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Independent Control of Immunoglobulin Switch Recombination at Individual Switch Regions Evidenced through Cre-"loxP"-Mediated Gene Targeting

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Summary

We have employed a method based on the Cre-"loxP" recombination system of bacteriophage P1 to generate a mouse strain in which the Jδ segments and the intron enhancer in the IgH locus are deleted. By analysis of immunoglobulin isotype switch recombination in heterozygous mutant B cells activated by lipopolysaccharide plus interleukin-4, we show that, on the mutant chromosome, switch recombination at the μ gene switch region is strongly suppressed, whereas the switch region of the γ1 gene is efficiently rearranged. These data demonstrate an independent control of switch recombination at individual switch regions and suggest that, in the process of switch recombination, the alignment of the recombining strands occurs independently of and probably after the introduction of double-strand breaks into the switch regions involved.

Introduction

Immunoglobulin genes are unique in the sense that a functional immunoglobulin (Ig) gene must be assembled through gene rearrangement from several individual gene segments during B lymphocyte development. In general, there are two types of gene rearrangements occurring at the Ig gene loci: V(D)-J gene rearrangement and Ig isotype switch recombination. The former refers to a process that generates the Ig variable region genes by sequential rearrangements of a Dν to a Jν segment and, subsequently, of a Vγ element to the DγJγ complex at the heavy chain locus (Igh) and of a Vε to a Jε segment at the light chain loci (reviewed by Alt et al., 1987). The latter is a rearrangement process at the heavy chain locus, which excises the μ and δ chain constant region (Cμ) genes together with adjacent DNA sequences and brings a downstream IgC gene proximal to the rearranged VμDμJμ gene (Honjo and Kataoka, 1978; Cory et al., 1980; Rabbits et al., 1980). The outcome of switch recombination is that a B cell can thereafter express, instead of μ and δ chains, another Ig isotype.

The molecular basis of Ig switch recombination has been extensively studied in the past (reviewed by Gritzner, 1989). In the mouse, the IgC genes are dispersed over a 200 kb distance on the chromosome and organized in the order of Jμ-(Eμ-Cμ)-Cμ-Cε-2-Cε-2-Cε-2 (Shimizu et al., 1982). It is now generally accepted that switch recombination usually occurs between defined Ig switch region (S region) sequences that are located upstream of each of the C genes except Cε (Honjo et al., 1989). Although the detailed molecular mechanism of switch recombination remains obscure, recent experiments strongly support the looping-out deletion model, which predicts that switch recombination involves the alignment of two S region sequences and then deletion, by looping out, of the intervening chromosomal DNA between two partner S region sequences on the same chromosome (Honjo and Kataoka, 1978; von Schwedler et al., 1990; Iwasu et al., 1990; Matsuoka et al., 1990).

In a given B cell, switch recombination is usually directed to the same S regions on both homologous chromosomes (Radbruch et al., 1986; Hummel et al., 1987). In this control, lymphokines appear to play a critical role. Thus, interleukin-4 (IL-4) promotes Ig isotype switching from μ to γ1 and ε (Layton et al., 1984; Coffman et al., 1986), and such isotype switching can be specifically inhibited by interferon γ (Snapper and Paul, 1987). At the molecular level, IL-4 specifically induces germine (sterile) transcripts of the C1, C2, and Cε genes in lipopolysaccharide (LPS)-activated B cells. This transcription can be inhibited by interferon γ, which, instead, induces sterile transcripts from the C2a gene (Rothman et al., 1986; Stavnezer et al., 1988; Esser and Radbruch, 1990). Recent experiments have further identified a presumed promoter/enhancer element located upstream of S1 region. This element can be activated to initiate the transcription of the C1 gene in B cells in the presence of LPS plus IL-4 (Xu and Stavnezer, 1992). These observations are consistent with a previous model that proposed that sterile transcription through the target IgH locus may render the S region of the corresponding Ig gene accessible for the switch recombination machinery (Stavnezer-Nordgren and Sirlin, 1986; Yancopoulos et al., 1986).

In the past, the unequivocal identification of cis-acting control elements involved in Ig gene rearrangements has been hampered by the lack of appropriate techniques. In the present study, we have developed a gene targeting strategy that allows the targeted deletion of genomic DNA sequences at high frequency and is based on the Cre-"loxP" system of bacteriophage P1 (Stemberg and Hamilton, 1981; Sauer and Henderson, 1988). Using this technique, we have deleted the Jμ-Eμ sequences of the IgH locus in mouse embryonic stem (ES) cells and generated mice carrying this mutation, which we refer to as JδT. The analysis of the mutant mice indicates that the JδT mutation profoundly affects class switch recombination in cis.

