

Mapping of a Functional Recombination Motif that Defines Isotype Specificity for $\mu \rightarrow \gamma 3$ Switch Recombination Implicates NF- κ B p50 as the Isotype-specific Switching Factor

Amy L. Kenter, Robert Wuerffel, Carmen Dominguez, Ananth Shanmugam, and Hongmei Zhang

Department of Microbiology and Immunology, University of Illinois College of Medicine, Chicago, IL 60612

Abstract

Ig class switch recombination (CSR) requires expression of activation-induced deaminase (AID) and production of germline transcripts to target S regions for recombination. However, the mechanism of CSR remains unclear. Here we show that an extrachromosomal S plasmid assay is AID dependent and that a single consensus repeat is both necessary and sufficient for isotype-specific CSR. Transfected switch substrates specific for $\mu \rightarrow \gamma 3$ and $\mu \rightarrow \gamma 1$ are stimulated to switch with lipopolysaccharide (LPS) alone or LPS and interleukin-4, respectively. An S $\gamma 3$ /S $\gamma 1$ substrate containing only three S $\gamma 3$ -associated nucleotides reconstituted LPS responsiveness and permitted mapping of a functional recombination motif specific for $\mu \rightarrow \gamma 3$ CSR. This functional recombination motif colocalized with a binding site for NF- κ B p50, and p50 binding to this site was previously established. We show a p50 requirement for plasmid-based $\mu \rightarrow \gamma 3$ CSR using p50-deficient B cells. Switch junctions from p50-deficient B cells showed decreased lengths of microhomology between S μ and S $\gamma 3$ relative to wild-type cells, indicating a function for p50 in the mechanics of CSR. We note a striking parallel between the effects of p50 and Msh2 deficiency on S μ /S $\gamma 3$ junctions. The data suggest that p50 may be the isotype-specific factor in $\mu \rightarrow \gamma 3$ CSR and epistatic with Msh2.

Key words: AID • B lymphocyte • immunoglobulin • NF- κ B p50 • class switch

Introduction

Ig class switch recombination (CSR) promotes the expression of antibody molecules with different constant (C_H) regions permitting diversification of effector function while maintaining the original antigen-binding specificity arising from V(D)J joining. CSR is mediated by an intrachromosomal DNA rearrangement that focuses on stretches of repetitive DNA sequences termed switch (S) regions, which are located upstream of all the C_H genes except C δ (for reviews see references 1, 2). It is clear that mechanism of CSR requires the expression of activation-induced deaminase (AID) (3–5, and for review see reference 6) and germline transcription (gt) through participating S regions (for reviews see references 1, 2). Evidence suggests that AID functions by deaminating cytosine residues and converting them to dU (7–9). Removal

of the uracil by the base excision repair pathway enzyme uracil-DNA glycosylase is required for CSR and somatic hypermutation (10). Staggered double strand breaks (DSBs) could emerge after deamination of closely spaced dC residues located on opposing strands in the S region. Blunt and staggered DSBs have been observed in S DNA, and their formation is both AID and uracil-DNA glycosylase dependent, demonstrating that they are intermediates in CSR (11–14). S DNA may become accessible to AID-generated lesions through gt expression (15, 16). Indeed, S regions have the unusual propensity to form R loops in vitro and in vivo when transcribed along the C-rich strand, which would provide ssDNA substrate to AID (17–20). Resection of the S/S junctions is likely to be dependent on the non-homologous end joining proteins, Ku70/Ku80, DNA-PKcs

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Address correspondence to Amy L. Kenter, Department of Microbiology and Immunology, University of Illinois College of Medicine, Chicago, IL 60612-7344. Phone: (312) 996-5293; Fax: (312) 996-6415; email: star1@uic.edu

A. Shanmugam's present address is Box 8134, Dept. of Psychiatry, Washington University, 660 S. Euclid Ave., Saint Louis, MO 63110-1010.

Abbreviations used in this study: α dex, anti- δ dextran; AID, activation-induced deaminase; CSR, class switch recombination; DC, Digestion circularization; DSB, double strand break; FRM, functional recombination motif; gt, germline transcription; MMR, mismatch repair; nAChR, non-rearranging acetylcholine receptor; RRL, relative recombination level; TR, tandem repeat.

(with the exception of $\mu \rightarrow \gamma 1$ CSR) (21–24), several of the mismatch repair (MMR) proteins (25–28), and the histone H2AX (29–31).

Although it is clear that *gt* expression contributes to isotype specificity in CSR, evidence suggests that other factors are also involved in directing isotype choice. B cells deficient for specific transcription factors express AID and appropriate *gts* but do not switch to particular isotypes (32–34), suggesting that the isotype specificity arises through other factors not yet enumerated. Isotype-specific switch substrates used in transient transfection studies provide evidence for four independent switching activities (35, 36). Switching activity at endogenous loci is strictly correlated with AID expression, the coordinate expression of the appropriate *gt*, and isotype-specific switch plasmid activity, indicating that transacting switching activities are integral to the process of CSR (36).

We report here that the extrachromosomal switch substrates do not function in AID-deficient LPS-activated B cells, confirming the physiological relevance of these switch substrates. We found that single $S\gamma 3$ or $S\gamma 1$ tandem repeats are sufficient to support $\mu \rightarrow \gamma 3$ or $\mu \rightarrow \gamma 1$ CSR, respectively, demonstrating that isotype specificity is encoded in a single repeat unit. Deletion mapping of the $S\gamma 3$ consensus repeat and analysis of chimeric $S\gamma 3/S\gamma 1$ repeats indicates that the specificity for $S\gamma 3$ in LPS B cells is dependent on the integrity of a binding site for NF- κ B p50. Previous studies showed that endogenous $\mu \rightarrow \gamma 3$ CSR is abolished in p50-deficient B cells (33, 34, 37). We demonstrate that the switch substrate specific for $\mu \rightarrow \gamma 3$ CSR is not functional in mitogen-activated p50-deficient B cells. Finally, a reduction of microhomology in $S\mu/S\gamma 3$ junctions was observed in p50-deficient but not in WT B cells, demonstrating that NF- κ B p50 plays a role in the mechanics of CSR. The data raise the intriguing possibility that p50 is the isotype-specific factor mediating $\mu \rightarrow \gamma 3$ CSR.

Materials and Methods

Cell Culture, Mice, Transfection, and Cloning of $S\mu/S\gamma 3$ Junctions. The 1.B4.B6 cell line was grown in culture as described previously (36). Splenic B cells derived from Balb/c nu/nu mice were prepared and activated with LPS + anti- δ -dextran ($\alpha\delta$ dex) + IL-4 + IL-5 + TGF β or LPS alone as previously described (34, 35). $\alpha\delta$ dex was a gift from Dr. C. Snapper (Uniformed Services University of the Health Sciences, Bethesda, MD). The *nfk1*^{+/−} (p50^{+/−}), *nfk1*^{−/−} (p50^{−/−}) mice were littermates and were backcrossed to C57Bl6/J (37). Enrichment of B cells from WT (129 × B6), *nfk1*^{+/−} (p50^{+/−}), *nfk1*^{−/−} (p50^{−/−}), and AID^{−/−} mouse spleens was accomplished using Collect Immunocolumns (Cedarlane Laboratories) according to the manufacturer's instructions. AID^{−/−} mice were a gift from T. Honjo (Kyoto University, Kyoto, Japan), and *nfk1* and WT mice were purchased from Jackson Labs. The purity of the cell population was confirmed by FACS[®] analysis. In transfections experiments, the cells were electroporated in the presence of 8 μ g of plasmid at 300 V/950 μ F on day 3 of culture. After an additional 3 d in culture, nuclei were isolated using the Blood and Cell Culture DNA Preparation kit (QIAGEN), and DNA was prepared with the

Puregene Genomic DNA Purification kit (Gentra Systems). PCR amplification and cloning of $S\mu/S\gamma 3$ hybrid molecules was performed as described previously (38) except that primer μ -1.2 (5'-GCTGGGGTGGAGCTCAGCTATGCTACGC-3') was used, which anneals to positions 5307–5333 at the 5' end of the germline $S\mu$ (MUSIGCD07).

