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V(D)J recombination in B cells is impaired but not blocked by targeted deletion of the immunoglobulin heavy chain intron enhancer

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We have assessed the importance of the immunoglobulin heavy chain (IgH) intron enhancer for recombination of variable gene segments (V, D and J) during B cell development. We generated chimeric mice with embryonic stem cells lacking the intron enhancer from one of their IgH loci. The IgH intron enhancer was substituted by a short oligonucleotide through homologous recombination using the 'Hit and Run' procedure. V(D)J recombination occurred less frequently on mutant alleles, but was not blocked completely. Quantitative polymerase chain reaction analyses demonstrated that 15–30% of the mutated loci in mature B cells were unrearranged, in striking contrast to the wild-type alleles. The remainder of the mutated loci underwent D–J (65–80%) as well as V–DJ rearrangements, although the latter were less frequent (3–6%). These results are in line with previous data which showed that the V(D)J recombination machinery is modulated through *cis*-regulatory elements within the intron enhancer. However, our data predict the existence of additional *cis*-regulatory element(s) which, together with the intron enhancer, are required to activate the V(D)J recombination machinery fully. Such *cis*-regulatory element(s) might function as an enhancer of recombination or as a locus control region regulating the accessibility of the IgH locus.

Key words: allelic exclusion/homologous recombination/immunoglobulin gene rearrangement/immunoglobulin heavy chain intron enhancer

Introduction

In the course of B lymphocyte development the assembly of immunoglobulin (Ig) variable gene segments (V, D and J) in pro- and pre-B cells is a prerequisite for the expression of functional immunoglobulins. The onset, timing and specificity of the recombination process must be strictly controlled. Based on the assumption that the V(D)J recombination machinery is active throughout early lymphocyte development, the accessibility of the Ig loci to the recombinase machinery might be a key factor in the control of the rearrangement process (Yancopoulos and Alt, 1985; for reviews see Alt *et al.*, 1992; Schatz *et al.*, 1992). This model predicts the existence of control elements within the Ig loci that modulate the accessibility of nearby gene segments. Since all Ig loci are transcriptionally active at the time of their rearrangement (Lennon and Perry, 1990; Schlissel *et al.*, 1991a), it has been proposed that transcription might be a prerequisite or a signal for the recombination process (Yancopoulos and Alt, 1985; Blackwell *et al.*, 1986; Blackwell and Alt, 1989). It has been shown that induction of germline transcription of κ-light chain loci (Schlissel and Baltimore, 1989) as well as IgH loci (Schlissel *et al.*, 1991b) was accompanied by an increased frequency of gene rearrangement. A direct involvement of *cis*-regulatory elements functioning as enhancers, silencers or promoters which control transcription of Ig genes, was demonstrated in transgenic mice by Ferrier *et al.* (1989, 1990). Artificial mini-loci, consisting of unrearranged T cell receptor Vβ, Dβ and Jβ gene segments linked to the cμ gene either in the presence or absence of the IgH intron enhancer, were employed as substrates for recombination in transgenic mice. The mini-loci lacking the IgH intron enhancer were neither transcribed nor rearranged in any lymphoid or non-lymphoid tissue and conversely, the IgH intron enhancer was sufficient to induce transcription as well as rearrangement of the mini-loci. D–Jβ rearrangement occurred in both T and B cells, but V–DJβ rearrangements were only detectable in T cells (Ferrier *et al.*, 1989, 1990). In contrast, a similar approach, utilizing transgenic κ gene mini-loci, demonstrated that Vκ–Jκ rearrangements occurred in the thymus even though the loci were not being transcribed there (Goodhardt *et al.*, 1989). In any case, it remains unclear whether transcription confers accessibility for V(D)J recombination or whether accessibility of the loci is a prerequisite for both transcription and recombination.

We have addressed this question by deleting the IgH intron enhancer by homologous recombination through the 'Hit and Run' procedure (Hasty *et al.*, 1991; Valancius and Smithies, 1991). This method allows the generation of deletions without introducing any selectable marker genes, which is advantageous when analyzing the effects of regulatory elements like enhancers. Embryonic stem (ES) cells lacking the IgH intron enhancer from one of the their IgH loci were generated and used to produce chimeric mice. Mature, surface IgD^a positive (sIgD^{a+}) B cells, derived from the mutant ES cells, were isolated from chimeric mice by fluorescence activated cell sorting and the rearrangement status of the wild-type and mutant IgH loci was determined by a quantitative polymerase chain reaction method (PCR; Saiki *et al.*, 1985). The wild-type alleles underwent V(D)J recombination in a normal fashion. In striking contrast, rearrangements of the mutant IgH loci, lacking the intron enhancer, occurred less frequently. About 15–30% of the mutant IgH loci were unrearranged. However, despite the absence of the IgH intron enhancer, most (65–80%) of the mutant loci underwent D–J rearrangements and a few (3–6%) V–DJ rearrangements.