Results

Generation of the JδT Mutation in ES Cells Using the Cre-"loxP" System

A general method, based on the Cre-"loxP" recombination system, has been used to delete a gene segment within precision in ES cells. This method involves two steps (Figure 1). In the first step, two loxP sites are introduced at
Figure 1. Strategy of the Cre–loxP-Based Deletional Gene Targeting
Shown from top to bottom are DNA structures of a germline gene to be deleted, the targeting construct, the predicted homologous recombinant, and the deletion product generated by the Cre enzyme. loxP sites are depicted as closed triangles.

Figure 2. Identification of the Targeted Cointegration of the loxP Site and HSV-tk–neo’ Gene in ES Cells
Shown is a Southern blot of genomic DNA from parental (lane 1) and two targeted ES cell lines, JC7 (lane 2) and JC9 (lane 3). The genomic DNA was digested with BamHI, and the genomic structure of the joining (J) and constant region of immunoglobulin heavy chain a gene is depicted under the blot. Exons are represented as closed boxes. The closed circle represents the IgH intron enhancer. loxP sites are depicted as triangles. B, BamHI. Probes A and B were used to identify the homologous recombination events at the 3’ (right) and 5’ (left) regions of the Jα–Eα fragment, respectively.

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the borders of the gene segment to be deleted, through homologous recombination (Thomas and Cappechi, 1987). In the second step, the Cre enzyme is transiently expressed in the targeted ES cells. Cre catalyzes a loxP site–dependent recombination, so that the DNA sequences between the two loxP sites are efficiently excised from the chromosome, leaving a single loxP site behind. To facilitate the selection of cells harboring an integrated targeting vector in the first step and of cells having undergone Cre-mediated DNA recombination in the second, tandemly linked bacterial neomycin resistance (neo’)) and herpes simplex virus thymidine kinase (HSV-tk) genes are used in the targeting vector, to serve as positive and negative selection markers.

To generate the JαT mutation in ES cells, the targeting vector pGH102 was used to introduce two loxP sites into the Jα–Eα locus, as shown in Figure 2. After transfection with the linearized vector and selection with G418, homologous recombinants were first identified by polymerase chain reaction (PCR) from the G418 clones and subsequently confirmed by Southern blot analysis. The targeted clones are predicted to give a 7.8 kb BamHI band in addition to a 8.7 kb germline band when hybridized with the probe B (Figure 2). From a total of 432 clones tested, we obtained 13 homologous recombinants, representing a frequency of 1 in 34 G418 clones. To determine whether these clones also carried a second loxP site upstream of the DQ52 element, we performed Southern blot analysis using probe A (Figure 2). Two clones, JC7 and JC9, showed the expected band of 1.8 kb (Figure 2), demonstrating that they carry the co-integrated loxP site 5’ of DQ52. The frequency of co-integration of the second loxP site and the neo–tk gene cassette was 1 in 216 G418 clones.

Two types of plasmids were used to express the Cre enzyme in ES cells: plasmid pCM-Cre contains the original cre gene (Stemberg et al., 1986), except that the DNA sequences surrounding the initiation ATG codon were changed according to Kozak (Kozak, 1986; see also Experimental Procedures); plasmid pMC-Cre carries an additional insertion of 21 bp 3’ of the initiation ATG codon of the cre gene, encoding the nuclear location signal peptide of the SV40 large T antigen (Kalderon et al., 1984). After transfection with supercoiled Cre-encoding plasmid DNA,
ES cells that had undergone Cre–loxP-mediated recombination were selected by gancyclovir. The surviving clones were screened by PCR and Southern blot analysis for the deletion event (Figure 3). In pIC-Cre transfection experiments, we consistently found that approximately 2% of plated ES cell clones had deleted the Jn–Ei fragment (Table 1). The deletion frequency increased 2-fold when the pMC-Cre was used for transfection (Table 1 and data not shown). The increased efficiency of pMC-Cre is presumably due to a rapid accumulation of the Cre enzyme in the cell nucleus. We did not detect additional bands in the Southern blot analysis when we used a 3' Ei DNA fragment (Figures 3 and 7) or a Jn fragment (data not shown) as probes. This argues against integration of the excised fragment elsewhere in the genome as well as an unexpected duplication of (part of) the locus. The 3' Ei fragment probe also did not reveal unexpected bands in the Southern blot analysis of hybridomas isolated from heterozygous mutant mice (data not shown).

To estimate the efficiency of transient transfection under our experimental conditions, we transfected the ES cells with a similar amount of pON405, a plasmid encoding Escherichia coli β-galactosidase (see Experimental Procedures). Staining for β-galactosidase activity repeatedly revealed that approximately 5% of the transfected ES cells expressed this protein (data not shown). Assuming that the transient transfection efficiency in ES cells is the same for the Cre- and β-galactosidase-encoding plasmids, this result implies that 40%–80% of the ES cells expressing the Cre enzyme had deleted the Jn–Ei fragment.