RT-PCR, Quantitative RT-PCR, Digestion Circularization-PCR, and Bacterial Transformation Assays. RT-PCR for $\gamma 3$ germline transcripts and GAPDH was performed as described (33, 34). Digestion circularization (DC)-PCR for detection of endogenous and plasmid-based $\mu \rightarrow \gamma 3$ and $\mu \rightarrow \gamma 1$ CSR was performed as described previously, respectively (34, 35). The primers used for plasmid-specific DC-PCR were the same for all the switch plasmids studied. Radioactivity was assessed using a PhosphorImager and ImageQuant software for quantitation. Bacterial transformation assays were performed as described (35, 36). Briefly, DNA recovered from nuclei of transfected cells was either left untreated or digested with EcoRI and then transformed into bacteria. The EcoRI-resistant (EcoRI^r) colonies denotes plasmid resistant to EcoRI digestion. EcoRI^r colonies were prepared as minipreps and analyzed by restriction mapping to identify S/S recombinant plasmids. The percentage of EcoRI^r is obtained by dividing the total number of EcoRI^r transformants by the number of Amp^r transformants and then multiplying by 100.

Construction of Plasmids. Construction of pG3.1 and pG3.01s were described previously (35). To obtain pG3.02s, pG3.025s, and pG3.045s a cassette was constructed that contained TK, I α , and the degenerate $S\gamma 3$ repeats found in pG3.01s (35). The cloned cassette is referred to as L34 and contains a BamHI site located between the I α and the degenerate $S\gamma 3$ repeats. Two partially complementary oligos containing the consensus $S\gamma 3$ repeat were synthesized: G3N1, 5'-TTGTGGGGACCAGGCTGAGCAGCTCTCAGG-GAGCTGGGGAGGTGGAGTTGTG-3' and G3N2, 5'-CAC-AATGAACCTACCCCAGCTCCCCCAGAGCTGCCCAGC-TTGGTCCCCACAACCTCCACC-3'. The oligos were annealed, filled-in with Klenow DNA polymerase (Promega), and cloned into the filled-in BamHI site of L34. Subclones containing 1, 2, or 6 $S\gamma 3$ consensus repeats were isolated, and DNA sequence was verified. Complementary consensus $S\gamma 1$ or chimeric $S\gamma 1.m1$ oligos were synthesized, annealed, and cloned into the Klenow filled-in BamHI site of L34. The G-rich strand for the $S\gamma 1$ consensus repeat was 5'-GGTGACCCAGGCAGAGCAGCTCCAGGGG-AGCCAGGACAGGTGGAAGTGT-3'. The G-rich strand for the $S\gamma 1.m1$ repeat was 5'-GGGGACCAGGCTGAGCAGCTC-CAGGGGAGCCAGGACAGGTGGAAGTGT-3'. The cassettes from the appropriate L34 subclones were isolated as EcoRI-NotI restriction fragments and then directionally cloned into a switch plasmid vector containing neo, μ intronic enhancer and V_H promoter, and $S\mu$ with EcoRI-NotI ends.

Online Supplemental Material. Fig. S1 shows a comparison of $S\mu/S\gamma 3$ junctions derived from recombinant pG3.1, pG3.02s, and pG1.m1.02s. Switch plasmids were transfected into LPS-activated B cells, and recombinant plasmids were recovered using the bacterial transformation assay. Switch junctions were identified by automated DNA sequence analysis. Fig. S2 shows that reduced microhomology is found in $S\mu/S\gamma 3$ junctions from p50-deficient mice. $S\mu/S\gamma 3$ junctions from p50^{+/+} (A), p50^{+/−} (B), and p50^{−/−} (C) mice were PCR amplified from B cells stimulated with LPS + $\alpha\delta$ dex + IL-4 + IL-5 + TGF β and cloned. At least two independent DNA samples were used as amplification substrates to generate junctions for each group analyzed. Switch junctions were identified by automated DNA sequence analysis. Fig. S3 shows that the expression of MMR transcripts is not al-

tered by NF- κ B p50 deficiency in activated B cells. Quantitative SYBER Green PCR assays were developed for Mlh1, Pms2, Msh2, Msh3, Msh6, and GAPDH. At least two independent RNA samples from each activation state and cell type were reverse transcribed to cDNA and used in the real-time PCR assay. Figs. S1–S3 are available at <http://www.jem.org/cgi/content/full/jem.20031935/DC1>.

Results

Switch Plasmid Recombination Is Dependent on AID Expression. Our previous studies using extrachromosomal switch substrates demonstrated distinct isotype switching activities in B cells capable of endogenous CSR (35, 36). AID is a critical mediator of CSR, and its deficiency is characterized by a profound block in CSR to all isotypes, whereas gt expression remains unaffected (3). To further explore the physiological relevance of the switch plasmid assay, the switch substrates were tested for AID dependence. AID^{+/+} and AID^{-/-} B cells were T cell depleted, LPS activated, and the induction of γ 3 gts and endogenous $\mu \rightarrow \gamma$ 3 CSR were examined by RT-PCR and DC-PCR

(Fig. 1 A), respectively. In the RT-PCR assay, GAPDH was used as an internal control for cDNA input (Fig. 1 B). After 72 h of activation with LPS, AID^{+/+} and AID^{-/-} B cells expressed the γ 3 gt equally well, indicating that all the cells were successfully stimulated. In the DC-PCR assay, the *nonrearranging acetylcholine receptor* (*nAChR*) gene was used as a control for the digestion and ligation reactions, and the *nAChR* DC-PCR product was found for all samples (Fig. 1 C). AID^{+/+} B cells activated with LPS for 5 d were positive for the S μ /S γ 3 DC-PCR product, whereas this product was undetectable in DNA isolated from AID-deficient B cells. The normal expression of gts and the absence of $\mu \rightarrow \gamma$ 3 switching is fully consistent with the AID-deficient phenotype described previously (3, 4).