Results

Targeted deletion of the IgH intron enhancer via the 'Hit and Run' procedure

The insertion type vector (p5'o3'ΔE/Tk/Neo) used for the targeted deletion of the IgH intron enhancer (E) via the 'Hit

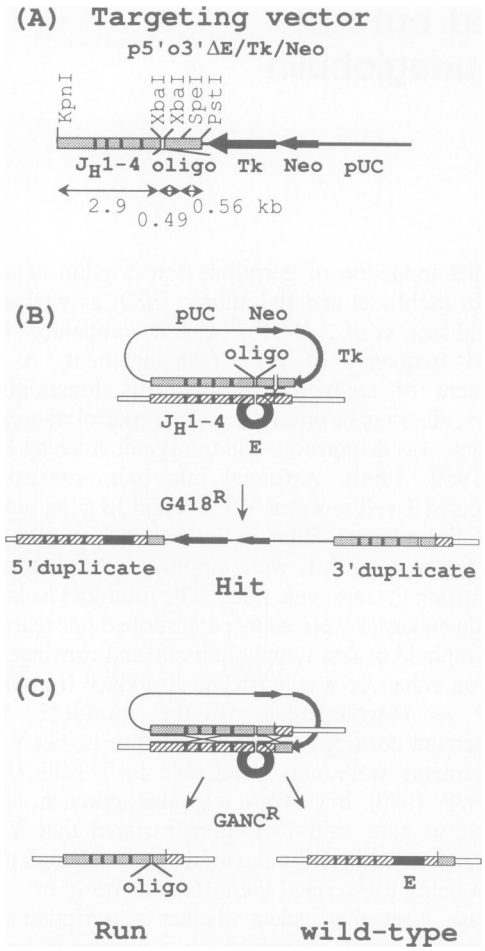

Fig. 1. Schematic description of the 'Hit and Run' procedure to target the deletion of the IgH intron enhancer. (**A**) Targeting vector. The IgH intron enhancer within a J_H fragment (shaded box) bearing the four J_H elements (black bars) is replaced by an oligonucleotide (oligo). Thymidine kinase (Tk, large arrow) and neomycin (Neo, small arrow) gene cassettes were inserted at unique sites of pUC18 plasmid. (**B**) Homologous recombination will lead to a duplication of the targeted J_H region (5′ and 3′ duplicate) and an insertion of the plasmid vector including the selectable marker genes (Tk and Neo). (**C**) Intrachromosomal homologous recombination within the duplicated J_H region will result in the loss of plasmid, Tk, Neo and one of the duplicates. $GANC^R$ clones should either be reverted to the wild-type (E; filled box) or should have the IgH intron enhancer replaced by the oligonucleotide (oligo), resulting in mutant Run clones.

and Run' procedure (Hasty *et al.*, 1991; Valancius and Smithies, 1991) is depicted in Figure 1A. The entire IgH intron enhancer [a 1 kb *Xba*I fragment (Banerji *et al.*, 1983; Gillies *et al.*, 1983; Mercola *et al.*, 1983; Neuberger, 1983; Queen and Baltimore, 1983)] was deleted from a genomic fragment bearing the four joining (J_H1-4) gene segments and replaced by an oligonucleotide (oligo) serving as a diagnostic nucleotide sequence. The neomycin resistance gene (Neo; Thomas and Capecchi, 1987) and thymidine kinase gene (Tk; Mansour *et al.*, 1988) cassettes were inserted into the vector backbone and the plasmid DNA was linearized within the region of homology at the *Spe*I site.

129/Sv derived ES cells (D3; Doetschman *et al.*, 1985) were electroporated with the targeting vector and G418 resistant ($G418^R$) clones were screened for homologous recombinants (Hit clones) by PCR using primers specific for the oligonucleotide ('oligo' in Figure 2B) and a region 3′ of the targeted J_H locus ('5′cμ' in Figure 2B). Homologous recombination leads to duplication of the targeted J_H locus inserting both selectable marker genes in between the duplicated J_H locus (Figure 1B). The deletion of the IgH intron enhancer will be introduced either into the 5′-, 3′- or both duplicates (Hasty *et al.*, 1991). However, due to the choice of the PCR primers described above, we selected for Hit clones lacking the enhancer in their 3′ duplicate (Figure 2C). The identification of four Hit clones (1/120 $G418^R$ clones) was verified by Southern blotting (Figure 2D and data not shown). In addition to the wild-type bands (13.7 kb *Kpn*I, 9.0 kb *Bam*HI and 6.5 kb *Eco*RI), the diagnostic bands for the targeted alleles of the Hit clones (6.0 and 2.9 kb for *Kpn*I; 5.4 and 1.3 kb for *Bam*HI and 14.8 kb for *Eco*RI) are present (Figure 2D).

Intrachromosomal recombination within the duplicated J_H locus of Hit cells can result in the loss of the Tk gene, selected as gancyclovir resistant ($GANC^R$; Mansour *et al.*, 1988) clones (Figure 1C). Depending upon the resolution of the recombination structure, either revertant wild-type clones or mutant Run clones lacking the intron enhancer will be created. $GANC^R$ clones were pre-screened by PCR (see Figure 2) and analyzed by Southern blotting (Figure 2E and data not shown). The hybridizing *Kpn*I fragments (13.7, 6.0 and 2.9 kb) of five $GANC^R$ clones (Figure 2E, lanes marked with H) were indistinguishable from the parental Hit cells (Figure 2D). The loss of the 6.0 and 2.9 kb bands indicated that two $GANC^R$ clones were reverted to wild-type (Figure 2E, lanes marked with WT). Six $GANC^R$ clones turned out to be Run clones (Figure 2E, lanes marked with R). They had lost the 6.0 kb *Kpn*I fragment but retained the 13.7 kb wild-type fragment and the 2.9 kb fragment which is specific for the targeted deletion of the IgH intron enhancer.

