ chain genes are either non-productively rearranged or deleted. To investigate the molecular mechanism for isotypic κ−λ exclusion, in particular the role of the Igκ intron enhancer, we replaced this enhancer by a neomycin resistance (neo^R) gene in embryonic stem (ES) cells. B cells heterozygous for the mutation undergo Vκ−Jκ recombination exclusively in the intact Igκ locus but not in the mutated Igκ locus. Homozygous mutant mice exhibited no rearrangements in their Igκ loci. However, splenic B cell numbers were only slightly reduced as compared with the wild-type, and all B cells expressed λ chain bearing surface Ig. These findings demonstrate that rearrangement in the Igκ locus is not essential for λ gene rearrangement. We also generated homozygous mutant mice in which the neo^R gene was inserted at the 3′ end of the Igκ intron enhancer. Unexpectedly, mere insertion of the neo^R gene showed some suppressive effect on Vκ−Jκ recombination. However, the much more pronounced inhibition of Vκ−Jκ recombination by the replacement of the Igκ intron enhancer suggests that this enhancer is essential for Vκ−Jκ recombination.

Key words: gene targeting/immunoglobulin gene enhancer/immunoglobulin gene rearrangement/light chain isotypic exclusion

Introduction

Two enhancers have been identified in the Igκ locus, the Igκ intron enhancer between Jκ (Igκ joining region) and Cκ (Igκ constant region) (Queen and Baltimore, 1983; Bergman *et al.*, 1984; Picard and Schaffner, 1984), and the Igκ 3′ enhancer situated 10 kb downstream of Cκ. In the MOPC11 myeloma cell line, the Igκ 3′ enhancer possesses ~7-fold stronger transcription enhancing activity than the Igκ intron enhancer (Meyer and Neuberger, 1989). Although the Igκ intron enhancer is not active in the plasmacytoma cell line S107, due to the absence of the NFκB activity, the cells actively transcribe their endogenous Igκ genes (Atchison and Perry, 1988). These data suggest that the Igκ 3′ enhancer is sufficient for Igκ gene transcription in mature B lymphocytes. Another function for the Igκ intron enhancer sequence might be the regulation of Vκ−Jκ rearrangement, in analogy to the situation in the Ig heavy (H) chain locus (Ferrier *et al.*, 1990; Serwe and Sablitzky, 1993).

Ig gene rearrangements occur in an ordered fashion during B cell development. The Ig H chain locus usually rearranges earlier, and Ig light (L) chain gene rearrangement follows (Tonegawa, 1983; Alt *et al.*, 1984). Evidence from Ig-transgenic mice and gene targeted mice and from pre-B cell lines transfected with Ig H chain genes indicates that the membrane form of Ig μ heavy chain (μm) is a signal for suppression of further $V_H-D_H-J_H$ rearrangements in the IgH locus (allelic exclusion of IgH) (Nussenzweig *et al.*, 1987; Manz *et al.*, 1988; Kitamura and Rajewsky, 1992), and for enhancement of L chain gene rearrangements in the Igκ locus (Reth *et al.*, 1987). A mouse B cell expresses a single L chain from the two L chain loci (κ and λ), which are located on different chromosomes (Tonegawa, 1983). This phenomenon is termed isotypic κ−λ exclusion. In addition, only κ or λ single gene is productively rearranged in a B cell (allelic exclusion of κ genes or λ genes). The molecular mechanisms of allelic and isotypic exclusion at IgL, which provide antibody monospecificity, have not been well characterized. Most κ expressing B cells retain their Igλ locus in germline configuration, while most λ expressing B cells have non-functional rearrangements in both copies of the Igκ genes (Coleclough *et al.*, 1981; Hieter *et al.*, 1981; Korsmeyer *et al.*, 1981; Lewis *et al.*, 1982). This observation indicates that the Igκ locus rearranges earlier and/or more frequently than the Igλ locus during B cell development.

To explain κ−λ isotypic exclusion, an ordered model and a stochastic model have been proposed. In its extreme form, the ordered model postulates that Igκ genes rearrange first, and only if both Igκ alleles are non-productively rearranged or deleted do Igλ genes become accessible for rearrangement (Hieter *et al.*, 1981). This activation of the Igλ locus is thought to occur through the recombination of the recombining sequence (RS), which has been found in both mouse (Durdik *et al.*, 1984) and in humans (Siminovitch *et al.*, 1985; Klobeck and Zachau, 1986). RS recombination, which is observed in most λ expressing B cells, leads to the deletion of part or all of the Jκ−Cκ region between the RS sequence located downstream of Cκ and heptamer signals located upstream of either Jκ or Cκ (Shimizu *et al.*, 1991).

The stochastic model postulates that Igλ rearrangement is independent of Igκ rearrangement (Coleclough *et al.*, 1981, 1983; Claverie and Langman, 1984). Slower activation of the Igλ locus or inefficient recombination signals in the Igλ locus (Ramsden and Wu, 1991) may be responsible. Thus, to ensure antibody monospecificity, both isotypic κ−λ