To evaluate the influence of AID on switch plasmid recombination, the relative frequency of CSR events was compared in AID^{+/+} and AID^{-/-} B cells using a previously devised plasmid-specific and semiquantitative DC-PCR assay (35). AID^{+/+} and AID^{-/-} B cells were activated with LPS for 3 d, transfected with pG3.1, which detects $\mu \rightarrow \gamma$ 3 CSR, and cultured in the presence of LPS for an additional 3 d. Amplification of the plas-

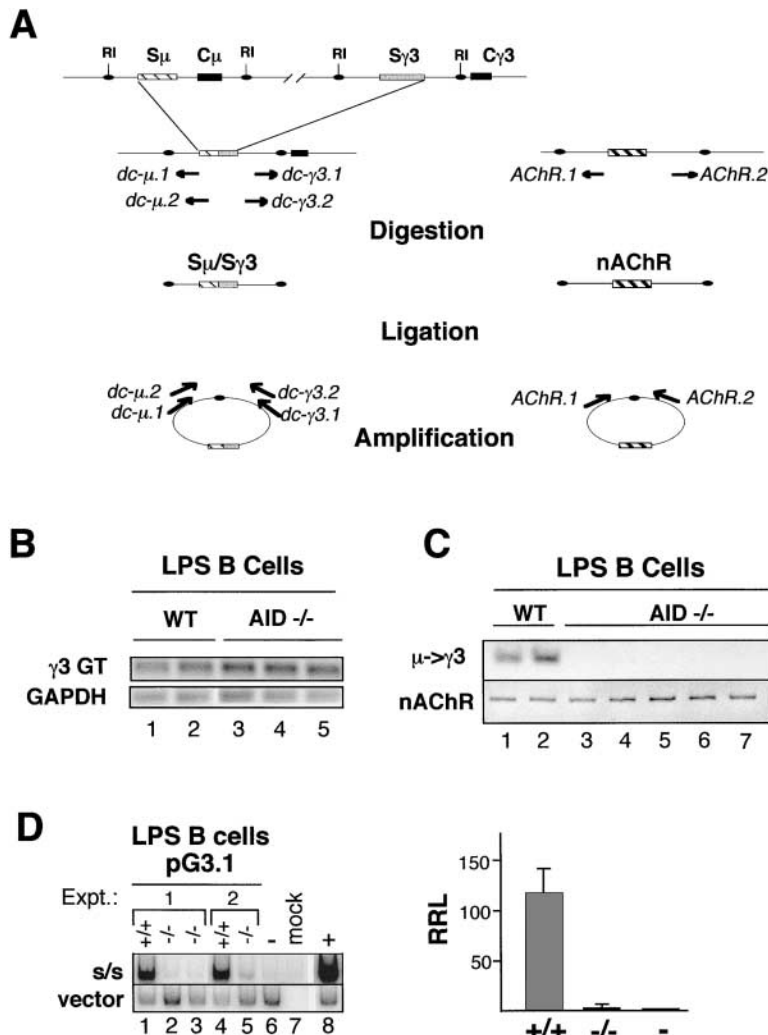


Figure 1. Switch plasmid-based $\mu \rightarrow \gamma$ 3 CSR in LPS B cells is AID dependent. (A) A diagram of the DC-PCR strategy for endogenous loci is shown. A portion of the IgH locus is diagrammed before and after $\mu \rightarrow \gamma$ 3 recombination. EcoRI sites (RI) flank the 5' and 3' ends of the S μ and S γ 3 regions, respectively, and are preserved after CSR. After digestion with EcoRI, the DNA is ligated. Nested primer sets specific for sites at the 5' end of S μ (*dc- μ .1*, *dc- μ .2*) and the 3' end of S γ 3 (*dc- γ 3.1*, *dc- γ 3.2*) amplify the region spanning the circle joint and yield a specific S/S DC-PCR product. The positions and orientations of the primer sets are shown before and after ligation. The *nAChR* gene serves as an internal control for digestion and ligation. (B–D) B cells from AID^{+/+} and AID^{-/-} spleens were activated in culture with LPS for 3 (B) or 5 d (C) or were activated with LPS for 3 d and then transfected with pG3.1 and grown in LPS culture for an additional 3 d (D). (B) RT-PCR for detection of GAPDH and the γ 3 germline transcript (γ 3 GT) was performed on RNA of LPS-activated B cells from AID^{+/+} (lanes 1 and 2) or AID^{-/-} (lanes 3–5) mice. (C) DC-PCR was used to detect endogenous $\mu \rightarrow \gamma$ 3 switching in DNA from LPS-activated AID^{+/+} or AID^{-/-} B cells (top). The *nAChR* gene was used as a positive control (bottom). (D) LPS activated B cells from AID^{+/+} or AID^{-/-} mice were transfected with the pG3.1 plasmid and used in a plasmid-based DC-PCR assay. The RRL for pG3.1 is the ratio of the radioactivity associated with the 180-bp (S/S) fragment to that of the 510-bp (vector) fragment. Intact pG3.1 (0.5 pg) demonstrated to be in the linear range of detection in the DC-PCR assay (not depicted) and used as a negative control (lane 6), and its RRL was set to 1 (right). The RRL values for the AID^{+/+} and AID^{-/-} transfections were normalized to the negative control. In the right panel AID^{+/+} and AID^{-/-} samples are designated +/+ and -/-, respectively. The intact negative control is indicated as -. The average RRLs from two independent experiments for pG3.1 is plotted (right), and SDs are shown.