Isolation of ES cell derived, splenic $sIgD^{a+}$ B cells from chimeric mice

To investigate the effects of IgH intron enhancer deletion on V(D)J recombination, a mutant Run clone was injected into blastocysts from C57BL/6 mice to produce chimeric animals. To isolate ES cell derived, mutant B cells from chimeric mice, the allotype specific monoclonal antibody 10-4-22 (Oi and Herzenberg, 1979) was used, which discriminates between $sIgD^{a+}$ (ES cell derived) and $sIgD^{b+}$ (C57BL/6 host derived) B cells. Spleen cells were prepared from two chimeric animals and the ES cell derived $sIgD^{a+}$ B cells were isolated by cell sorting utilizing a magnetic activated cell sorter (MACS; Miltenyi *et al.*, 1990) and, subsequently, a fluorescence activated cell sorter (FACStar plus; Becton-Dickinson). Starting from a population of spleen cells comprising ~2.5% $sIgD^{a+}$ B cells, the sorted population contained >95% $sIgD^{a+}$, ES cell derived, mutant B cells (data not shown). As a control, wild-type $sIgD^{a+}$ B cells were isolated in the same way from the spleen of a 129/Sv mouse (data not shown).

Deletion of the IgH intron enhancer impairs but does not block V(D)J recombination

DNA rearrangements of control $sIgD^{a+}$ B cells [carrying two wild-type (WT) IgH loci] and of ES cell derived

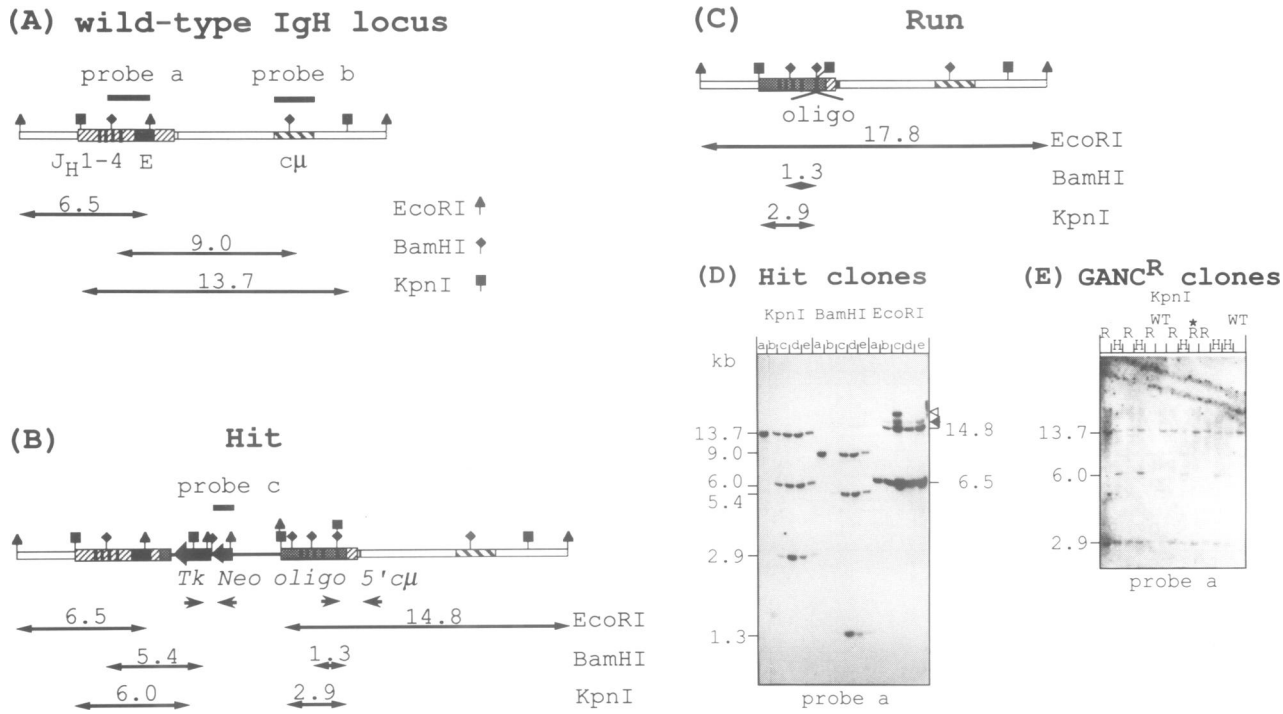

Fig. 2. Molecular analysis of the targeted Hit and Run clones. Genomic structure of the J_H-cμ gene locus in the wild-type configuration (**A**), of the predicted targeted genome of Hit (**B**) and Run (**C**) clones. The probes used for Southern blot analyses and the lengths of the diagnostic restriction fragments for *Eco*RI, *Bam*HI and *Kpn*I are shown. Small arrows indicate primers used for PCR to identify Hit and Run clones, respectively. (**D**) Southern blot analysis of Hit clones. Genomic DNA isolated from D3 (lane a) and four PCR positive Hit clones (lanes b, c, d and e) was digested with either *Kpn*I, *Bam*HI or *Eco*RI. Unexpected bands present in the *Eco*RI digest are due to incomplete digestion (marked with a filled arrow) or due to an additional copy of the targeting vector in one of the Hit clones (lane b; marked with an open arrow). (**E**) Southern blot analysis of $GANC^R$ clones. DNA of 13 $GANC^R$ clones, derived from Hit clone e, was digested with *Kpn*I (Run, R; WT, wild-type; H, Hit). The Run clone marked with R* was used to generate chimeric mice.

$sIgD^{a+}$ B cells [carrying a wild-type and a mutant (ΔE) IgH locus] were analyzed by quantitative PCR and subsequent Southern hybridization using a J_H specific probe (probe a in Figure 2A) to visualize the PCR products (Figures 3 and 4). As schematically shown in Figure 3A, DNA of the wild-type and/or mutant J_H loci can be amplified using the primers E (WT specific), oligo (ΔE specific) or 3′E (detecting both alleles) in combination with primer Mu0 (Schlissel *et al.*, 1991a), primer D_HL (Schlissel *et al.*, 1991a) or the V_H-specific primers (V_HJ558, V_HQ52 or V_H7183; Schlissel *et al.*, 1991a) to distinguish between unrearranged J_H loci and rearranged DJ_H or VDJ_H loci, respectively.