**Generation of the JnT Mouse Mutant**

Expression of the Cre enzyme in ES cells might affect the potential of the ES cell to contribute to germ cell formation. To determine whether the JnT mutation could still be transmitted to the germline, we produced chimeric mice from 4 mutant ES cell clones. From 1 clone we obtained only one male chimeric mouse, which was sterile. Chimeras from the other 3 clones successfully gave germline transmission.

We expected deletion of the Jn–Ei loci to abolish B cell generation completely, since the mutant mice cannot make functional Ig molecules. To prove that this was indeed the case, we performed a flow cytometric analysis of the B cell compartment in heterozygous mutant mice (IgG–, JnT/IgG–, +). Wild-type F1 mice (IgG–, +/IgG–, +) were used as a control.

As shown in Figure 4, all of the B cells in the peripheral blood from the heterozygous mutant mice expressed only surface IgM (from the wild-type allele), in contrast with wild-type F1 animals, in which IgM– and IgM+ cells were equally represented. The analysis of the bone marrow B cell compartment in homozygous mutants indicated that B cell development in the mutant mice is blocked at the pre-B cell stage (Ehlich et al., 1993). These results are consistent with the previous finding that the expression of membrane-bound immunoglobulin is essential for early B cell development (Kitamura et al., 1991).

**Switch Recombination of Ss to S1 Is Partially Blocked on the JnT Chromosome**

Because of the deletion of the Jn–Ei locus, it is obviously impossible for B cells to generate any functional V-D-Jn–Ei gene rearrangement on the JnT chromosome. Consequently, B cells from the heterozygous mutant mice must always carry a nonproductive chromosome lacking the Jn–Ei segment. In contrast, B cells from normal animals

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**Table 1: Deletion of the Jn–Ei Region via Cre–loxP-Mediated Recombination**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Plasmids</th>
<th>Gancyclovir Colonies/Plated Colonies</th>
<th>PCR-Positive Colonies/Colonies Tested</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JC7</td>
<td>pIC-Cre</td>
<td>292/3,250</td>
<td>9/48</td>
<td>1.7</td>
</tr>
<tr>
<td>JC9</td>
<td>pIC-Cre</td>
<td>894/40,000</td>
<td>20/24</td>
<td>2.0</td>
</tr>
<tr>
<td>JC7*</td>
<td>pMC-Cre</td>
<td>1,600/40,000</td>
<td>10/10</td>
<td>4.0</td>
</tr>
</tbody>
</table>

* A subclone of ES cell line JC7.
* Colonies with Jn–Ei deletion as determined by PCR.
* Percentage of colonies with Jn–Ei deletion among total plated colonies. Total plated colonies exclude background colonies carrying wild-type IgG loci, which might derive from contaminating wild-type ES cells.
generally have, at the corresponding position, either a $V_{\lambda}D_{\lambda}J_{\lambda}E_{\lambda}$ or a $D_{\lambda}J_{\lambda}E_{\lambda}$ complex on the nonproductive chromosome (All et al., 1987).

To determine whether, in the absence of a $V_{\alpha}D_{\alpha}J_{\alpha}E_{\alpha}$ complex, the $S_{\gamma}$ region of the $J_{\lambda}$ chromosome can still participate in switch recombination, we analyzed switch recombination in splenic B cells cultivated in vitro in the presence of LPS and IL-4. These culture conditions have been shown to specifically stimulate isotype switching from $\mu$ to $\gamma$1 and $\gamma$2 (Layton et al., 1984; Coffman et al., 1986). B cells expressing $\mu$ or $\gamma$1 chains in the cytoplasm were isolated from the cultures by fluorescence-activated cell sorting (FACS). Rearrangement of $S$ regions juxtaposed to various $C_{\gamma}$ genes was then examined by Southern blot analysis. As can be seen in Figure 5, $\gamma$1-producing B cells from wild-type $F_{1}$ mice show an almost complete disappearance of the germline bands of both the $a$ and $b$ allotype $S_{\gamma}$ regions ($S_{\gamma}^{a}$ and $S_{\gamma}^{b}$), consistent with the earlier observation that switch recombination occurs in both productively and nonproductively rearranged IgH loci (Radbruch et al., 1986; Hummel et al., 1987). In contrast, B cells from the heterozygous mutants show a depletion of the germline band for $S_{\gamma}^{a}$ (on the wild-type chromosome), whereas the $S_{\gamma}^{b}$ band is largely preserved. This result demonstrates that the $J_{\lambda}$ mutation interferes with switch recombination at $S_{\gamma}$ in cis and thus suggests that the $V_{\alpha}D_{\alpha}J_{\alpha}E_{\alpha}$ complex plays an important role in conferring on $S_{\gamma}$ accessibility for switch recombination.