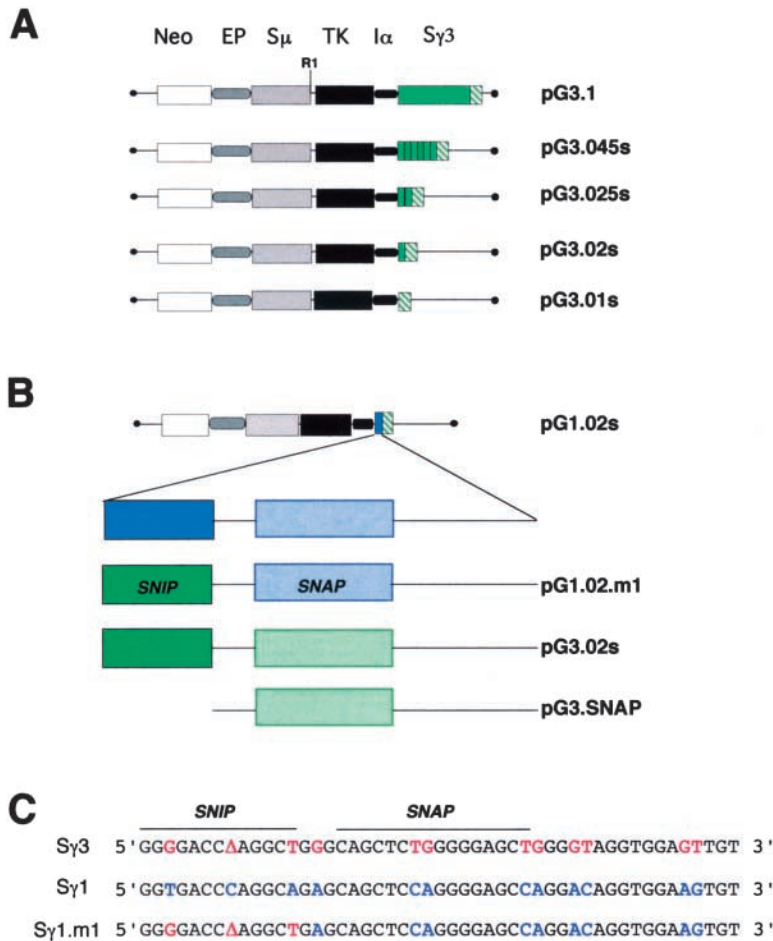


Figure 2. Structure of switch plasmids related to pG3.1. (A) Intact pG3.1 contains a *neomycin-resistance gene* (*neo*), the *Ig* μ intronic enhancer (*E*), an *IgH* variable region promoter (*P*), a *thymidine kinase* (*TK*) gene, the promoter for α *gts* (*I* α), and the *S* μ and *S* γ 3 regions. PG3.01s is identical to pG3.1 except for the deletion of the *S* γ 3 region and retention two nonconsensus tandem repeats from the extreme 3' end of genomic *S* γ 3 (35). The plasmids pG3.02s, pG3.025s, and pG3.045s contain 1, 2, and 6 consensus *S* γ 3 tandem repeats, respectively. The nonconsensus and consensus tandem repeats located in *S* γ 3 are indicated as green hatched and solid green boxes, respectively. (B) PG1.02s contains an *S* γ 1 consensus repeat, which is depicted by the solid blue box. The *S* γ 1 consensus repeat is expanded to show two subsections, SNIP (solid) and SNAP (speckled), separated by a short and long spacer, as indicated. *S* γ 1 and *S* γ 3 DNA sequences are shown in blue and green, respectively. PG1.02.m1 contains a chimeric repeat including the *S* γ 1 repeat backbone and an *S* γ 3 SNIP site. PG3.SNAP contains a 30-bp truncated *S* γ 3 consensus repeat centered on the SNAP site. (C) The DNA sequence for *S* γ 3 and *S* γ 1 consensus repeats are shown. In the chimeric *S* γ 1.m1 repeat, the *S* γ 1 and *S* γ 3 SNIP sites differ at three nucleotide positions.

mid-specific vector and S/S recombinant fragments after *SacI* digestion and ligation yields a 510- and a 180-bp PCR product, respectively. The linear range of detection was established using twofold serial dilutions of pG3.1 into 1 μ g of genomic DNA followed by DC-PCR in the presence of radiolabeled nucleotides (unpublished data) as described previously (35). S/S recombinant and intact pG3.1 spiked into genomic DNA as well as mock-transfected DNA were used as positive, negative, and specificity controls for the DC-PCR, respectively (Fig. 1 D). In two independent transfection experiments, the 510-bp vector-associated DC-PCR product was found for all the cells analyzed, whereas the 180-bp product resulting from the presence of composite *S* μ /*S* γ 3 DNA was found only in plasmid transfected into *AID*^{+/+} B cells but not *AID*^{-/-} B cells (Fig. 1 D, left). The relative recombination level (RRL) is expressed as the ratio of the S/S signal to the vector signal. Comparison of RRLs for pG3.1 indicates a <80-fold recombinational activity in *AID*^{+/+} B cells than in the *AID*^{-/-} B cells (Fig. 1 D, right). These findings demonstrate the *AID* dependency of the switch plasmid assay and provide important confirmation that the switch substrates, analyzed in a transient transfection format, reflect physiological CSR.

A Single Tandem Repeat Supports CSR in Switch Substrates. The detection of isotype-specific switching activities suggests that molecular recognition of S regions may be a feature of CSR. The pG3.1 plasmid contains a 2.0-kb insert of *S* γ 3 DNA, which represents the complete genomic version of this S region. Our recent studies indicated that *S* γ 3 can be reduced from 44 tandem repeats to 5 repeat units without adversely affecting plasmid-based CSR frequency (36). In contrast, pG3.01s, which contains two degenerate tandem repeats comprised of 125 bp derived from the 3' end of the *S* γ 3 region, does not support CSR, indicating that the presence of consensus S DNA is crucial for CSR (35). To define the minimum S DNA target required for recombination, structural variants of pG3.01s were constructed which include 1, 2, or 6 consensus tandem repeats and are referred to as, pG3.02s, pG3.025s, and pG3.045s, respectively (Fig. 2 A).

DC-PCR analysis was used to determine the level of switching for the minimal plasmids as compared with pG3.1 in 1.B4.B6 cells and in LPS-activated normal splenic B cells (Fig. 3, A and B). 1.B4.B6 cells were shown previously to support endogenous and pG3.1-based $\mu \rightarrow \gamma$ 3 CSR (35, 36). PCR amplification of the S/S composite fragment after *SacI* digestion and ligation yields 131- and 180-bp fragments for the minimal substrates and for pG3.1,

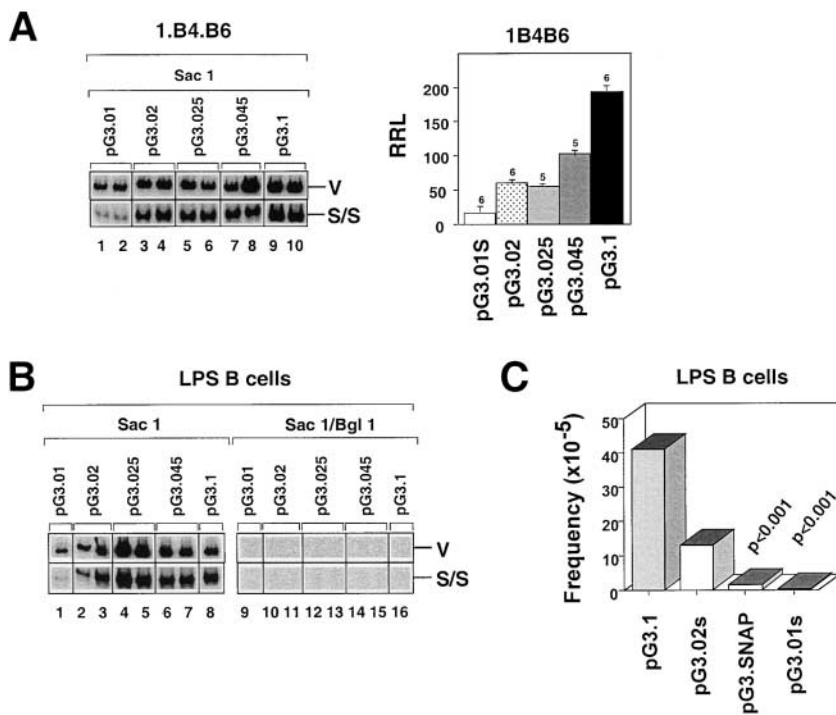


Figure 3. Switch substrates require a single Sy consensus repeat to support CSR. (A) Switch plasmids, as indicated, were transfected into 1.B4.B6 cells and analyzed for CSR using the plasmid-based DC-PCR assay (left). The RRLs are the results of five to six transfections from at least two independent experiments, and SDs are shown (right). The RRL is calculated for each plasmid as the ratio of radioactivity associated with the 180-bp S/S fragment to that of the 510-bp vector-associated fragment. The RRLs are not normalized. (B) Switch plasmids were transfected into LPS-activated B cells and analyzed by plasmid-based DC-PCR using either SacI or SacI and BglI digestion. (C) The switching activity of pG3.1, pG3.02s, pG3.SNAP, and pG3.01s were compared in LPS-activated B cells using the bacterial transformation assay. DNA recovered from nuclei of the transfected cells was untreated or digested with EcoRI and then transformed into bacteria. S/S recombinant frequency was as follows: pG3.1 (17/41,540); pG3.02s (10/110,060); pG3.01s (0/231,800); and pG3.SNAP (6/570,400). Switch frequency was obtained by dividing the number of S/S recombinant transformants by the total number of transformants and multiplying by 10^5 . Results are summarized from at least three to six transfections from two to three independent experiments. p-values, derived by χ^2 analysis, are positioned above the histograms and indicate the confidence level that the plasmid switch frequency in the pG3.02s was significantly different from that obtained from pG3.01s and pG3.SNAP.