© Oxford University Press

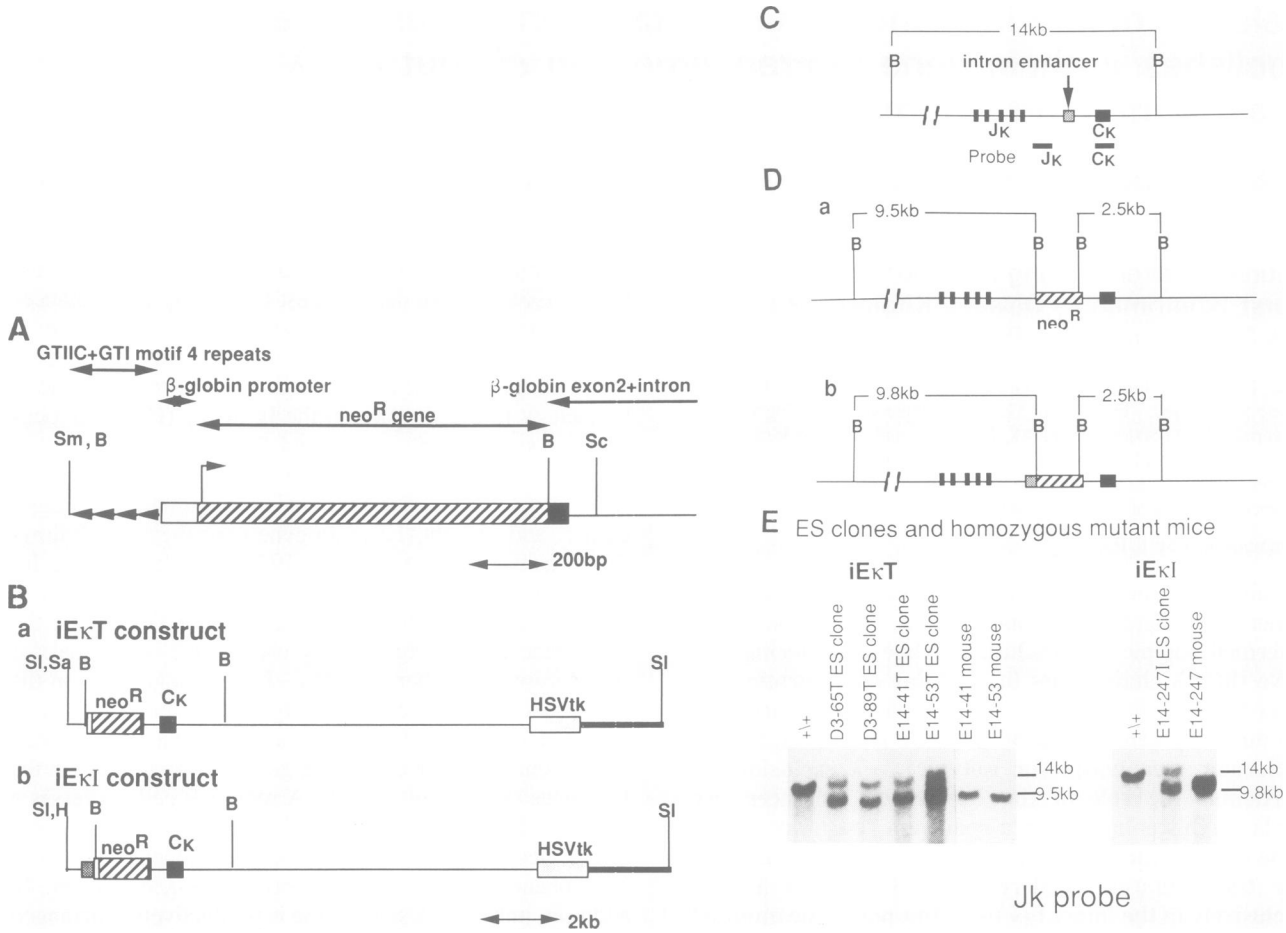

Fig. 1. Strategy for the disruption of the Igκ intron enhancer sequence. (**A**) Structure of the GTIIC+GTI neo^R gene. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Sa, *Sac*II; Sc, *Sca*I; Sl, *Sal*I; Sm, *Sma*I. Only relevant sites are shown. (**B**) Gene targeting constructs. For the iEκT construct, the Igκ intron enhancer sequence from position 69 to 504 was replaced by the neo^R gene fragment. Numbering of the Igκ intron enhancer sequence is according to Altenburger *et al.* (1981). The iEκI construct has the neo^R gene inserted at the position 504. The position 504 is 80 bp downstream from the κE3 site (Queen and Baltimore, 1983), which locates most proximal to the neo^R gene among the described transcription factor binding sites. The transcript from the neo^R gene is spliced between the rabbit β-globin exon 2 splice donor site and the splice acceptor site of Cκ. Before introduction into ES cells, the constructs were linearized by *Sal*I digestion. (**C**) Genomic structure of the Igκ wild-type locus. Exons are represented by black bars. The lengths of diagnostic restriction fragments and location of probes used for Southern blot analysis are shown. (**D**) Predicted structure of the Igκ locus targeted by the iEκT construct (a) and the iEκI construct (b). (**E**) Southern blot analysis of ES cell clones and tail DNAs from mutant mice. *Bam*HI-digested genomic DNAs were hybridized to the Jκ probe. The ES clones have a 9.5 kb fragment (iEκT) or a 9.8 kb fragment (iEκI) from the targeted Igκ locus in addition to the 14 kb fragment from the intact Igκ gene.

exclusion and κ allelic exclusion could be explained by a single mechanism, which may be mainly mediated through feedback control by membrane-bound Ig (Alt *et al.*, 1980; Coleclough *et al.*, 1981; Hagman *et al.*, 1989; Neuberger *et al.*, 1989).

To investigate the role of the Igκ intron enhancer during B cell development, we introduced defined mutations into ES cells. We generated two different kinds of ES clones, one containing a neomycin resistance (neo^R) gene replacing the Igκ intron enhancer, the iEκT mutation; the other containing the neo^R gene at the 3′ end of the Igκ intron enhancer, the iEκI mutation. Mutant mice generated from these ES clones were then used to study gene rearrangements in the L chain gene loci.

Results

DNA constructs and experimental strategy

We produced two DNA constructs: an iEκT construct and an iEκI construct (Figure 1B). In these constructs the expression of the neo^R gene is controlled by the rabbit β-globin promoter plus an enhancer element containing four tandem repeats of the GT-IIC+GT-I motif. This element is active in ES cells but seems to be inactive in lymphoid tissues (Davidson *et al.*, 1988; Fromental *et al.*, 1988) (Figure 1A). In the iEκT construct, the neo^R gene replaced the Igκ intron enhancer sequence (Queen and Baltimore, 1983) containing the octamer site (Currie and Roeder, 1989), the NFκB binding site (Atchison and Perry, 1987), κE1, κE2, κE3 (Queen and Baltimore, 1983), and the kappa silencer (Pierce *et al.*, 1991), but a nearby nuclear matrix association site (Cockerill and Garrard, 1986) remains intact. In the iEκI construct the neo^R gene was inserted at the 3′ end of the enhancer sequence (Figure 1B). In both constructs the herpes simplex virus thymidine kinase gene (HSVtk) was placed at the 3′ end of the genomic sequence to allow negative selection against random integration (Mansour *et al.*, 1988).

The linearized DNA constructs were introduced by electroporation into ES cells, D3 (Doetschman *et al.*, 1985) or E14.1 (Kühn *et al.*, 1991), a subclone of E14 (Hooper *et al.*, 1987), and drug-resistant colonies were selected with