Figure 4. Flow Cytometric Analysis of Peripheral Blood B Lymphocytes from Heterozygous Mutant and Wild-Type $F_{1}$ Mice
Peripheral blood lymphocytes from wild-type ($IgH^{a}, IghT^{a}$) and heterozygous mutant ($IgH^{b}, J_{\lambda}T^{b}$) mice were stained with phycoerythrin-coupled anti-IgM and fluorescein isothiocyanate-coupled anti-IgM antibodies. The results are presented as two-dimensional dot plots and represent only the data from cells in the lymphocyte gate as defined by light scatter. The percentage of cells in a given quadrant is indicated in the dot plots.

Figure 5. Analysis of Switch Recombination of Ig Genes in Wild-Type $F_{1}$ and Heterozygous Mutant Mice by Genomic DNA Southern Hybridization
Total splenic B cells from wild-type $F_{1}$ ($IgH^{a}, IghT^{a}$) and heterozygous mutant ($IgH^{b}, J_{\lambda}T^{b}$) mice were used for the analysis. Genomic DNA isolated from $1 \times 10^{6}$ IgM and IgG1 blast B cells (sorted by FACS) was digested with EcoRI or HindIII enzymes as indicated. The restriction maps of the corresponding IgH loci are shown at the right side of each blot. The probes are depicted as closed bars below each restriction map. The germline bands of individual IgH loci are indicated in the figure. The relative weakness of the $S_{\gamma}^{a}$ and $C_{\gamma}^{a}$ bands compared with the $S_{\gamma}^{b}$ and $C_{\gamma}^{b}$ bands might reflect sequence polymorphisms between the $IgH^{a}$ and $IgH^{b}$ alleles at the regions homologous to the probes.

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Table 2. Extent of DNA Rearrangements at the IgM Loci of γ1-Producing B Cells from Heterozygous JγT Mutant and Wild-Type F1, Mice

<table>
<thead>
<tr>
<th>Region</th>
<th>Wild-Type (IγH+, +IγH+, +) (%)</th>
<th>Mutant (IγH+, JγT/IγH+, +) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IγH+</td>
<td>IγH+</td>
</tr>
<tr>
<td>S,</td>
<td>~100</td>
<td>~100</td>
</tr>
<tr>
<td>C,3</td>
<td>97</td>
<td>95</td>
</tr>
<tr>
<td>S,1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>C,1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S,</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 The percentage of rearrangement was determined by densitometric scanning. To correct for differences in DNA loading, the band intensities in a given lane were standardized on the basis of the band corresponding to the Cγ gene, under the assumption that in IgM-expressing cells S regions and Cγ genes are not rearranged.
2 IγH+ and IγH+ haplotypes were indistinguishable.

To evaluate the extent of the suppression of Sγ recombinaction on the JγT chromosome, the residual germline Sγ DNA of the γ− or μ chain–expressing cells on the Southern blot was quantified by densitometric analysis. According to this analysis, ~30% of the γ1-expressing cells from the heterozygous mutant mice have rearranged Sγ, on the JγT (nonproductive) chromosome (Table 2); the 30% value was confirmed in an independent Southern blot analysis in which the DNA was cut with HindIII and hybridized to a Sγ probe and a Sγ Cγ probe for standardization [data not shown], whereas in wild-type F1 mice close to 100% of the cells recombined the Sγ region on both productive and nonproductive IgM loci. Hybridization of the same blot with a Cγ probe shows that about 80% of the γ1-expressing B cells from the mutant mice carry a germline Cγ gene on the JγT allele, in striking contrast with γ1-producing B cells from wild-type mice, in which only 15% of the nonproductive IgM loci contain a germline Cγ gene.

To assess the accessibility of the Sγ region on the JγT chromosome for switch recombination in vivo, we isolated IgG-producing hybridomas from spleen cells of a heterozygous mouse after immunization with a T cell–dependent antigen (see Experimental Procedures). Southern blot analysis of such hybridomas indicated that in 2 out of 16 clones the Sγ region on the mutant chromosome was re-arranged (data not shown). The quantitative difference between the in vitro and in vivo data may be due to difference in the strength of the triggering signals to which the cells were exposed in the two cases. Collectively, the results show that the block of class switch recombination at Sγ of the JγT chromosome is severe but not complete.

The Sγ Region of the JγT Chromosome Is Efficiently Rearranged after LPS Plus IL-4 Induction

Switch recombination involves two partner S sequences: Sγ and the S region of a downstream C region gene. The down-regulation of switch recombination at Sγ in the JγT chromosome therefore raises the question of whether the Sγ region on the same chromosome is similarly affected.

Switch recombination at Sγ was determined by Southern hybridization with an Sγ-specific probe (Figure 5). Most γ1-producing B cells from wild-type F1 mice have rearranged the Sγ on both a and b alleles. The residual amount of germline Sγ DNA in these cells presumably derived from those nonproductive chromosomes that did not participate in switch recombination. As expected, in γ1-producing B cells from the heterozygous mutant mice, the germline Sγ, band (corresponding to the productive IgM locus) disappeared completely. Surprisingly, however, the germline Sγ band (from the JγT chromosome) was also virtually absent. A quantitative scanning analysis demonstrated that the remaining amount of germline Sγ DNA of the JγT chromosome (7%) approximately equaled that of germline Sγ plus Sβ in normal γ1-expressing B cells (Table 2). This result thus indicates that the regulation of Sγ rearrangement in switch recombination is independent from that of Sγ and not under the control of the Eγ element on the same chromosome.