Within the control $sIgD^{a+}$ B cells (WT/WT) all J_H loci were recombined either to DJ (Figure 3B; lane 4) or VDJ (Figure 3B; lanes 1–3). Using the co-amplified *Id2* gene (Sun *et al.*, 1991) as an internal standard, quantification of the hybridization signals revealed that ~35–37% of the J_H loci carried a DJ rearrangement and 63–65% a VDJ one. Considering that we analyzed most (the J558 V_H gene family is by far the largest one) but not all members of the 14 V_H gene families (Kofler *et al.*, 1992), these data are in good agreement with the theoretically expected and previously determined ratio of 30% DJ to 70% VDJ rearrangements (Alt *et al.*, 1984; Hardy *et al.*, 1991). A residual germ line band (<2%) seen in Figure 4c (lane 5) is most probably due to a few contaminating non-B cells.

The rearrangement pattern detected in ES cell derived, mutant $sIgD^{a+}$ B cells was dependent upon the presence (wild-type) or absence (mutant) of the IgH intron enhancer.

Quantitative PCR analysis (Figure 3 and 4; summarized in Table I) demonstrated that almost all (95–99%) of the wild-type alleles within the mutant B cells carried a VDJ rearrangement (Figure 3B; lanes 5–7) and ~1–5% of them showed a DJ rearrangement (Figure 3B; lane 8). A residual germ line band (<2%) seen in Figure 4c (lane 10) is again most likely due to some contaminating non-B cells.

In striking contrast to the wild-type alleles from either control (Figure 4b and c; lane 5) or ES cell derived (Figure 4b and c; lane 10) $sIgD^{a+}$ B cells, a substantial fraction (~15–30%) of the mutant ΔE alleles remained in germline configuration (Figure 4a and b; lane 10). This result indicates that the V(D)J recombination machinery is less efficient in the absence of the IgH intron enhancer and implies a regulatory function of the intron enhancer for the joining mechanism. On the other hand, the mutant alleles did undergo V(D)J rearrangements despite the absence of the intron enhancer. About 65–80% of the mutant ΔE alleles underwent D–J rearrangements (Figure 3B; lane 12). V–DJ rearrangements could also be detected with all three V_H specific primers (Figure 3B; lanes 9–11). However, they were much less abundant (~3–6%) than DJ rearrangements and were only clearly visible after prolonged PCR amplification (data not shown). These results indicate that the deletion of the intron enhancer did not lead to a total block of the joining mechanism. Apparently, the accessibility of the IgH loci for the joining machinery is regulated via the intron enhancer in combination with other *cis*-regulatory element(s) which might function like an enhancer of recombination or a locus control element.