respectively. This analysis demonstrates CSR of pG3.1 in 1.B4.B6 cells and in LPS-activated B cells, whereas essentially no CSR was found for the pG3.01s plasmid, consistent with previous findings (35). The switch substrates, pG3.02s, pG3.025s, and pG3.045s, were active for CSR (Fig. 3, A and B), where pG3.045s showed twofold lower switching activity, and pG3.025s and pG3.02s had about fourfold lower activity than the pG3.1 plasmid as assessed by PhosphorImager analysis (Fig. 3 A, right). As a control, transfected DNA isolated from LPS-activated splenic B cells was digested with the combination SacI and BglI. Under intramolecular ligation conditions, BglI digestion will abolish the 510-bp vector-associated fragment because there are two BglI sites located in the vector backbone. All the switch plasmids also contain a BglI site at the 3' end of the Sy3 region, and digestion with SacI and BglI will abolish the S/S DC-PCR product. The absence of the vector and S/S-associated fragments after SacI and BglI digestion indicates that the switch substrates are digested to completion and that ligation is exclusively intramolecular (Fig. 3 B, compare lanes 1–8 and 9–16). These results demonstrate that the efficiency of switching on switch substrates is related to the number of tandem repeats in the Sy3 region and that a single consensus repeat is sufficient for CSR.

To further evaluate CSR on pG3.02s, the previously described bacterial transformation assay was used to compare plasmid-based CSR frequencies for pG3.1, pG3.02s, and pG3.01s (35). The frequency of CSR for pG3.02s was approximately sixfold lower than pG3.1, whereas no switching was detected for the pG3.01s plasmid (Fig. 3 C). This

finding confirms the DC-PCR analysis indicating that a single tandem repeat is sufficient to enable CSR. To determine the minimum length requirements for S DNA in the switch reaction, a new plasmid termed pG3.SNAP was constructed in which the 49-bp consensus repeat found in pG3.02s was truncated to 30 bps and analyzed in LPS-activated B cells (Fig. 2 B). The switching frequency of pG3.SNAP was 41-fold lower than pG3.1 and sixfold lower than pG3.02s, thus demonstrating that reduction of S DNA to less than a single tandem repeat results in a severe diminution of CSR efficiency (Fig. 3 C). The reduced frequency of CSR for pG3.SNAP suggests that sequence important to CSR is located in either or both the SNIP site at the 5' end and several nucleotides at the 3' end of the repeat unit.

S/S junctions in recombinant pG3.1 and pG3.02s recovered in the bacterial transformation assay were analyzed by DNA sequence analysis (Fig. S1, A and B, available at <http://www.jem.org/cgi/content/full/jem.20031935/DC1>). These composite S/S regions were found to have characteristics associated with switch junctions derived from the endogenous locus (39). Similar to physiological switch junctions, the switch plasmid-derived recombination breakpoints were scattered across the breadth of the S μ and S γ 3 DNA sequences. The structure, degree microhomology, and presence of mutations in the switch junctions derived from pG3.02s conform to the usual parameters associated with CSR and confirms that this minimal switch plasmid is capable of supporting bona fide switching (40–42).

Minimal Switch Substrates Display Isotype Specificity. Previous studies indicate that a switch substrate specific for

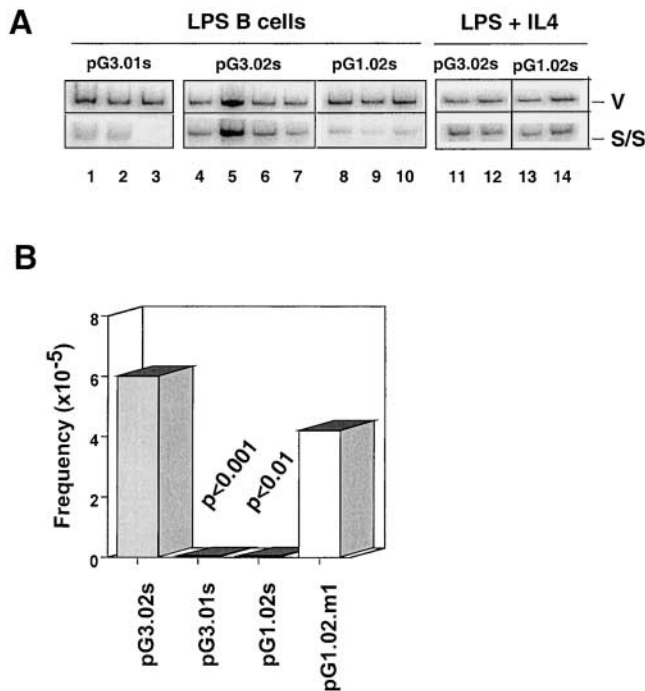


Figure 4. Substitution of three nucleotides in the Sy1 SNIP site leads to reconstitution of switching in LPS B cells for the pG1.02s.m1 plasmid. (A) The plasmids, pG3.01s, pG3.02s, and pG1.02s, were transfected into B cells activated with LPS in the presence or absence of IL-4 and then harvested and analyzed by DC-PCR. The 510-bp vector (V) and the 180-bp S/S PCR fragments are shown. (B) The plasmids, pG3.01s, pG3.02s, pG1.02s, and pG1.02s.m1, were transfected into B cells activated with LPS then harvested and analyzed using the bacterial transformation assay. S/S recombinant frequency was as follows: pG3.02s (10/110,060); pG3.01s (0/231,800); pG1.02s (0/138,800); and pG1.02.m1 (9/212,080). Switch frequency was obtained by dividing the number of S/S transformants by the total number of transformants and multiplying by 10⁵. Results are summarized from at least three to six transfections from two to three independent experiments. p-values are positioned above the histograms. Values of p were derived by χ^2 analysis.

$\mu \rightarrow \gamma 1$ CSR is induced to recombine in splenic B cells activated with LPS + IL-4 but not with LPS alone, demonstrating distinct switching activities for $\mu \rightarrow \gamma 3$ and $\mu \rightarrow \gamma 1$ CSR (36). To determine whether isotype specificity is retained in minimal switch plasmids, pG1.02s was constructed and is identical to pG3.02s except for the presence of an Sy1 consensus repeat (Fig. 2 B). Previous studies indicated that the Sy tandem repeats contain SNIP and SNAP recognition motifs, which are shown here to provide a point of reference in mapping functional recombination motifs (FRMs) on the switch plasmids (43, 44). To ascertain whether pG1.02s is functional and displays IL-4 dependency for CSR, pG3.02s and pG1.02s were transfected into normal B cells activated with LPS in the presence or absence of IL-4. The DC-PCR assay indicates that pG3.02s undergoes CSR in LPS-activated B cells, whereas switching was at background levels for pG1.02s and pG3.01s under these conditions (Fig. 4 A). Furthermore, the absence of CSR in the pG1.02s and pG3.01s and its presence in pG3.02s after transfection into LPS B cells was

confirmed using the bacterial transformation assay (see Materials and Methods). In contrast, both pG3.02s and pG1.02s undergo CSR in B cells stimulated with LPS + IL-4. These results demonstrate that Sy1-specific switching activity is IL-4 inducible, distinct from Sy3 switching activity, and that switch substrates containing a single consensus repeat recapitulate full-length switch plasmid function.