To examine further whether the observed Sγ rearrangement in the JγT chromosome involved other Sγ region sequences on the same chromosome, we hybridized the Southern blots with a Cγ, a Cγ, or an Sγ probe (Figure 5). As shown in Table 2, nearly 100% of the JγT chromosomes from γ1-producing B cells still carried a Cγ and a Cγ gene. Hybridization with the Sγ probe reveals no detectable switch recombination between Sγ and Sγ in the γ1-expressing B cells of the heterozygous mutant mice. Thus, switch recombination at Sγ of the JγT chromosome does not seem to involve S regions of the downstream C genes. Considering that the majority of γ1-expressing B cells of the mutant mice have germline Cγ and Cγ loci on the JγT chromosome (which excludes rearrangement between Sγ and upstream S sequences), the rearrangement of Sγ on the JγT chromosome can most easily be interpreted to reflect the deletion of internal Sγ sequences.

No Detectable Cγ Gene Transcripts from the JγT Chromosome

B cells from heterozygous mutant mice were cultivated in vitro for 3 days together with LPS plus IL-4. Since the mutant allele (IγH+, JγT) is always nonproductive in these cells, we used surface μ−positive B cells (sorted by FACS) from wild-type F1 animals (IγH+/IγH+) as the control, in which all IγH+ alleles are also nonfunctional. μ chain cDNA was prepared from total cellular RNA extracted from the cultured cells and amplified with primers specific for the Cγ gene (Figure 6). The amplified PCR products were hybridized with an oligonucleotide probe detecting only the Cγ.

As shown in Figure 6, B cells from wild-type F1 animals
produce at least $10^5$ times more $\mu^+$ transcripts from the nonproductive chromosome than their mutant counterparts. In fact, the hybridization signal of $\mu^+$ transcripts from B cells of the mutant mice is indistinguishable from that of C57BL6J mice ($\mu^+$), which do not express $\mu^+$ mRNA at all. Because there is no obvious reason that transcripts initiated upstream of $S_\mu$ should not go through the $C_\mu$ gene, the level of $C_\mu$ transcripts from the $J_\mu T$ chromosome should reflect that of transcripts through the $S_\mu$ region on the same chromosome.

**Alteration of DNA Methylation near the $S_\mu$ Region of the $J_\mu T$ Chromosome**

A number of experiments have suggested that DNA methylation might play a role in the control of V-D-J gene rearrangement (Hsieh and Lieber, 1992) and Ig switch recombination (Burger and Rabcewicz, 1990). For this reason we examined the DNA methylation pattern of the $S_\mu$ region in splenic B cells from wild-type F1 and mutant animals, using the method of Akira et al. (1984). Demethylation of CpG at the H1 site (Figure 7) is predicted to yield 4.8 kb EcoRI-HpaII and 6.8 kb fragments from IgH$^+$ and IgH$^-$ alleles, respectively. We focussed on the H1 site, because this site is located at the border of $S_\mu$ region, so its methylation status may reflect that of the $S_\mu$ region per se.

In B cells from normal animals, the H1 site is completely demethylated on both the productive (IgH$^+$) and nonproductive (IgH$^-$) chromosomes, as evidenced by the complete disappearance of the EcoRI fragments on the blot and, instead, the emergence of 4.8 kb and 6.8 kb fragments (Figure 7). This result thus suggests a hypomethylation status of the $S_\mu$ sequences on both the productive and nonproductive chromosome in normal B cells. In contrast, B cells from the mutant mice exhibit complete digestion of the EcoRI fragment by HpaII enzyme only on the productive chromosome (IgH$^+$) (Figure 7). Interestingly, approximately 20% of the mutant B cells carry a demethylated H1 site on the $J_\mu T$ chromosome, as determined by densitometric scanning, a number roughly matching that of the $\gamma$-producing B cells with rearranged $S_\mu$ on the $J_\mu T$ chromosome (Table 2). These results indicate that the $V_{\mu}D_{\mu},(D_{\mu}),E_{\mu}$ complex controls $S_\mu$ region methylation in B cells and suggest that demethylation might be involved in the process conferring on the $S_\mu$ region accessibility for switch recombination.