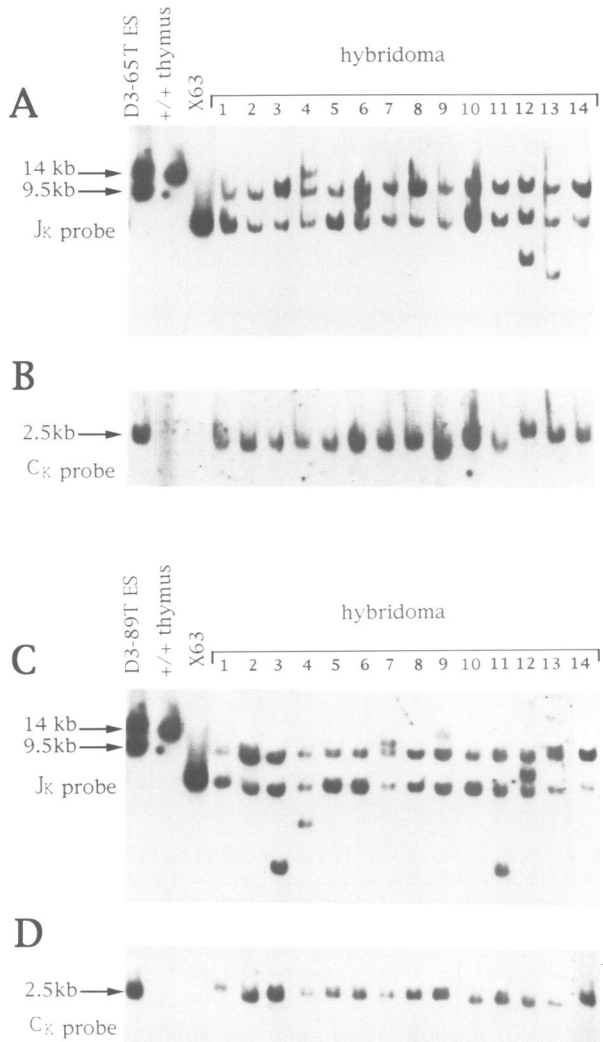

Fig. 2. Southern blot analysis of hybridoma clones from chimeric mouse splenocytes. (**A**) and (**B**) 14 hybridomas from the chimeric mice of the D3−65T clone. (**C**) and (**D**) 14 hybridomas from the chimeric mice of the D3-89T clone. *Bam*HI-digested DNAs were hybridized to the Jκ probe (A and C) and then to the Cκ probe (B and D). The ES clones show 9.5 kb (A and C) and 2.5 kb (B and D) bands derived from the mutated Igκ gene as well as the 14 kb band (A and C) derived from the intact Igκ gene. X63 represents the fusion partner for the generation of hybridomas.

G418 (neomycin) and Gancyclovir (GANC) (Mansour *et al.*, 1988). ES cell clones carrying the expected mutation on one allele through a homologous recombination event were identified by polymerase chain reaction (PCR), and the structure of the targeted locus was verified by Southern blot analysis (Figure 1E). We isolated four positive ES clones (D3H-65T, D3H-89T, E14-41T and E14-53T) targeted by the iEκT construct and one clone (E14-247I) from the iEκI construct. We then generated chimeric mice by injecting these clones into C57BL/6 blastocyst embryos. Chimeric offspring were crossed with C57BL/6 females. We obtained germline transmission of the mutations in the cases of ES clones E14-41T, E14-53T and E14-247I. We show here results of the analysis of B cells from chimeric mice generated from ES clones D3H-65T and D3H-89T and of B cells from homozygous mutant mice generated from ES clones E14-41T, E14-53T and E14-247I.

In B cells heterozygous for iEκT mutation, Vκ−Jκ rearrangements take place exclusively in the intact allele

To compare Vκ−Jκ rearrangements in the iEκT Igκ allele with those in the wild-type locus, we generated hybridoma clones from splenic B cells from D3H-65T and D3H-89T derived chimeric mice. Hybridoma clones derived from ES cells were identified by detecting the μ^a allotype of secreted antibody. Vκ−Jκ recombination in these clones was then examined by Southern blot hybridization. All clones showed a 2.5 kb band hybridizing to the Cκ probe, indicating that they have retained the mutated Igκ locus (Figure 2B and D). We then hybridized the same filters with the Jκ probe and found that all hybridoma clones retained the 9.5 kb fragment (the iEκT Igκ gene in germline configuration) and had lost the 14 kb fragment from the unmutated copy of the Igκ gene (Figure 2A and C). These data indicate that Vκ−Jκ rearrangements had taken place exclusively in the intact, not the mutant allele. The absence of new 'rearranged' bands hybridizing with the Jκ probe in some of the hybridoma clones is presumably due to overlap with other bands or deletion by RS recombination. We analysed a total of 50 hybridoma clones from each of the two chimeric mice derived from D3H-65T and D3H-89T (data not shown): all hybridoma clones which performed Vκ−Jκ rearrangements used the intact and not the mutant Igκ locus. It is known from earlier studies (Coleclough, 1983) that in normal mice some 30% of κ chain expressing normal spleen B lymphocytes carry Vκ−Jκ rearrangements on both chromosomes. Therefore, the absence of rearrangements in Igκ loci carrying the iEκT mutation indicates that the replacement of the Igκ intron enhancer by the neo^R gene abolishes Vκ−Jκ rearrangement.

Lack of rearrangements in the Igκ locus of iEκT but not iEκI homozygous mutant mice

We obtained germline transmission from three independent chimeric mice derived from ES clones, E14-41T, E14-53T and E14-247I, using C57BL/6 females. This allowed us to assess Vκ−Jκ rearrangements and RS recombination in the B cells of homozygous mutant mice. To do this, we enriched B220 positive splenic B cells by magnetic sorting and prepared DNA from them for Southern blot analysis. Typically, this enrichment procedure gave us ~90% pure B cell populations (Figure 3A). Interestingly, the spleen sizes of the iEκT and iEκI homozygous mutants were normal and 30−50% of splenic mononuclear cells were B cells as in control mice.

No rearrangements of the Igκ loci could be detected by Southern blotting with the Jκ probe in the B cells of the iEκT homozygous mutant mice (Figure 3B, lanes 5 and 7). In the iEκI homozygous mutant mice, ~30% of the Igκ alleles were rearranged, as shown by the diminution of the intensities of the germline bands, compared with thymus samples after normalization of the amount of DNA loaded using the β-actin probe (Figure 3B, lanes 9 and 11). As expected, ~60% of the Igκ alleles had rearranged in normal splenic B cells (Figure 3B, lane 2), while purified λ chain bearing splenic B cells from wild-type mice had <5% of their Igκ alleles in germline configuration (Figure 3B, lane 3).

We also analysed RS recombination. Splenic B cells from the iEκT mutants (Figure 3C, lanes 5 and 7) showed intensities of the germline RS band similar to those of thymus