Mapping Functional Recombination Motifs Using Sy3/Sy1 Chimeric Switch Substrates. Retention of isotype specificity by minimal switch plasmids demonstrates that all of the information required for molecular recognition of the Sy3 and Sy1 regions is encoded in a single consensus tandem repeat. Comparison of the Sy3 and Sy1 tandem repeats reveals differences at only 12 out of 49 nucleotides (Fig. 2 C). Systematic substitution of Sy3-associated nucleotides into the Sy1 sequence might lead to reconstitution of the motifs necessary for $\mu \rightarrow \gamma 3$ plasmid-based CSR in LPS B cells. To test this hypothesis, a new minimal plasmid, referred to as pG1.02.m1, was constructed and contains a Sy1 repeat altered at three nucleotide positions (T \rightarrow G, T \rightarrow A, and C \rightarrow Δ , where Δ represents a deletion) located at the 5' end of the repeat (Fig. 2 C). Several other constructs containing additional combinations of S/S chimeric repeats were attempted and proved to be unclonable.

The pG3.02s, pG3.01s, pG1.02s, and pG1.02.m1 plasmids were transfected into LPS B cells and analyzed using the bacterial transformation assay for CSR activity (Fig. 4 B). In this study, pG3.02s and pG3.01s functioned as positive and negative controls, respectively. The pG1.02s plasmid did not undergo CSR in LPS-activated B cells, confirming the DC-PCR analysis of this switch plasmid, whereas pG1.02.m1 was able to recombine under these conditions (Fig. 4 B). The S/S junctions derived from pG1.02.m1 were isolated in the bacterial transformation assay, submitted to DNA sequence analysis, and found to have features similar to those found for pG3.02s, demonstrating that this plasmid is capable of bona fide CSR (Fig. S1 C). Together these studies show that limited nucleotide changes of the Sy1 sequence are sufficient to reconstitute $\mu \rightarrow \gamma 3$ CSR competence on switch plasmids in LPS B cells and suggest that the SNIP binding site or DNA sequence surrounding this site operates as an isotype-specific FRM.

Sy3 Recombination Breakpoints Are Nonrandom in pG3.02s and pG1.02.m1. The truncated Sy regions found in pG3.02s and pG1.02.m1 provide a limited target for CSR. Sy recombination breakpoints derived from pG3.02s and pG1.02.m1 associated switch junctions were found to be nonrandomly distributed (Fig. S1 D). The breakpoints are located in several subregions found in both the Sy3 consensus and degenerate repeats and are closely flanked by RGYW hotspots. Most of the breakpoints are located in a section of the degenerate repeat replete with RGYW motifs. The absence, truncation, or replacement of the Sy3-associated SNIP site in pG3.01s, pG3.SNAP, and pG1.02s, respectively, led to loss of CSR in response to LPS in activated B cells, demonstrating that the SNIP motif, located in the consensus repeat, is involved in a critical step of the

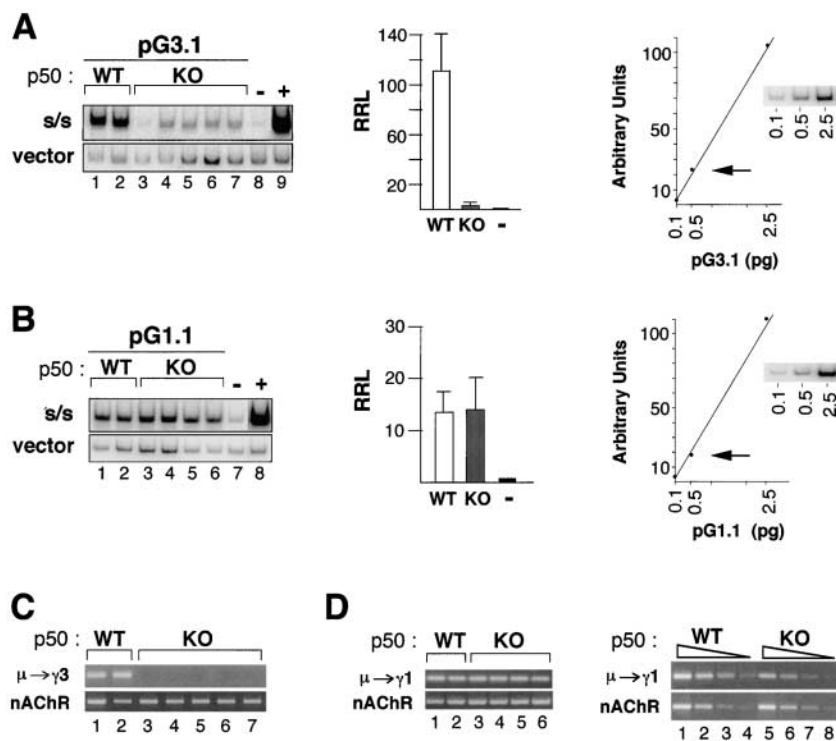


Figure 5. Plasmid-based $\mu \rightarrow \gamma 3$ CSR is abolished in NF- κ B p50-deficient B cells. (A and B) B cells from NF- κ B p50 WT and knockout (KO) spleens were T cell depleted, activated with LPS or LPS + α ddex + IL-4 + IL-5 + TGF β for 3 d, then transfected with pG3.1 (A, left) or pG1 (B, left), respectively, grown in culture for an additional 3 d, and then used in a plasmid-based DC-PCR assay. The PCR products representing the switched plasmid (S/S) and the vector backbone are shown from independent transfected samples. The average RRLs from two independent experiments for pG3.1 (A) and pG1 (B) is plotted (middle), and SDs are shown. Standard curves (fivefold dilutions) for vector DC-PCR products are shown (right). The arrow indicates the concentration of plasmid (0.5 pg) used in the DC-PCR assays shown here. (C) Endogenous DC-PCR assays for $\mu \rightarrow \gamma 3$ CSR in LPS + α ddex + IL-4 + IL-5 + TGF β activated B cells from p50 WT (lanes 1 and 2) and KO (lanes 3–7) are shown. The nAChR locus is used as a control for sample loading and ligation. (D) Endogenous DC-PCR assays for $\mu \rightarrow \gamma 1$ CSR in LPS + α ddex + IL-4 + IL-5 + TGF β activated B cells are shown for p50 WT (lanes 1 and 2) and KO (lanes 3–6) in the left panel. Fourfold serial dilution of representative DC-PCR samples are shown in the right panel for WT (lanes 1–4) and KO (lanes 5–8). The nAChR locus is used as a control for sample loading and ligation.

recombination reaction. The location of recombination breakpoints in the downstream degenerate repeats implies that if CSR initiates in the consensus repeat then processing of staggered DSBs must occur. Genetic evidence suggests that MMR proteins are involved in processing of broken DNA ends in CSR (26, 27, 45) and modulate the efficiency of switching (25, 46).