**Discussion**

The Cre–loxP System as an Efficient Tool for Gene Manipulation in ES Cells

The Cre–loxP recombination system has been shown to be able to mediate loxP site-specific recombination in both mammalian cell lines (Sauer and Henderson, 1988) and transgenic mice (Lakso et al., 1992; Orban et al., 1992). By combining this system with the conventional gene targeting technique, an efficient method for the introduction of deletions of defined length into the mouse germline is at hand. Using a modified cre expression vector, we find that a major fraction of ES cells transiently transfected by the cre vector undergo Cre–loxP-mediated gene deletion (which is not the case in our hands if the related FLP/FRT system from yeast is used [Jung et al., 1993, unpublished data]) and that the procedure does not interfere with germline transmission of the mutation. This approach has several advantages over the conventional targeting technique: The deletions are of predefined length, and we expect on the basis of earlier work (Orban et al., 1992; Sauer and Henderson, 1988) that this length may vary over a broad range. No selection marker gene but only a single loxP site of approximately 50 bp length is left behind in the genome. Targeting vectors can easily be designed such that subtle mutations are introduced into a gene, providing an alternative to the "hit and run" strategy of Hasty et al. (1991) and Valancius and Smithies (1991). Finally, the method should allow not only cell lineage–specific gene deletion through the use of cre transgenic mice, in analogy to the work of Lakso et al. (1992) and Orban et al. (1992), but also insertion of genetic material into a loxP site targeted within the genome (Sauer and Henderson, 1990).

The $J_\mu T$ Mutant Exhibits Complete B Cell Deficiency

We have previously obtained a B cell-deficient mouse mutant through targeted inactivation of the membrane exon of the $C_\mu$ gene (Kitamura et al., 1991). While in this mutant ($\mu$MT) the occasional generation of an antibody producing cell through the usage of a $C$ gene other than
Control of Immunoglobulin Switch Recombination

Figure 7. DNA Methylation Analysis of the μ Chain Gene Locus in B Cells from Wild-Type F1, and Heterozygous Mutant Mice

Genomic DNA was isolated from μ− splenic B cells sorted by FACS. Each lane represents DNA of 10⁶ cells from wild-type F1, or the heterozygous mutant mice as indicated. The restriction maps of the a and b haplotype μ− gene loci are depicted under the blot. Arrowheads indicate MspI (HpaII) sites, the H1 site corresponds to the arrowhead on the extreme left. E, EcoRI; M, MspI; H, HpaII. The probe is a 0.9 kb EcoRI-PstI fragment downstream of the IgH intron enhancer.

Cμ cannot a priori be excluded. JμT mice can under no circumstances generate functional B cells, since in this mouse strain the formation of heavy chain V region genes through somatic Vα/Dμ–Jμ recombination is impossible. Thus the JμT strain offers itself as an ideal animal model of complete B cell deficiency.

Switch Recombination Is Controlled by the Concerted Action of Independent, Cis-Acting Regulators Initiating Recombination at Individual S Regions

There is considerable evidence suggesting that the targeting of class switch recombination to particular S regions involves cis-acting control elements upstream of S (Gritzmacher, 1989; Xu and Stavnezer, 1992; Leung and MaizeL, 1992). That such an element can be essential for class switching to a particular isotype in vivo has recently been demonstrated in experiments in which switching to IgG1 expression was abrogated by the targeted deletion of sequences upstream of S1 in the mouse germ line (Jung et al., 1993).

The present data show that the similar principle also operates in the case of the “donor” Sμ region, which is involved in the switching from IgM expression to that of all other isotypes. Efficient switch recombination at Sμ requires the presence of the upstream intron enhancer (Eμ) and/or sequences upstream of the latter, e.g., a DμJμ or VμDμJμ gene. More importantly, however, our results demonstrate that the initiation of switch recombination at a particular S region (e.g., Sμ1) results in DNA rearrangements within that same S region and is limited to it if the process has not concomitantly been initiated at a partner S region (i.e., Sδ). Since class switch recombination often occurs in regions of short sequence homologies at the ends of the recombining DNA strands (Szudek et al., 1985; Winter et al., 1987; Iwasata et al., 1990), as it is typical for the repair of double-strand breaks in higher cells (Roth and Wilson, 1986), it is tempting to speculate (see also Winter et al., 1987) that class switch recombination also involves double-strand breaks. These would be targeted to the S regions involved by the S region–associated cis-acting control elements. If this happens at both a donor (Sμ) and an acceptor (S1) S region, recombination between the two S regions could occur. If only one S region is activated, as is the case for the Sμ1 of the JμT chromosome in B cells activated by LPS plus IL-4, recombination would be limited to that same S region. The process would be expected to involve extensive DNA rearrangement, as is seen experimentally, if the introduction of double-strand breaks is accompanied by endonucleolytic “trimming” or if breaks occur in multiple positions.