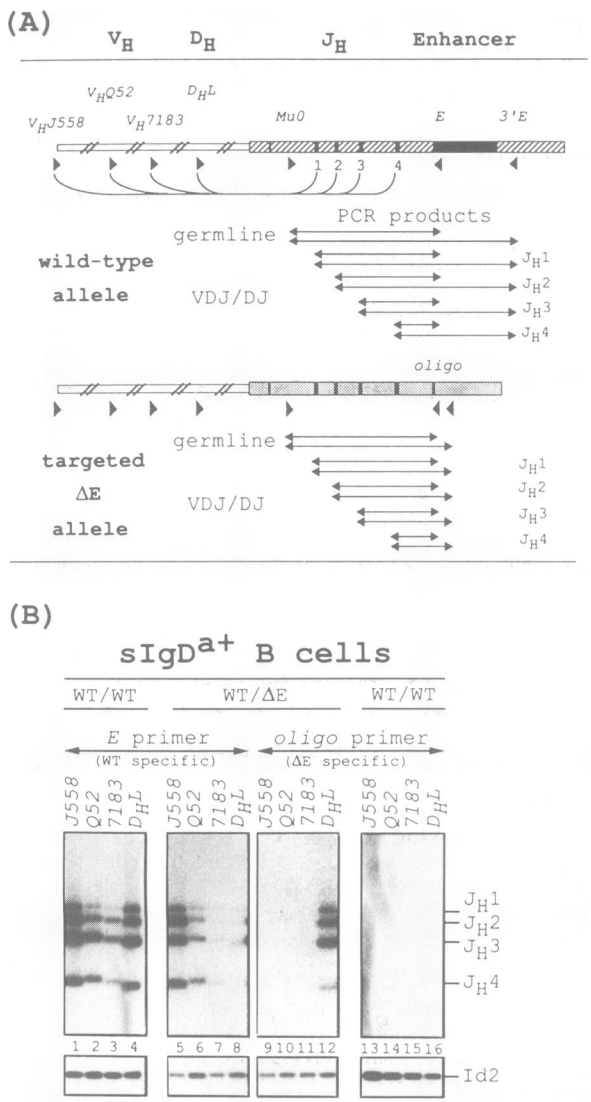

Fig. 3. Quantitative PCR analysis of V(D)J recombination in control (129/Sv derived) and mutant (ES cell derived) surface IgD^{a+} B cells. (**A**) Schematic representation of wild-type (hatched) and targeted ΔE (stippled) IgH alleles in germline configuration of ES cell derived sIgD^{a+} B cells. The J_H1-4 gene elements, the IgH intron enhancer and the oligonucleotide replacing the IgH intron enhancer are indicated as in Figures 1 and 2. The primers used for PCR analysis are depicted as filled triangles. The PCR products expected for the various primer combinations are indicated below the IgH alleles as double-headed arrows. (**B**) Quantitative PCR analysis of the rearrangement status present in control, 129/Sv derived (WT/WT; lanes 1–4 and 13–16) and mutant, ES cell derived (WT/ΔE; lanes 5–12) sIgD^{a+} B cells. The enhancer specific primer E (lanes 1–8) or the deletion specific primer oligo (lanes 9–16) were used in combination with either the V_H specific or D_H specific primers as indicated. The resulting four PCR products which hybridized to the J_H specific probe a (see Figure 2A) correspond to the various VDJ or DJ rearrangements. As an internal standard for the quantification, genomic DNA of the *Id2* gene (Sun *et al.*, 1991) was co-amplified. A subclone of the *Id2* gene (Inge van Crüchten and F.Sablitzky, unpublished) was used for hybridization of the same, stripped filter.

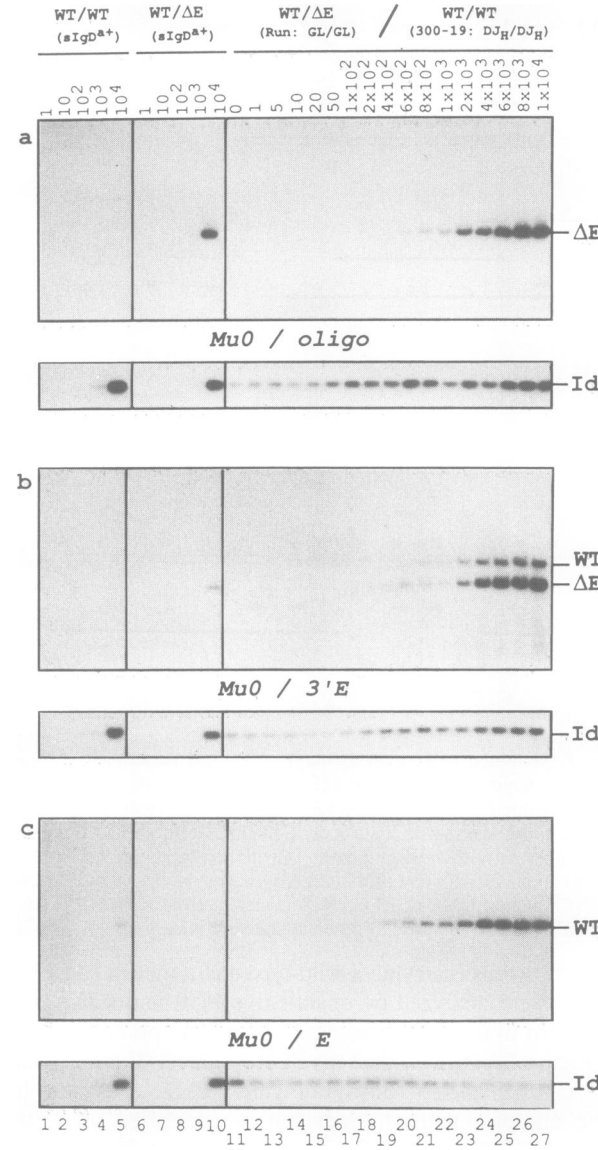

Fig. 4. Quantitative PCR analysis determining the amount of unrearranged germline configuration in control (129/Sv derived) and mutant (ES cell derived) surface IgD^{a+} B cells. 129/Sv derived (WT/WT; lanes 1–5) and mutant, ES cell derived (WT/ΔE; lanes 6–10) sIgD^{a+} B cells were analyzed using primer Mu0 in combination with the primers oligo (**a**), 3'E (**b**) or E (**c**) respectively. Depending upon which primer was used, wild-type (WT) and/or mutant (ΔE) J_H loci will be amplified. As a defined standard a dilution of mutant Run cells (WT/ΔE; both IgH loci unrearranged) in 300-19 cells (WT/WT; both IgH loci carry a DJ rearrangement; Reth *et al.*, 1986) was used. The number of unrearranged mutant and wild-type alleles present in a constant amount of DNA (equivalent to 10^4 cells) are indicated (lanes 11–27). As a internal standard for the quantification, genomic DNA of the *Id2* gene (Sun *et al.*, 1991) was co-amplified as described above.

Functional VDJ rearrangements might be expressed independently of the IgH intron enhancer

Since we analyzed mature, sIgD^a expressing B cells, it is evident from our data that almost all of the functionally rearranged and expressed VDJ joints were derived from the wild-type rather than the mutant allele. However, some DJ rearrangements (1–5%) could be detected on the wild-type allele (Figure 3B; lane 8). In order to test whether these DJ rearrangements are indeed present in ES cell derived sIgD^{a+} B cells or whether they are due to contaminating C57BL/6 derived sIgD^{b+} B cells, PCR products of the amplified DJ rearrangements were digested with *Dde*I which allows the distinction between the *a* and *b* alleles of the J_H locus (F.Sablitzky, unpublished). Subsequent analysis by Southern hybridization using a J_H3 specific probe (Figure 5) indicated that most of the detected DJ rearrangements were indeed due to contamination. However, a small