NF- κ B p50 Expression Is Required for Plasmid-based $\mu \rightarrow \gamma 3$ CSR. The localization of the FRM to a site containing a NF- κ B p50 binding site suggests that p50 may be a mediator of $\mu \rightarrow \gamma 3$ CSR. To further explore the functional involvement of p50 in $\mu \rightarrow \gamma 3$ CSR, WT and p50-deficient B cells were activated with LPS or with LPS + α ddex + IL-4 + IL-5 + TGF β (34) for 3 d and then transfected

with the pG3.1 or the pG1 plasmids, respectively. The LPS + α ddex + IL-4 + IL-5 + TGF β conditions were used, since these provided robust switching $\mu \rightarrow \gamma 1$ (47), whereas LPS + IL-4 stimuli did not work well for the p50 $^{-/-}$ B cells. Sha et al. reported that resting B cells respond poorly to LPS (37). However, we have found that unfractionated splenic B cells from WT and p50-deficient mice proliferate equally well in response to LPS for at least the first 3 d of culture. Thereafter, p50-deficient B cells do not survive as well as WT so that at the end of 6 d survival of the p50 $^{-/-}$ B cells is about half that of the p50 $^{+/+}$ B cells as assessed by trypan blue exclusion (unpublished data). Nonetheless, plasmid-based CSR should be easily detectable even in cell cultures with a 50% reduction of cell sur-

Table I. Microhomology Is Reduced in S μ /S $\gamma 3$ Junctions Derived from p50 $^{-/-}$ Mice

	Percentage of S μ /S $\gamma 3$ junctions with indicated microhomology				p-value ^a	Number of junctions	Reference
Mouse	0 bp	1–3 bp	4–8 bp	9–11 bp			
p50 $^{+/+}$ ^b	28	52	16	4		25	This paper
p50 $^{+/-}$ ^b	33	28	39	0	NS	18	This paper
p50 $^{-/-}$ ^b	50	50	0	0	0.017	22	This paper
Msh2 $^{+/+}$ ^c	28	56	16	0		32	Reference 27
Msh2 $^{-/-}$ ^c	50	50	0	0	0.004	32	Reference 27

NS, not significantly different.

^aThe significance of difference between $+/+$ and $+/-$ to $-/-$ was calculated using a one-tail Student's *t* test.

^bCells were activated with α ddex + LPS + IL-4 + IL-5 + TGF β for 5 d, and DNA was isolated. The percentage of junctions with designated overlaps is shown.

^cCells were activated with LPS for 5 d, and DNA was isolated.

vival if switching frequency is unperturbed. Standard curves indicating that the concentration of switch plasmid in the DC-PCR (0.5 pg indicated by the arrow) produces a vector signal in the linear range of detection are shown (Fig. 5, A and B, rightmost panels). These standards are included in all the DC-PCR assays reported here. DC-PCR and RRL analysis of transfected pG3.1 and pG.1 indicates that whereas p50 deficiency has a profound negative impact on $\mu \rightarrow \gamma 3$ plasmid-based switching, $\mu \rightarrow \gamma 1$ CSR is intact (Fig. 5, A and B). As an additional control, endogenous $\mu \rightarrow \gamma 3$ and $\mu \rightarrow \gamma 1$ CSR was evaluated by DC-PCR in the transfected samples. We found that the WT samples support switching to both loci, whereas the p50-deficient samples are capable of $\mu \rightarrow \gamma 1$ but not $\mu \rightarrow \gamma 3$, as expected (Fig. 5, C and D). Endogenous $\mu \rightarrow \gamma 1$ DC-PCR samples were compared after fourfold serial dilution and show that CSR is reduced three- to fourfold in p50^{-/-} B cells as shown in representative examples (Fig. 5 D, right). Nonetheless, the p50^{-/-} B cells still clearly undergo CSR. These findings demonstrate a striking parallel in the isotype specificity and p50 dependency of both plasmid-based and endogenous CSR and confirm that $\mu \rightarrow \gamma 3$ plasmid-based CSR is dependent on the presence of p50 as predicted.

Switch Junctions Exhibit Reduced Microhomology in p50-deficient B Cells. Switch junctions display characteristic features including short stretches of identity or microhomology between the S μ donor and S γ acceptor in ~50% of junctions. To determine whether p50 plays a role in the mechanics of endogenous $\mu \rightarrow \gamma 3$ CSR, B cells were cultured in the presence of LPS + α ddex + IL-4 + IL-5 + TGF β for 5 d, and S μ /S $\gamma 3$ junctions isolated from p50 WT (+/+), heterozygous (+/-), and deficient (-/-) B cells were examined by DNA sequence analysis (36, 38). Under these activation conditions, the absence of $\mu \rightarrow \gamma 3$ switching in the p50^{-/-} B cells was not due to a proliferative insufficiency or reduced expression of $\gamma 3$ gt (34). Despite the significant reduction of $\mu \rightarrow \gamma 3$ CSR in p50-deficient B cells, rare S μ /S $\gamma 3$ junctions were successfully amplified and analyzed by automated DNA sequence analysis (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20031935/DC1>). Comparison of the lengths of identity between S μ and S $\gamma 3$ indicates that junctions from p50^{+/+} and p50^{+/-} mice have significantly longer overlaps than those from the p50^{-/-} mice ($P = 0.017$) (Table I). Mismatch repair proteins (MMR) were shown recently to reduce the frequency of CSR (two- to fourfold) and alter the length of microhomology at switch junctions (27). It is striking that S μ /S $\gamma 3$ junctions derived from the Msh2^{-/-} B cells display a similar reduction of microhomology as found in the p50^{-/-} junctions (Table I).

NF- κ B p50 is a transcription factor that is important in B cell activation responses, thus raising the possibility that p50 deficiency leads to reduction of MMR gene expression. This is important since the MMR proteins form heterodimers with distinct functions (for reviews see references 48, 49) and individual protein levels can influence heterodimer formation (48–51). Studies in human cell lines in-

dicate a linear relationship between MMR mRNA and protein, suggesting that the level of each protein is determined by transcription (52). To determine whether p50 deficiency influences MMR levels, real-time RT-PCR assays were devised for Msh2, Msh3, Msh6, Pms2, and Mlh1 transcripts (Fig. S3, available at <http://www.jem.org/cgi/content/full/jem.20031935/DC1>). There was no discernible difference in the level of these transcripts for p50^{+/+} and p50^{-/-} B cells, indicating that alteration of MMR gene expression is unlikely to have caused the reduction of microhomology at S μ /S $\gamma 3$ junctions derived from p50-deficient B cells.