This model is compatible with the earlier observation by Winter et al. (1987), who showed intra-Sμ deletions in IgM-producing hybridoma cells obtained by fusion of a nonproducer myeloma with B cells shortly after in vitro activation by LPS plus IL-4. In general terms, it is strictly analogous to the model developed by Roth et al. (1992) for V-D-J recombination. With respect to the looping-out deletion model of class switch recombination (Honjo and Kataoka, 1978), it suggests, again in analogy to what Roth et al. (1992) have demonstrated for V-D-J recombination, that the formation both of circular DNA excision products (Iwasata et al., 1990; von Schuwedler et al., 1990; Matsuoka et al., 1990) and of S region alignment may occur after the introduction of double-strand breaks at the S region to which switch recombination is targeted.

What is the Molecular Basis of S Region Accessibility for Switch Recombination?

That transcription correlates with switch recombination has led to the proposals that either the change of the chromatin configuration induced by transcription (Stavnezer et al., 1984) or the transcripts themselves (Reaban and Griffin, 1990) might be responsible for making an S region accessible for isotype switching. Our analysis of the JμT mutant mice revealed that a significant number of B cells from these animals are still able to rearrange Sμ region sequences on the JμT chromosome, despite the lack of μ chain transcript as detected by a sensitive PCR assay. This result seems to exclude the possibility that Cμ transcripts starting upstream of Sμ control switch recombination in cis, although they may do so in trans. The data also argue against a requirement of transcription per se for
the initiation of switching. However, to exclude such a requirement rigorously, "run on" determinations of transcription of the mutant as distinct from the wild-type S region in the heterozygous cells would be needed, but they are difficult to perform.

The increased level of DNA methylation in B cells at a site (H1) close to the S region on the J_{H}T as compared with the wild-type Igh locus and the fact that, in y1-expressing heterozygous mutant B cells, the fraction of J_{H}T chromosomes with a rearranged S region is roughly the same as that of J_{H}T chromosomes carrying a demethylated H1 site, are both consistent with earlier work suggesting that DNA methylation might be an inhibitory factor in the control of switch recombination in LPS-activated B cells (Burger and Radbruch, 1990) and of V-D-J recombination in Abelson virus-transformed pre-B cells (Hsieh and Lieber, 1992). It is therefore possible that the cis-acting regulators of switch recombination exert their function through the control of DNA methylation, which could directly determine S region accessibility for switch recombination.

**Experimental Procedures**

**Targeting Vector and cre-Expressing Vectors**

To make the targeting vector, a single loxp site excised from pBS30 (Sauer, 1987) was first cloned into the EcoRI site of a pGEM-7z vector to generate pGEM-30 vector. Head-to-tail linked neomycin resistance (neo) and Hsv-tk genes (Thomas and Capecchi, 1987) were then inserted into the HindIII-Sall sites of the pGEM-30 to get the pGHI-1 vector carrying a neo-tk-loxp cassette. A 1.3 kb DNA fragment homologous to the region 3′ of the EcoRI site downstream of immunoglobulin heavy chain intron enhancer (Eh) was isolated by PCR from plasmid pSV1-1 (Neuberger, 1983) and cloned into the pGHI-1 vector as a Sall-ClaI fragment (pGHI-3). The following primers were used for amplification: 5′ primer (CTTGTACGGATCCAATTGCTTATTTT) and 3′ primer (AACAATGACCTCGGTTGATGATTGAAGAT). The resulting vector was designated pGHI-4. Finally, the neo-tk-loxp cassette together with the 1.3 kb genomic fragment was recovered from pGHI-3 as a XhoI-Sall fragment and cloned into the pGEM-7z vector together with a 3.5 kb XhoI-EcoRI fragment containing J_{H}Lz-E segments, by three-fragment ligation. The resulting vector was designated pGHI-4. Finally, the neo-tk-loxp cassette together with the 1.3 kb genomic fragment was recovered from pGHI-3 as a XhoI-Sall fragment and cloned into the pGEM-7z vector to generate the targeting vector pGHI-102 (Figure 2).

Nucleotide sequences surrounding the translation initiation codon of the cre gene (Stemberg et al., 1986) were modified according to Kozak's consensus sequences (Kozak, 1986), using a PCR method. The PCR primers used to introduce the modifications were as follows: 5′ primer (GGACTGCCGGATCATGCTAATTCTG) and 3′ primer (CTGGAATGATAGAAGAT). The modified cre gene was cloned into the PstI-NasI sites of pMC1-neo (pMc1) (Thomas and Capecchi, 1987) to obtain the pCE-Cre plasmid, in which the transcription of cre is driven by a synthetic HSV-tk promoter and enhancer. To generate the plasmid pMC-Cre, the cre gene was modified using the same PCR method, except that an oligonucleotide (TATAGCTGCCAGCTGCCCAAGAGAAG AGGAAGGTGGCTCCATTACTGAC) was used as a 5′ primer for PCR amplification, by which the nuclear localization signal peptide of the SV40 large T antigen (Kalendar et al., 1984) was introduced into the N-terminus of the Cre enzyme. The nucleotide sequences of the modified cre genes were confirmed by sequence determination.