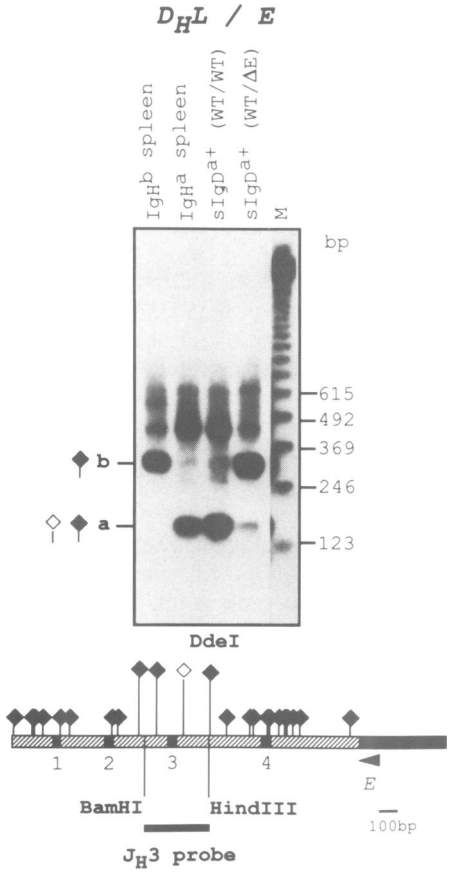

Fig. 5. Discrimination of DJ rearrangements derived from either IgH^a or IgH^b alleles. DJ rearrangements of 129/Sv (IgH^a) and C57Bl/6 (IgH^b) spleen cells and control sIgD^{a+} (WT/WT) and mutant sIgD^{a+} (WT/ΔE) sorted B cells were amplified (35 cycles) using primer D_HL in combination with the wild-type specific primer E. The PCR products were cut with the restriction enzyme *Dde*I (filled and open rhombi) prior to Southern analysis. Due to a nucleotide difference, the IgH^b allele lacks a *Dde*I site 3′ of J_H3 (open rhombus) which is present in the IgH^a allele (F.Sablitzky, unpublished) allowing the distinction of the *a* and *b* alleles of the J_H locus. Using a J_H3 probe (*Bam*HI−*Hin*dIII fragment) for hybridization, the *b* allele gives rise to a 327 bp fragment (indicated with b) whereas the *a* allele gives rise to two similar sized fragments of 163 and 164 bp, respectively (indicated with a). The common smaller fragment (103 bp) hybridizes weakly and is only visible on longer exposures (data not shown). As a molecular weight marker (M) a 123 bp ladder (Gibco-BRL) was used.

fraction (estimated to be <0.1%) of the DJ rearrangements in the wild-type allele were contributed by ES cell derived sIgD^{a+} B cells. Together with the finding that some mutant ΔE alleles carried a VDJ rearrangement, these results suggest that even in the absence of the IgH intron enhancer, a functional VDJ rearrangement can occur, is transcriptionally activated and is expressed. Similarly, earlier data demonstrated that the spontaneous deletion of the IgH intron enhancer in variants of Ig expressing cell lines did not reduce the expression of the Ig loci (Klein *et al.*, 1984, 1985; Wabl and Burrows, 1984; Zaller and Eckhardt, 1985).

Discussion

The IgH intron enhancer modulates V(D)J recombination in B cells

Our present results, summarized in Table I, are in agreement with the data obtained by Ferrier *et al.* (1989, 1990) and demonstrate an important regulatory function of the IgH intron enhancer for the process of V(D)J recombination. Deletion of the intron enhancer resulted in a clear reduction of V(D)J recombination. 15−30% of the mutant IgH loci remained in a germline configuration, in striking contrast to the wild-type alleles. Deletion of the *Xba*I fragment abolished all known transcriptional *cis*-regulatory elements within the intron enhancer, including the promoter for the Iμ sterile transcript (Lennon and Perry, 1985; Su and Kadesch, 1990). It remains to be shown whether this loss of transcriptional regulation was the cause of the impaired V(D)J recombination. Alternatively, *cis*-regulatory elements within the intron enhancer might function as recombination enhancers by binding proteins involved in the rearrangement process. Finally, deletion of the *Xba*I fragment also removed the matrix association regions (MARs) which surround the enhancer core element (Cockerill *et al.*, 1987). Presumably this deletion of the MARs resulted in an alteration of the chromatin structure which might have decreased the accessibility of the mutant IgH loci.

Table I. Summary of the quantitative PCR analyses

	sIgD^{a+} splenic B cells		
	Control	ES cell derived	
	(WT/WT)	(WT)	(ΔE)
Germline	<2%[a]	<2%[a]	15−30%
DJ	35−37%[b]	1−5%[c]	65−80%
VDJ	63−65%[b]	95−99%	3−6%

[a]Germline J_H loci are contributed by a few contaminating non-B cells.
[b]Obtained values are in accord with the analyses of Alt *et al.* (1984) and Hardy *et al.* (1991)
[c]Less than 0.1% of the DJ rearrangements are derived from sIgD^{a+} B cells (Figure 5)

The IgH intron enhancer is not essential for D−J recombination

The targeted deletion of the IgH intron enhancer demonstrated that the V(D)J recombination machinery is functional, albeit less efficient, in the absence of the intron enhancer. 65−80% of the mutant IgH loci underwent D−J rearrangements. Additional *cis*-regulatory element(s) outside the intron enhancer have to be postulated which together with the intron enhancer regulate the accessibility of the IgH locus for the joining mechanism. This element could regulate transcription. One candidate involved in the regulation of V(D)J recombination is the recently identified 3′ enhancer (Pettersson *et al.*, 1990; Dariavach *et al.*, 1991; Lieberson *et al.*, 1991). However, the 3′ enhancer, which would have to function over a very long distance, seemed to be activated at late stages of B cell development (Dariavach *et al.*, 1991).