Discussion

In studies reported here, recombination of a switch plasmid specific for $\mu \rightarrow \gamma 3$ switching was demonstrated to be AID dependent in LPS-activated splenic B cells. AID is a crucial mediator of CSR and has been shown to be the sole B cell-specific gene required to support CSR in non-B cells (53). Thus, the requirement for AID expression to enable successful substrate-based CSR confirms the physiological relevance of the switch plasmid assay. Four distinct switching activities for $\mu \rightarrow \gamma 3$, $\mu \rightarrow \gamma 1$, $\mu \rightarrow \epsilon$, and $\mu \rightarrow \alpha$ CSR have been detected based on the differential capacity of isotype-specific switch substrates to undergo recombination in switching B cell lines and in mitogen-activated splenic B cells (35, 36). In switching B cell lines, endogenous CSR was strictly correlated with coordinate expression of the appropriate gt and isotype-specific switching activity, indicating that transacting switch factors are integral to the recombination reaction.

In an effort to define parameters governing isotype-specific recognition of S regions, we noted that S $\gamma 3$ can be reduced from 44 to 5 repeat units without adversely affecting plasmid-based CSR frequency, whereas substrates containing two degenerate tandem repeats were incapable of CSR (35, 36). Our current studies demonstrate that a single 49-bp repeat unit is necessary and sufficient for plasmid-based CSR, whereas reduction of the S $\gamma 3$ consensus repeat from 49 to 30 bps severely diminishes plasmid-based switching. This leads to the question: to what extent is endogenous CSR dependent on S region sequence? Analysis of endogenous switching in the S μ ^{-/-} mouse, which retains 15 GAGCT motifs located in the S μ flanking regions, demonstrates reduced but detectable CSR, indicating that a very limited number of S μ motifs are sufficient to support CSR (54). The region upstream of the S μ tandem repeats (TRs) is also a target for AID catalyzed dC deamination, as assessed by the accumulation of mutations, and may account for the residual recombination in the absence of the S μ TRs (29, 31, 55, 56), whereas these mutations are essentially undetectable in germline S $\gamma 3$ and germline S $\gamma 1$ (57). Deletion of the entire endogenous I $\gamma 1$ -C $\gamma 1$ intron containing the S $\gamma 1$ region led to undetectable $\mu \rightarrow \gamma 1$ CSR (58). Replacement of S $\gamma 1$ with a 1-kb random G-rich sequence by targeted homologous recombination produced a 93% reduction of $\mu \rightarrow \gamma 1$ CSR, indicating that non-S re-

gion sequence could only marginally support CSR. It is possible that the reintroduced G-rich sequence contains rare motifs capable of supporting CSR, thus explaining the barely detectable switching for that locus. The strict requirement of $\mu \rightarrow \gamma 1$ switching on the presence of the S $\gamma 1$ TRs strongly argues that there is sequence dependency for the downstream S regions.

Isotype specificity is a distinctive feature of our switch substrates, which contain extensive tracts of S region DNA (36). It is noteworthy that switch substrates containing a single S $\gamma 1$ consensus repeat retain isotype specificity since pG1.02s undergoes $\mu \rightarrow \gamma 1$ CSR in B cells activated with LPS and IL-4 but not with LPS alone. This observation demonstrates that the minimal switch plasmids recapitulate functions associated with full-length switch substrates (36). The pG3.SNAP plasmid, containing a truncated consensus repeat, which excludes the SNIP site and several nucleotides from the 3' end, shows a severe diminution of CSR frequency. This phenotype is consistent with the localization of an FRM within either or both ends of the consensus repeat. A chimeric repeat was constructed in which three nucleotide substitutions were introduced into the S $\gamma 1$ SNIP site transforming it into a canonical S $\gamma 3$ SNIP site. Restoration of switching function to the chimeric switch plasmid in LPS B cells identifies the S $\gamma 3$ SNIP motif as containing an isotype-specific FRM. These studies also provide the first clear evidence that S DNA sequence is a major contributing factor in isotype-specific CSR.

Targeted disruption of the NF- κ B p105 gene is associated with loss of $\mu \rightarrow \gamma 3$ CSR (33, 34, 37). We report here that plasmid-based $\mu \rightarrow \gamma 3$ switching is essentially abolished in p50-deficient B cells. The finding that S μ /S $\gamma 3$ junctions from p50-deficient B cells contained reduced microhomology compared with WT provides genetic evidence that p50 has a role in determining the mechanics of the recombination reaction. Furthermore, in vivo footprinting studies demonstrate p50-dependent protein interactions at S $\gamma 3$ in B cells (34, 43). These findings support the hypothesis that p50 is a mediator of $\mu \rightarrow \gamma 3$ CSR but do not indicate the level of p50 involvement. Deletion mapping and mutational analysis of S $\gamma 3$ DNA in switch substrates demonstrate that an isotype-specific FRM colocalizes with the SNIP site, a p50 recognition motif. NF- κ B p50 homodimer binding to S $\gamma 3$ SNIP sites was demonstrated previously using nuclear extracts from LPS-activated splenic B cells in gel shift, supershift, and chemical footprinting studies (43). However, it is still possible that DNA binding proteins other than p50 could interact with the FRM. Nonetheless, the weight of evidence supports the view that p50 functions directly in the $\mu \rightarrow \gamma 3$ reaction.

Single-stranded S DNA is substrate for AID (8, 15, 16, 59, 60). Recombination on our switch plasmids is not correlated with the level of transcription of S regions (35, 61). It is possible that the use of superhelically coiled plasmids in our transient transfection experiments provides sufficient ssDNA target for AID to promote switching in the absence of high levels of transcription. Thus, it is unlikely that the

absence of p50 leading to transcription insufficiency is the reason for the loss of plasmid switching in p50^{-/-} B cells.

MMR proteins have been implicated in regulating both the frequency and the process of CSR (25–28, 46). Msh2 deficiency is associated with reduced microhomology at S μ /S $\gamma 3$ junctions (27), and this protein can participate in the removal of nonhomologous DNA ends during DSB repair in yeast (62, 63). Our findings highlight a striking reduction in the extent of microhomology at S μ /S $\gamma 3$ junctions from p50^{-/-} and Msh2^{-/-} B cells and are consistent with the hypothesis that p50 is epistatic with Msh2. However, p50 deficiency has a much greater impact on CSR frequency than does Msh2. These results support a model in which p50 recruits Msh2 and other components of the recombination machinery to S $\gamma 3$ DNA.

Given that parameters defining the mechanism of CSR have begun to emerge, it is useful to consider the role FRMs and isotype-specific factors might play in the CSR reaction (for review see reference 6). It is likely that AID initiates CSR by deaminating cytosine residues in genomic S DNA and converting them to dU (7–9, 15). The mutagenic potential of uracil is very high since it can be efficiently replicated like normal thymine to yield C to T transition mutations (64). Indeed, ectopic expression of AID in non-B cells leads to a promiscuous mutator phenotype and tumorigenesis (65–67) and indicates that B cells must have highly specific targeting mechanisms to avoid the introduction of mutations by capricious AID. These considerations point to two scenarios in which isotype-specific FRMs may function in the mechanism of CSR. First, it is possible that isotype-specific factors are adaptor proteins that tether AID to S regions via FRMs. Gt expression, which renders an S target accessible and isotype-specific adaptors used to recruit AID to the FRMs, would together initiate CSR and provide two levels of protection against the promiscuous mutator function associated with AID. Second, isotype-specific activities and their FRMs might be involved in the resolution phase of CSR in which DNA lesions located in S regions are targeted to repair foci specifically engineered for S/S recombination. More work is needed to define the function of FRMs and isotype-specific activities in the mechanism of CSR.

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