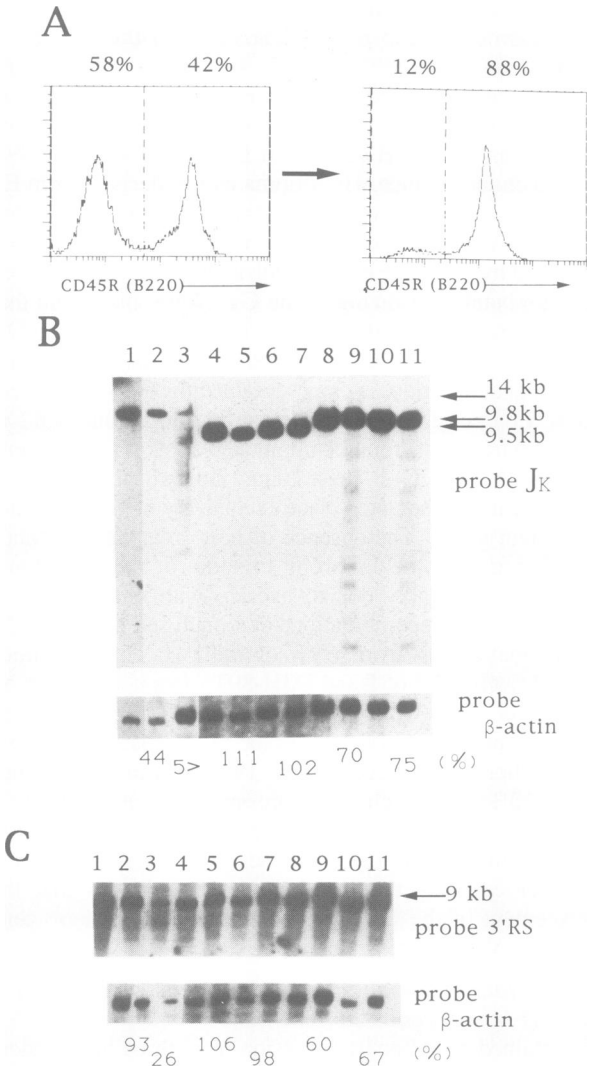

Fig. 3. Analysis of the rearrangement activity of the mutated Igκ loci in splenic B cells of homozygous iEκT and iEκI mutant mice. (**A**) Representative FACS histogram of splenic mononuclear cells stained with anti-B220 antibody before (left) and after (right) enrichment for B cells. (**B**) and (**C**) Southern blot analysis of *Bam*HI-digested DNAs of whole thymus or enriched splenic B lymphocytes. The blots were hybridized with the Jκ (**B**) and the RS probe (**C**). Lanes 1, 2 and 3 contain DNA from normal control mice; thymus (lane 1), enriched splenic B cells (lane 2) and purified λ-positive splenic B cells (lane 3). Samples from iEκT mice E14-41T (lanes 4 and 5) and E14-53T (lanes 6 and 7) or from two different iEκI mice (lanes 8 and 9 and 10 and 11). Lanes 4, 6, 8 and 10, thymus; lanes 5, 7, 9 and 11, enriched splenic B cell DNAs. Hybridization to the β-actin probe is shown below each blot. The signal intensities of the germline loci in the various B cell populations are also indicated as percentages of those present in the corresponding thymus samples after normalization to the intensity of the β-actin signal. The arrows indicate the sizes of different κ loci; 14 kb germ-line, 9.5 kb iEκT, 9.8 kb iEκI and 9 kb RS.

DNAs. Accordingly, there was little, if any, RS recombination in the iEκT homozygous mutants. To summarize these results, it seems that the Igκ loci are inactive in the B cells of the iEκT homozygous mutant mice, while the Igκ loci of the iEκI homozygous mutant mice show reduced activity, compared with those of normal splenic B cells.

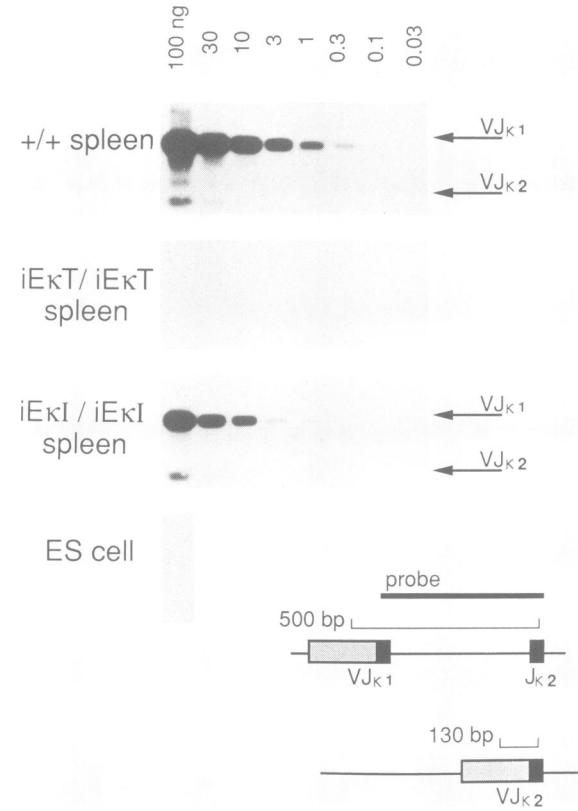

Fig. 4. Quantification of Vκ−Jκ rearrangements by PCR. Serially diluted chromosomal DNA samples from spleens of normal and mutant mice were amplified for 30 cycles as indicated. The arrows show the sizes of the expected amplification products. At the bottom two possible rearrangements are schematically shown which are detected by the genomic DNA probe located between Jκ1 and Jκ2.

Deletion of the Igκ intron enhancer abolishes Vκ−Jκ rearrangement

To quantitate further Vκ−Jκ rearrangements, we examined DNA from spleen cells of the iEκT and iEκI homozygous mutants by PCR (Schlissel and Baltimore, 1989). Figure 4 shows a representative Southern blot analysis of step-wise diluted DNA samples probed with a genomic DNA fragment located between Jκ1 and Jκ2. Since signal intensities were still in the logarithmic phase after 30 cycles of amplification (data not shown), Vκ−Jκ rearrangements could be assessed quantitatively. In spleen cells from wild-type mice, there was ~10-fold more amplified product than in those from the homozygous iEκI mutant, and at least 1000-fold more amplified product than in the case of the homozygous iEκT mutant. These data are consistent with the Southern blot analysis (Figure 3). While the insertion of the neo^R gene only moderately reduced Vκ−Jκ rearrangements in the homozygous iEκI mouse, gene rearrangements in the Igκ locus of the homozygous iEκT mouse were completely inhibited. These data indicate that the Igκ intron enhancer is essential for Vκ−Jκ recombination.

Splenic B cells express λ chain bearing Ig in the absence of rearrangement in the Igκ locus

Since the B cells of the homozygous mutant mice showed either a complete (iEκT) or a partial (iEκI) block in Vκ−Jκ rearrangements, we studied surface Ig expression of these B cells by two colour flow cytometry (Figure 5). At the age