Alternatively, the accessibility of the IgH locus for the recombination machinery could be controlled by as yet unknown *cis*-regulatory elements which might function as an enhancer of recombination or a locus control element. The data established by Ferrier *et al.* (1989, 1990) using artificial mini-loci as substrates for the recombination machinery in transgenic mice are compatible with this interpretation, assuming that the recombination substrates lacked these putative *cis*-regulatory elements.

V−DJ rearrangements might depend more upon the IgH intron enhancer than D−J rearrangements

Within the ES cell derived, mature sIgD^{a+} B cells, almost all (95−99%) of the wild-type IgH loci underwent VDJ rearrangements. The fraction of sIgD^{a+} B cells carrying VDJ joints in the mutant alleles is rather small (3−6%). Even though the mutant IgH loci are less frequently rearranged, one would expect to find more VDJ rearrangements in the mutant loci. On the other hand, a functional VDJ joint in the mutant loci lacking the intron enhancer might not be expressed sufficiently to give rise to sIg positive B cells. We detected, however, a small fraction of sIgD^{a+} B cells (estimated to be $<0.1\%$; see Figure 5) that carried DJ joints on the wild-type loci. This result predicts functional VDJ rearrangements in the mutant alleles which, despite the lack of the intron enhancer, were sufficiently transcribed to give rise to sIgD^{a+} B cells. Transcription of the IgH loci was in fact not reduced in variants of Ig expressing plasma cell lines which had spontaneously deleted the IgH intron enhancer (Klein *et al.*, 1984, 1985; Wabl and Burrows, 1984; Zaller and Eckhardt, 1985). At present we do not know whether transcription of IgH loci lacking the intron enhancer is generally sufficient to give rise to sIg expressing B cells. If this was the case, our data suggest that V−DJ joining is more dependent upon the presence of the IgH intron enhancer than D−J joining. Rearrangements of the mutant IgH loci would be locked once a DJ joint had occurred. This interpretation is in line with the results obtained by Takeda *et al.* (see accompanying paper) who demonstrated that Vκ−Jκ rearrangements were completely abolished through replacement of the Igκ intron enhancer by a Neo gene cassette.

IgH loci lacking the intron enhancer appear to be normal targets for allelic exclusion

We have shown that deletion of the IgH intron enhancer resulted in a clear reduction of V(D)J rearrangements in the mutant IgH loci. A substantial fraction (15−30%) of the mutant alleles within mature sIgD^{a} expressing B cells remained in the germline configuration. 65−80% of the mutant alleles, however, underwent D−J rearrangements, indicating that the IgH loci are still accessible to the recombination machinery in the absence of the IgH intron enhancer. These results are quantitatively different from the one obtained by J.Chen, F.Young and F.Alt (in preparation) who exchanged the IgH intron enhancer by homologous recombination with a Neo gene cassette and analyzed Abelson virus transformed pre-B cell lines derived from chimeric mice (Alt *et al.* 1992; F.W.Alt, personal communication). Within the collection of pre-B cell lines, ~90% of the mutant IgH loci remained in the germline configuration and ~10% were rearranged (Alt *et al.* 1992; F.W.Alt, personal communication). The different results could be due to the experimental approaches used. Compared with a deletion of the IgH intron enhancer, the replacement by a Neo gene cassette could distort the resulting effects on V(D)J recombination. Experiments by Takeda *et al.* (see accompanying paper) demonstrated that the insertion of a Neo gene cassette at the 3′-end of the Igκ intron enhancer resulted in a partial reduction of Vκ−Jκ joining whereas the replacement of the Igκ intron enhancer by the Neo gene blocked Vκ−Jκ rearrangements completely.

Alternatively, the observed differences could be due to the different subsets of B cells analyzed. The deletion (or replacement) of the IgH intron enhancer clearly alters the IgH loci in such a way that they are less frequently rearranged than the wild-type alleles. This will result in the generation of pre-B cells which carry a DJ joint at the wild-type locus and are unrearranged at the mutant locus ($DJ_{WT}/J_{\Delta E}$) as was observed by J.Chen, F.Young and F.Alt (in preparation). Many of these pre-B cells will continue to rearrange the wild-type ($V-DJ_{WT}$) rather than the mutant ($D-J_{\Delta E}$) locus. In the case of a functional rearrangement the resulting genotype will be $VDJ^{+}{}_{WT}/J_{\Delta E}$. This situation closely resembles the one of μ-transgenic mice (reviewed by Storb, 1987). Despite the presence of a functional heavy chain encoded by a μ transgene, endogenous IgH loci underwent D−J rearrangements. About 63% of the endogenous IgH loci of μ-transgenic hybridomas were arrested at the DJ stage (Rusconi and Köhler, 1985). Assuming that the deletion of the IgH intron enhancer did not affect the regulation of allelic exclusion, pre-B cells with the genotype $VDJ^{+}{}_{WT}/J_{\Delta E}$ will continue to rearrange the mutant IgH loci and thereby would fill up the pool of cells with a $VDJ^{+}{}_{WT}/DJ_{\Delta E}$ genotype. At this point rearrangement of the mutant allele would stop due to allelic exclusion. Mature sIgD^{a} expressing B cells will accordingly carry more DJ joints on the mutant IgH loci than do the Abelson virus transformed pre-B cell lines analyzed by J.Chen, F.Young and F.Alt (in preparation).