**Deletion of the J_{H}Lz-E Segment Gene**

In ES Cells

E14-1 ES cells (Kühn et al., 1991) were cultured according to the conditions described previously (Kühn et al., 1991). Linearized pGHI-102 vector was introduced into E14-1 cells by electroporation, and neo`-colonies were selected in the presence of 300 μg of G418 per ml. Homologous recombinants were first identified by PCR and then confirmed by genomic Southern hybridization. The primers used for PCR were as follows: 5′ primer (TGGACTGCCGGATCATGCTAATTCTG) located 190 bp upstream of the ATG codon of the neo gene and a 3′ primer (GGACTGCCGGATCATGCTAATTCTG) complementary to sequences 1.3 kb downstream of the E element. The strategy of the Southern hybridizations is shown in Figure 2. Probe A is a 1.3 kb EcoRI-PstI fragment (downstream of the E element) isolated from plasmid pSV1-1. Probe B was a 0.9 kb PstI fragment (upstream of the DQ52 element) obtained from plasmid pBS1-6.5RI.

To delete the J_{H}Lz-E region, 105 targeted ES cells were transfected with 30 μg of supercoiled Cre-encoding plasmid DNA by electroporation according to Kitamura et al. (1991). The transfected cells were plated on embryonic fibroblasts for 48 hr and then replated at proper density. At day 3 after replating, 0.75 × 10^6 to 1 × 10^6 M ganclovir was included in the medium, and selection was continued for 3 days. Colonies surviving the selection were picked at day 9-10, and deletion events were identified by PCR and Southern hybridization. The following primers were used for the PCR: 5′ primer (ATGGACCTGACCTGGCTCGTCT) located 200 bp upstream of the DQ52 element and the 3′ primer used for the identification of the targeted integration (see above). For Southern blot analysis, genomic DNA was digested with BamHI, and the blot was hybridized with the 0.9 kb KpiI fragment as indicated in Figure 3.

**Generation of the J_{H}T Mutant Mice**

ES cell clones carrying the J_{H}T mutation were injected into blastocysts of C57BL/6 mice (Robertson, 1987; Bradley, 1987), and the resulting male chimeras were mated to C57BL/6 females to generate heterozygous mutant and wild-type F1 mice.

**Cell Culture, Cell Sorting, and Flow Cytometric Analysis**

Total splenic B cells from heterozygous mutant and wild-type F1 mice were cultivated in RPMI 1640 medium (GIBCO BRL) supplemented with 40 μg LPS/ml and 10% IL-4 containing supernatant (Jung et al., 1993) for 6 days. The cells were then fixed and stained with fluorescein isothiocyanate-labeled R33-24-12 (anti-μ; Grützmann, 1981) and phycoerythrin-conjugated anti mouse-αμ (BDA, Inc.). Cell sorting was performed using a FACStar Plus cell sorter (Becton-Dickinson).

For cell surface staining, cells from peripheral blood and bone marrow of the mice were stained with monoclonal antibodies and analyzed by FACSscan (Becton-Dickinson). The following antibodies were used: fluorescein isothiocyanate–conjugated MB66 (anti-μ; Nakakawa et al., 1986) and phycoerythrin-labeled RS3.1 (anti-μ; Schüpple et al., 1987).

**Hybridomas**

Splenic B cells from a 6-month-old J_{H}T heterozygous mouse, immunized with 4-hydroxy-3-nitro-phenacyl (NP) coupled to chicken γ globulin, were used for cell fusion. The immunization and fusion were done essentially as previously described (Siekevitz et al., 1987). IgG-producing hybridomas were screened for anti-NP IgG antibodies in the culture supernatants by enzyme-linked immunosorbent assay, using goat anti-mouse γ antibodies as a developing reagent.

**Southern Hybridization**

Switch recombination at individual switch regions was determined by Southern hybridization according to Wang et al. (1987). The intensities of the hybridization signals were determined by scanning using a chromoscan 3 (Joyce Loeb). Switch recombination at S_{H}T chromosome in the μ-producing hybridomas was determined by Southern hybridization using a 1.3 kb EcoRI-PstI fragment downstream of the Ig heavy chain intron enhancer as a probe. This approach was used to determine the position of the C and gene in a 3′ primer (ACACGCGACTGTCGACATT) located at 250 bp from the C region of the second C exon. To detect PCR products of μ transcripts, the oligonucleotide AAACAGA-
GATCTGG was used as probe for the hybridization. The probe was labeled with 3P using T4 kinase according to Sambrook et al. (1989). The blot was hybridized at 38°C for 2 hr in 50 ml of hybridization buffer (6 x SSC, 0.5% SDS, 200 μg of salmon sperm DNA per ml, and 20 μM 3P-labeled probe). After hybridization, the blot was washed once in 6 x SSC at 38°C for 5 min, and then twice in 1 x SSC at 38°C for 1.5 min each before autoradiography.

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References