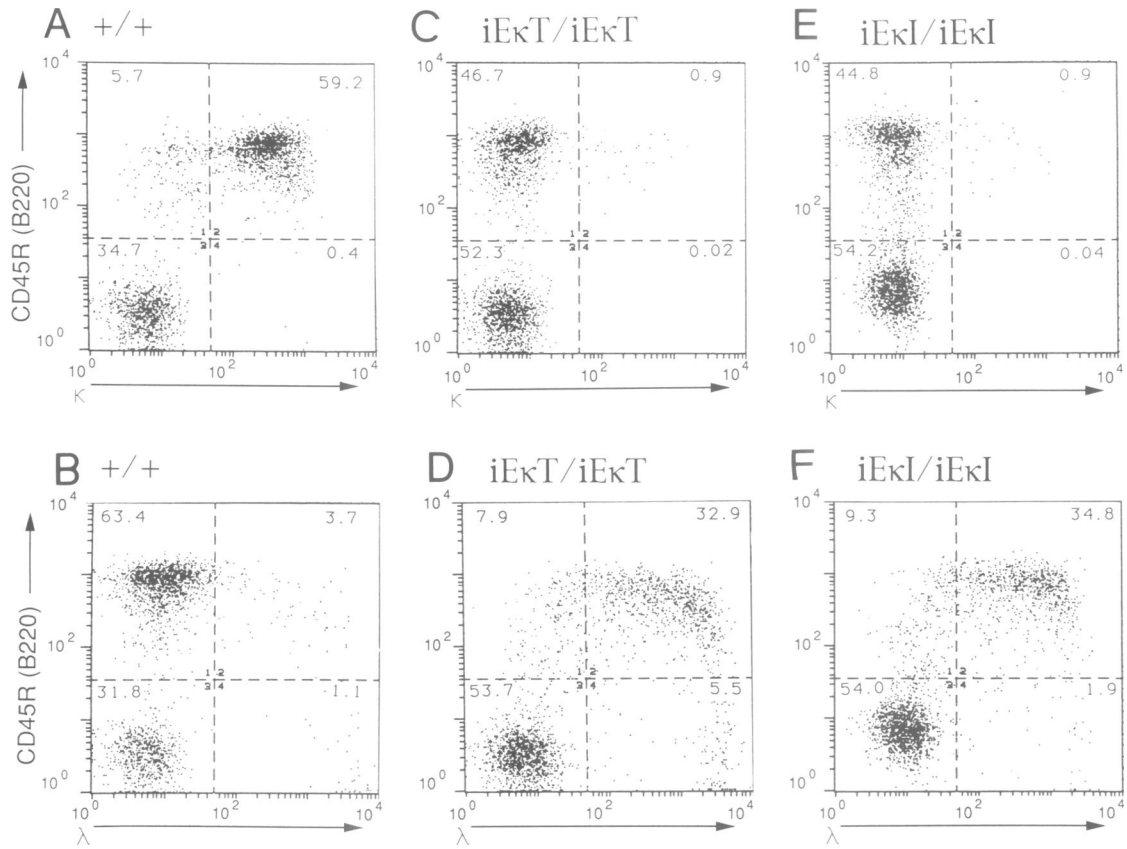

Fig. 5. Spleen B cells from iEκT and iEκI homozygous mutant mice express only λ chains. Spleen cells from 4 week old mice were stained with phycoerythrin-coupled anti-CD45R/B220 plus either fluorescein isothiocyanate coupled anti-κ or anti-λ antibody. Only data from cells in the lymphocyte gate as defined by light scatter were collected. The percentages of cells in a given quadrant are indicated in the figure.

of 4 weeks the spleen sizes of these mice were normal (data not shown), and the relative numbers of B cells showed only a slight diminution from ~60% of total lymphocytes in controls to 45% in the mutants. Interestingly, both types of mutant mice had only λ chain bearing B cells (C, D, E and F) while typically, ~5% of B cells from a normal mouse express λ chains (A and B).

The absence of κ chain expression in the iEκT homozygous mutants was confirmed by Northern blot analysis (Figure 6). The iEκT homozygous mutant spleen did not give a Cκ probe hybridizing signal of normal size. When compared with the band hybridizing with the Cκ probe from a wild-type mouse, the signals of the iEκI homozygous mutants corresponded to much larger mRNAs. Since they also hybridized with the neo^R probe, they would not result in κ chain expression in the iEκI homozygous mutant mice. These aberrant mRNAs could be due to abnormal splicing events involving neo^R sequences. Consistently, λ chain gene mRNAs were much more abundant in the same samples of the mutant mice than those of a normal mouse. These data indicate that neither Vκ−Jκ nor RS recombination is required for Igλ rearrangement.

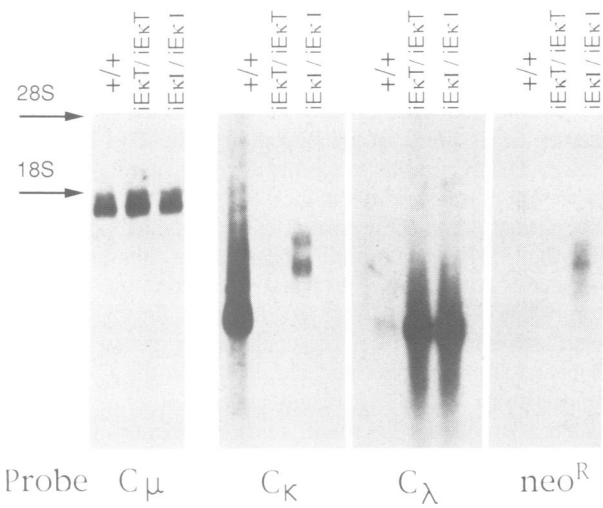

Fig. 6. B cells of homozygous mutant mice do not express normal κ but abundant λ transcripts. Four identical Northern blot filters were hybridized with radioactive probes as indicated.

Discussion

Here we have shown that the intact Igκ intron enhancer region is required for normal rearrangements in the Igκ locus, and that Igλ chain gene rearrangements can proceed efficiently in the absence of prior Vκ−Jκ and RS rearrangements.

In wild-type mice, only 30% of κ expressing B cells carry Vκ−Jκ rearrangements on both chromosomes (Coleclough, 1983). Furthermore, according to the analysis of circular DNA derived from the Igκ locus (Shimizu *et al.*, 1991), sequential Vκ−Jκ rearrangements in the Igκ locus are frequent events. These data suggest that a limiting factor is responsible for the induction of Vκ−Jκ rearrangements through a *cis*-acting element in the Igκ locus. B cell

hybridomas from chimeric mice, which were derived from the D3H-65T and D3H-89T ES clones, contained rearrangements only in their intact Igκ loci, while the alleles carrying the iEκT mutation were always in germline configuration (Figure 2). Furthermore, we were unable to detect any Vκ−Jκ rearrangements in the splenic B cells of the homozygous iEκT mutant mice, even when sensitive PCR assays were employed (Figures 3 and 4). Together with the moderate effect of the iEκI mutation on Vκ−Jκ rearrangements, these results suggest that it is the Igκ intron enhancer, through which gene rearrangements in the Igκ locus are activated, e.g. by enhancing transcription in accordance with the proposal of Yancopoulos and Alt (1985).