Materials and methods

Construction of the p5′o3′ΔE/Tk/Neo targeting vector

A 1 kb *Xba*I fragment containing the IgH intron enhancer was deleted from a 4.9 kb *Kpn*I−*Pst*I fragment [nucleotide positions 1−4906 of MUSIGCD07 of the GenBank/EMBL sequence data library (Devereux *et al.*, 1984)] bearing the gene elements J_H1-4 and replaced by a 20 bp oligonucleotide ('oligo', 5′-CTAGACTGGATCCGGTACCT-3′). A *Xho*I−*Hin*dIII fragment from pIC19R/MC1-TK (Mansour *et al.*, 1988) containing the Tk gene cassette was inserted into the unique *Nar*I site of pUC18 and a *Xho*I−*Hin*cII fragment from pMC1NeopA (Thomas and Capecchi, 1987) containing the Neo gene cassette was inserted into the unique *Nde*I site of the plasmid. The insertion type targeting vector was linearized at the *Spe*I site within the region of homology.

Isolation of mutant ES cells lacking the IgH intron enhancer and generation of chimeric mice

Growth and G418 selection of electroporated D3 cells (Doetschman *et al.*, 1989) was performed as described by Kitamura *et al.* (1991). To isolate Hit clones, G418^{R} cells were analyzed by PCR essentially as described by Brady *et al.* (1990) and Kitamura *et al.* (1991) using the primers oligo (see above) and 5′cμ (5′-GCGCTCAGAGAAGCCCACCC-3′) which is located 3′ of the J_H locus (nucleotide position 5064 in MUSIGCD07; Devereux *et al.*, 1984).

To isolate Run clones, 5×10^{5} Hit cells were directly plated in GANC (8 μM Cymeven; Syntex) and after 10−14 days of selection individual GANC^{R} clones were screened by PCR for the loss of Tk and Neo genes by using a Tk-specific primer (5′-CGCGAGAACGCGCAGCCTG-3′) and a Neo-specific primer (5′-TCCATCATGGCTGATGCAATGC-3′).

The PCR conditions were as follows: 10 mM Tris−HCl pH 8.3, 50 mM KCl, 0.01% gelatin, 1.5 mM $MgCl_2$, 0.15 μM each primer, 0.1 mM each dNTP and 1 U *Taq* polymerase (Gibco-BRL). After denaturation (10 min at 92°C) amplification was performed in 40 cycles [1.5 min at 92°C, 3 min at 64°C (Tk and neo primers) or 65°C (oligo and 5′cμ primers), 3.5 min at 72°C)] with an additional cycle at the end (1.5 min at 92°C, 3 min at 64°C or 65°C, 15 min at 72°C).

Mutant ES cells were injected into C57BL/6 derived blastocysts to generate chimeric mice as described by Bradley *et al.* (1984).

Isolation of ES cell derived mutant B cells by flow cytometry

1.9×10^{8} spleen cells from two 18 week old chimeric animals were labelled with the biotin conjugated monoclonal antibody 10-4-22 (Oi and Herzenberg,

1979) at an optimal concentration of 10 μg/ml. Labelled cells were incubated with streptavidin conjugated magnetic microbeads (MACS no. 181-01; Miltenyi *et al.*, 1990) and subsequently with fluorescein isothiocyanate (FITC) conjugated streptavidin (BAS; 6 μg/ml). ES cell derived, $sIgD^{a+}$ B cells were enriched on an A1 MACS column (Miltenyi *et al.*, 1990) and subsequently isolated by cell sorting using a FACStar plus (Becton-Dickinson).

Quantitative PCR analysis of V(D)J rearrangements

Genomic DNA from 5×10^4 sorted $sIgD^{a+}$ B cells was prepared as described by Brady *et al.* (1990) and DNA, equivalent to various amounts of cells, was amplified as described by Brady *et al.* (1990) and Schlissel *et al.* (1991a). The primers used are as follows: The 5′-primers are specific for either the third framework region of members of three V_H gene families (V_HJ558, V_HQ52 and V_H7183; Schlissel *et al.*, 1991a; Kofler *et al.*, 1992), for the 5′-end of all members of the DFl16 and DSP2 gene families (D_HL; Kurosawa and Tonegawa, 1982; Schlissel *et al.*, 1991a) or for a region 5′ of J_H1 (Mu0; Schlissel *et al.*, 1991a; nucleotide position 700 in MUSIGCD07; Devereux *et al.*, 1984). The 3′-primers are specific for either the IgH intron enhancer (E: 5′-GCAGGCTCCACCAGACCT-3′; nucleotide position 2884 in MUSIGCD07), a region 3′ of the IgH intron enhancer (3′E: 5′-GGCAGAAGCCACAACCATAC-3′; nucleotide position 4066 in MUSIGCD07) or the oligonucleotide (oligo: 5′-AGGTACCGGATCCA-GTCTAG-3′). As an internal standard for the quantification, genomic DNA of the *Id2* gene (Sun *et al.*, 1991) was co-amplified using a primer pair specific for its 5′-end (5′-GGTGGATCCACCATGGCAATTCAGGG-ATGC-3′) and its 3′-end (5′-GGCGGATCCTTATTTAGCCACAGAG-TAC-3′). To assure linear amplification the numbers of cycles was limited to 23 (V_H or D_HL primers in combination with either the oligo primer or the E primer), 20 (Mu0 primer in combination with either the oligo primer or the E primer) or 22 (Mu0 primer in combination with the 3′E primer).

Southern blots of the amplified PCR products were hybridized first with a J_H3-4 specific fragment (probe a in Figure 2) and subsequently with a subclone of the *Id2* gene (Inge van Crüchten and F.Sablitzky, unpublished). Quantification was performed by scanning (UltroScan Laser Densitometer LKB 2202) autoradiographs of different exposure times. Relative intensities of the bands were equalized using the *Id2* gene as a reference.

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