Unexpectedly, we found that the insertion of the neo^R gene next to the Igκ intron enhancer (the iEκI Igκ gene) somewhat reduces the frequency of Vκ−Jκ rearrangements in the B cell population. Splenic B cells of the iEκI homozygous mutant showed 20−30% of the Vκ−Jκ rearrangements seen in normal splenic B cells (Figure 3). Since there seems to be competition between Igκ and Igλ rearrangements during B cell development (Chen *et al.*, 1993; Zou *et al.*, 1993), this decreased frequency of Vκ−Jκ rearrangements could be due to a minor interference of the neo^R gene insertion with Vκ−Jκ rearrangements. No mutations were found by sequence analysis in the Igκ intron enhancer of the iEκI Igκ gene in the E14-247I ES clone (data not shown). There are three possible reasons for a negative effect of the neo^R gene on Vκ−Jκ rearrangements. First, it has been reported that CpG methylation correlates negatively with V(D)J recombination (Engler *et al.*, 1991): there is a nine times higher density of CpG (78/874 bases) in the coding sequence of the neo^R gene than the average density in vertebrate genome, one CpG in every 100 bases (Bird, 1986). Second, there is evidence that the coding sequence of the neo^R gene has a suppressive effect on transcription (Artelt *et al.*, 1991). Hence, proximity of the neo^R coding sequence to the Jκ region may negatively affect the accessibility of the Jκ region in the iEκI Igκ gene to the V(D)J recombinase, either through methylation or suppression of transcription in the Jκ region (Leclercq *et al.*, 1989; Schlissel and Baltimore, 1989). The third possibility is that the GTIIC+GTI motif repeats in the enhancer of the neo^R gene could have a negative effect on Vκ−Jκ recombination during B cell development. In Cκ-targeted mice, the presence of pMC1neo containing a mutant polyoma enhancer (Thomas and Capecchi, 1987) in the Cκ region did not disturb Vκ−Jκ rearrangements (Zou *et al.*, 1993).

During B cell development, rearrangements in the Igλ locus usually follow those in the Igκ locus, and a single B cell expresses either κ or λ chains but not both. These observations have lead to an 'ordered model', which postulates that λ genes become accessible for recombination only after both κ alleles are either non-productively rearranged or deleted (Hieter *et al.*, 1981). Here we have shown that adult homozygous iEκT mutant mice have numbers of splenic B cells comparable with wild-type littermates, and that these B cells express λ chains, although they carry neither Vκ−Jκ nor RS recombination in their Igκ loci. These findings demonstrate that Igκ rearrangements are not required for λ chain gene rearrangements, in accord with the observation that in a fraction of κ chain expressing B cells, λ chain genes are non-productively rearranged (Zou *et al.*, 1993). While this is in accord with the stochastic model of light chain gene rearrangements, one has to keep in mind that κ and λ gene rearrangements appear not to be totally independent of each other in that the efficiency of the generation of λ chain expressing B cells is significantly increased in κ chain deficient mice (Chen *et al.*, 1993; Zou *et al.*, 1993).

Our data do not fully exclude the ordered model because of the following two possibilities. First, there could be two different pathways for the activation of λ chain gene rearrangements, only one of which would be dependent on Igκ rearrangements. In the periphery of the iEκT homozygous mutant, λ chain expressing B cells, which had developed through the other pathway could have expanded. Nadel *et al.* (1990), who described that 23% of λ chain producing B cells have no RS recombination, proposed two pathways for the activation of λ chain gene rearrangements, one activated through RS recombination, the other independent of rearrangements of Igκ. A quantitative analysis of B lymphopoiesis in iEκT mice and in mice in which the Cκ gene was inactivated by gene targeting (and in which rearrangements in the Igκ loci still occur) argues against this model. In the two mouse strains, equal and large numbers of λ chain expressing B cells are found already in the compartment of newly generated B cells in the bone marrow (Zou *et al.*, 1993). A second possibility is that a suppressive signal from the Igκ loci acting on Igλ loci is responsible for the ordered rearrangements from κ to λ chain genes in wild-type mice, and that both the iEκT mutation and the inactivation of Cκ (Zou *et al.*, 1993) disturb this signal.

Materials and methods

Construction of the Igκ intron enhancer targeting vector

A neo^R gene was generated by replacing the *Pvu*II−*Bam*HI fragment of pG1GTIIC+GTI (Fromental, 1988) with the *Mlu*I−*Bam*HI fragment of pMC1neo (Thomas and Capecchi, 1987), so that the neo^R gene was transcribed from the promoter of pG1GTIIC+GTI. The *Sma*I−*Sca*I fragment of the neo^R gene (Figure 1A) was ligated with *Not*I linkers after Klenow enzyme treatment for further manipulation. For the construction of the targeting vectors, two *Not*I restriction enzyme sites (GCGGCCGC) were introduced at the Igκ intron enhancer region by using an *in vitro* mutagenesis kit (Bio-Rad). For the iEκT construct, *Not*I sites were introduced at the 5′ end (69−76) and the 3′ end (498−504) of the enhancer sequence to delete the 429 bp enhancer sequence. Numbering of the Igκ intron enhancer sequence is according to Altenburger *et al.* (1981). The *Not*I fragment of the neo^R gene was then inserted between the two *Not*I sites. The 14.5 kb fragment composed of the 550 bp *Sac*II−*Not*I genomic DNA, the *Not*I fragment of the neo^R gene and the 12.5 kb *Not*I−*Eco*RI genomic DNA was finally inserted into the *Hin*dIII site of pIC19R/MC1-tk (Mansour *et al.*, 1988) by a blunt-end ligation. For the iEκI construct, the *Not*I fragment of the neo^R gene was inserted into the unique *Not*I site, which was identical to the 3′ *Not*I site of the iEκT construct. The neo^R gene flanked by the *Hin*dIII−*Not*I (750 bp) and the *Not*I−*Eco*RI (12.5 kb) genomic DNAs was inserted into the *Hin*dIII site of pIC19R/MC1-tk as in the case of the iEκT construct.

Generation of ES cells carrying mutations in the Igκ locus

D3 ES cells (Doetschman *et al.*, 1985) or E14.1 ES cells (Kühn *et al.*, 1991) were cultivated following the method described by Kitamura *et al.* (1992). Targeting vectors linearized by *Sal*I were transfected into ES cells by electroporation, and transfected cells were selected following a protocol described by Mansour *et al.* (1988). Homologous recombination events were screened by PCR (Doetschman *et al.*, 1988) under conditions recommended by the supplier (Perkin Elmer Cetus) in 50 ml containing 3 pmol of each primer (Igκ, TGCCAGCCATTTGGCGTTCA; neo, CCTGCGTGCAATCCATCTTG). PCR was run for 50 cycles using a thermal cycler (Techne). Denaturation was performed for 1 min at 94°C, annealing for 0.6 min at 61°C and extension for 2 min at 72°C. Positive clones were verified by Southern blot analysis: genomic DNA from PCR-positive clones was digested

with *Bam*HI, size fractionated and hybridized to the Jκ probe as shown in Figure 1(E).

Generation of chimeric mice and their progeny

ES cell clones carrying the expected mutation were injected into blastcysts of C57BL/6 mice, and male chimeric mice were mated to C57BL/6 females. Agouti offspring were examined by Southern blotting for the presence of the mutation. Heterozygous mice were intercrossed, and homozygous mice were identified by Southern blotting. Mice were kept in a conventional breeding facility.

Generation of hybridomas from chimeric mice

Four week old chimeric mice were sacrificed to fuse their spleen cells with X63-Ag8.653, an Ig-non-producer myeloma cell line (Kearney *et al.*, 1979). B cell hybridomas derived from ES cells were identified by detecting secreted IgM of the $C\mu^a$ allotype, using an enzyme-linked immunosorbent assay performed as described (Kendall *et al.*, 1983). Plastic plates were coated by either monoclonal antibody (MoAb) RS3.1, anti-μ^a (Schüppel *et al.*, 1987) or MoAb MB86, anti-μ^b (Nishikawa *et al.*, 1986). Supernatants of hybridoma clones were then added, followed by the addition of alkaline phosphatase conjugated goat anti-mouse-IgM (Southern Biotechnology).

Preparation of spleen cells for Southern blot analysis

From 4 week old mutant mice, splenic B cells were enriched by a high gradient magnetic separation column as described by Miltenyi *et al.* (1990). The spleen cells which had been stained with anti-B220-Ab coupled micromagnetic particles (Miltenyi Biotec) were retained on the separation column under a high gradient magnetic field: these cells were subsequently eluted from the column. The purity of splenic B cells was examined by flow cytometric analysis after staining with fluorescein isothiocyanate conjugated RA3-6B2 (anti-CD45R/B220; Coffman, 1982). Mononuclear cells defined by light scatter were analysed, dead cells were excluded by propidium iodine staining. Surface λ chain positive (λ^+) B cells were enriched from 4 week old C57BL/6 spleens by a high gradient magnetic separation column, after the cells had been stained with Biotin-labelled goat anti-mouse λ chain antibody (Southern Biotechnology) plus streptavidin fluorescein (Boehringer) and subsequently with biotin-coupled micromagnetic particles (Miltenyi Biotec). The eluted cells were stained with phycoerythrin-conjugated goat anti-mouse κ chain antibody (Southern Biotechnology): $\lambda^+\kappa^-$ B cells were further purified by a cell sorter (Becton Dickinson). The purity of the sorted cells was 98%.

Southern and Northern blot analysis and probes

We followed the protocol of Kitamura *et al.* (1992) for chromosomal DNA purification, RNA extraction, Southern blot and Northern blot hybridization. About 10 μg of chromosomal DNA or total RNA was electrophoresed. Band intensities were measured by a phosphor image scanner (Molecular Dynamics) as described by Johnston *et al.* (1990). The Jκ and Cκ probes were the *Hin*dIII–*Xba*I fragment and the *Hpa*I–*Bgl*II fragment of the Jκ–Cκ region respectively (Sakano *et al.*, 1979). The RS probe, kindly provided by M.Reth, was the 0.8 kb *Sau*3A fragment from the RS region (Durdik *et al.*, 1984). The β-actin 3′ untranslated region probe came from the sequence, 1639–1892 bp (Tokunaga *et al.*, 1986). The Cλ1 probe was the 1.8 kb *Bam*HI–*Bgl*II fragment from the $C_{\lambda 1}$ region (Bernard *et al.*, 1978). The Cμ probe was the 530 bp *Pst*I genomic fragment including exon 3 (Kawakami *et al.*, 1980). The neo^R probe was the 910 bp *Eco*RI–*Bam*HI fragment of pMC1neo (Thomas and Capecchi, 1987).

PCR analysis to quantitate Vκ–Jκ recombination

We followed the protocol of Doetschman *et al.* (1988) to isolate total spleen cell DNA. We serially diluted the DNA from 100 ng to 30 pg, and subsequently added ES cell DNA so that the total amount of DNA was 100 ng. PCR was done under conditions recommended by the supplier (Perkin Elmer Cetus) in 50 ml with degenerate primers as described (Schlissel and Baltimore, 1989). PCR was run for 30 cycles using a thermal cycler (Techne). Denaturation was performed for 1 min at 94°C, annealing and extension for 1.5 min at 72°C, as described by Kitamura and Rajewsky (1992). Amplified samples were run on a 2% agarose gel, followed by Southern blotting. The filter was hybridized to a genomic DNA probe located between Jκ1 and Jκ2 positions 86–481; numbering according to Sakano *et al.* (1979).

Flow cytometric analysis

Cells from spleen were stained with phycoerythrin conjugated RA3-6B2 (anti-CD45R/B220) in addition to either fluorescein isothiocyanate conjugated goat anti-mouse κ chain antibody (Southern Biotechnology) or fluorescein isothiocyanate conjugated goat anti-mouse λ chain antibody (Southern Biotechnology). Cells present in the lymphocyte gate as defined by light scatter (Förster *et al.*, 1989) were analysed. Dead cells were excluded by propidium iodine staining.

Acknowledgments

We thank Drs Klaus Karjalainen, Alexandra Livingstone, Jean-Marie Buerstedde, Michael V.Wiles, Marie Kosco, Rick Wetsel and Olga Bezzubova for reading the manuscript; Gholam Reza Dastoornikoo for excellent technical help, and Yolande Lang and Pascale Renard for the generation of chimeric mice. Human leukemia inhibitory factor-producing cells were provided by the Genetics Institute (Cambridge, MA). The Basel Institute for Immunology is founded and supported by Hoffmann-La Roche. The work at the Institute for Genetics in Cologne was supported by the Deutsche Forschungsgemeinschaft through SFB 243, the Human Frontier Science Program and the Fazit Foundation.

References

Alt,F.W., Enea,V., Bothwell,A.L.M. and Baltimore,D. (1980) *Cell*, **21**, 1–12.

Alt,F.W., Yancopoulos,G.D., Blackwell,T.K., Wood,C., Thomas,E., Boss,M., Coffman,R., Rosenberg,N., Tonegawa,S. and Baltimore,D. (1984) *EMBO J.*, **3**, 1209–1219.

Altenburger,W., Neumaier,P., Steinmetz,M. and Zachau,H.G. (1981) *Nucleic Acids Res.*, **9**, 971–981.

Artelt,P., Grannemann,R., Stocking,C., Friel,J., Bartsch,J. and Hauser,H. (1991) *Gene*, **99**, 249–254.

Atchison,M.L. and Perry,R.P. (1987) *Cell*, **48**, 121–128.

Atchison,M.L. and Perry,R.P. (1988) *EMBO J.*, **7**, 4213–4220.

Bergman,Y., Rice,D., Grosschedl,R. and Baltimore,D. (1984) *Proc. Natl Acad. Sci. USA*, **81**, 7041–7045.

Bernard,O., Hozumi,N. and Tonegawa,S. (1978) *Cell*, **15**, 1133–1144.

Bird,A.P. (1986) *Nature*, **321**, 209–213.

Chen,J., Trounstine,M., Kurahara,C., Young,F., Kuo,C.C., Xu,Y., Loring,J.F., Alt,F.W. and Huszar,D. (1993) *EMBO J.*, **12**, 821–830.

Claverie,J.-M. and Langman,R. (1984) *Trends Biochem. Sci.*, **9**, 293–296.

Cockerill,P.N. and Garrard,W.T. (1986) *Cell*, **44**, 273–282.

Coffman,R.L. (1982) *Immunol. Rev.*, **69**, 5–23.

Coleclough,C. (1983) *Nature*, **303**, 23–26.

Coleclough,C., Perry,R.P., Karjalainen,K. and Weigert,M. (1981) *Nature*, **290**, 371–378.

Currie,R.A. and Roeder,R.G. (1989) *Mol. Cell. Biol.*, **9**, 4239–4247.

Davidson,I., Xiao,J.H., Rosales,R., Staub,A. and Chambon,P. (1988) *Cell*, **54**, 931–942.

Doetschman,T.C., Eistelter,H., Katz,M., Schmidt,W. and Kemler,R. (1985) *J. Embryol. Exp. Morphol.*, **87**, 27–45.

Doetschman,T., Maeda,N. and Smithies,O. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 8583–8587.

Durdik,J., Moore,M.W. and Selsing,E. (1984) *Nature*, **307**, 749–752.

Engler,P., Haasch,D., Pinkert,C.A., Doglio,L., Glymour,M., Brinster,R. and Storb,U. (1991) *Cell*, **65**, 939–947.

Ferrier,P., Krippl,B., Blackwell,T.K., Furley,A.J.W., Suh,H., Winoto,A., Cook,W.D., Hood,L., Costantini,F. and Alt,F.W. (1990) *EMBO J.*, **9**, 117–125.

Förster,I., Vieira,P. and Rajewsky,K. (1989) *Int. Immunol.*, **1**, 321–331.

Fromental,C., Kanno,M., Nomiyama,H. and Chambon,P. (1988) *Cell*, **54**, 943–953.

Hagman,J., Lo,D., Doglio,L.T., Hackett,J.,Jr, Rudin,C.M., Haasch,D., Brinster,R. and Storb,U. (1989) *J. Exp. Med.*, **169**, 1911–1929.

Hieter,P.A., Korsmeyer,S.J., Waldmann,T.A. and Leder,P. (1981) *Nature*, **290**, 368–372.

Hooper,M., Hardy,K., Handyside,A., Hunter,S. and Monk,M. (1987) *Nature*, **326**, 292–295.

Johnston,R.F., Pickett,S.C. and Barker,D.L. (1990) *Electrophoresis*, **11**, 355–360.

Kawakami,T., Takahashi,N. and Honjo,T. (1980) *Nucleic Acids Res.*, **8**, 3933–3945.

Kearney,J.F., Radbruch,A., Liesegang,B. and Rajewsky,K. (1979) *J. Immunol.*, **123**, 1548–1550.

Kendall,C., Ionesco-Matiu,I. and Dreesman,G.R. (1983) *J. Immunol. Methods*, **56**, 329–339.

Kitamura,D. and Rajewsky,K. (1992) *Nature*, **356**, 154–156.

Kitamura,D., Kudo,A., Schaal,S., Müller,W., Melchers,F. and Rajewsky,K. (1992) *Cell*, **69**, 823–831.

Klobeck,H.-G. and Zachau,H.G. (1986) *Nucleic Acids Res.*, **14**, 4591–4603.
Korsmeyer,S.J., Hieter,P.A., Ravetch,J.V., Poplack,D.G., Waldmann,T.A. and Leder,P. (1981) *Proc. Natl Acad. Sci. USA*, **78**, 7096–7100.
Kühn,R., Rajewsky,K. and Müller,W. (1991) *Science*, **254**, 707–710.
Leclercq,L., Butkeraitis,P. and Reth,M. (1989) *Nucleic Acids Res.*, **17**, 6809–6819.
Lewis,S., Rosenberg,N., Alt.F. and Baltimore,D. (1982) *Cell*, **30**, 807–816.
Mansour,S.L., Thomas,K.R. and Capecchi,M.R. (1988) *Nature*, **336**, 348–352.
Manz,J., Denis,K., Witte,O., Brinster,R. and Storb,U. (1988) *J. Exp. Med.*, **168**, 1363–1381.
Meyer,K.B. and Neuberger,M.S. (1989) *EMBO J.*, **8**, 1959–1964.
Miltenyi,S., Müller,W., Weichel,W. and Radbruch,A. (1990) *Cytometry*, **11**, 231–238.
Nadel,B., Cazenave,P.-A. and Sanchez,P. (1990) *EMBO J.*, **9**, 435–440.
Neuberger,M.S., Caskey,H.M., Pettersson,S., Williams,G.T. and Surani,M.A. (1989) *Nature*, **338**, 350–352.
Nishikawa,S., Sasaki,Y., Kina,T., Amagai,T. and Katsura,Y. (1986) *Immunogenetics*, **23**, 137–139.
Nussenzweig,M.C., Shaw,A.C., Sinn,E., Danner,D.B., Holmes,K.L., Morse,H.C.,III and Leder,P. (1987) *Science*, **236**, 816–819.
Picard,D. and Schaffner,W. (1984) *Nature*, **307**, 80–82.
Pierce,J.W., Gifford,A.M. and Baltimore,D. (1991) *Mol. Cell. Biol.*, **11**, 1431–1437.
Queen,C. and Baltimore,D. (1983) *Cell*, **33**, 741–748.
Ramsden,D.A. and Wu,G.E. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 10721–10725.
Reth,M., Petrac,E., Wiese,P., Lobel,L. and Alt,F.W. (1987) *EMBO J.*, **6**, 3299–3305.
Sakano,H., Hüppi,K., Heinrich,G. and Tonegawa,S. (1979) *Nature*, **280**, 288–294.
Schlissel,M.S. and Baltimore,D. (1989) *Cell*, **58**, 1001–1007.
Schüppel,R., Wilke,J. and Weiler,E. (1987) *Eur. J. Immunol.*, **17**, 739–741.
Serwe,M. and Stablitsky,F. (1993) *EMBO J.*, **12**, in press.
Shimizu,T., Iwasato,T. and Yamagishi,H. (1991) *J. Exp. Med.*, **173**, 1065–1072.
Siminovitch,K.A., Bakhshi,A., Goldman,P. and Korsmeyer,S.J. (1985) *Nature*, **316**, 260–262.
Thomas,K.R. and Capecchi,M.R. (1987) *Cell*, **51**, 503–512.
Tokunaga,K., Taniguchi,H., Yoda,K., Shimizu,M. and Sakiyama,S. (1986) *Nucleic Acids Res.*, **14**, 2829.
Tonegawa,S. (1983) *Nature*, **302**, 575–581.
Yancopoulos,G.D. and Alt,F.W. (1985) *Cell*, **40**, 271–281.
Zou,Y.-R., Takeda,S. and Rajewsky,K. (1993) *EMBO J.*, **12**, 811–820.

Received on January 4, 1993; revised on March 3, 1993