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Inducible DNA breaks in Ig S regions are dependent on AID and UNG

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Class switch recombination (CSR) occurs by an intrachromosomal deletion whereby the IgM constant region gene (C\textsubscript{H}\textsubscript{10}) is replaced by a downstream constant region gene. This unique recombination event involves formation of double-strand breaks (DSBs) in immunoglobulin switch (S) regions, and requires activation-induced cytidine deaminase (AID), which converts cytosines to uracils. Repair of the uracils is proposed to lead to DNA breaks required for recombination. Uracil DNA glycosylase (UNG) is required for most CSR activity although its role is disputed. Here we use ligation-mediated PCR to detect DSBs in S regions in splenic B cells undergoing CSR. We find that the kinetics of DSB induction corresponds with AID expression, and that DSBs are AID- and UNG-dependent and occur preferentially at G:C basepairs in WRC/GYW AID hotspots. Our results indicate that AID attacks cytosines on both DNA strands, and staggered breaks are processed to blunt DSBs at the initiating ss break sites. We propose a model to explain the types of end-processing events observed.

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Abbreviations used: AID, activation-induced cytidine deaminase; AP, apurinic/apyrimidic; C\textsubscript{H}, heavy chain constant; CSR, class switch recombination; ds, double-stranded; DSB, double-strand break; LM-PCR, ligation-mediated–PCR; NHEJ, nonhomologous end-joining; S, switch; ss, single-stranded; SSB, single-strand break; UNG, uracil DNA glycosylase.

Upon activation by antigen and accessory signals, naive IgM\textsuperscript{+}IgD\textsuperscript{+} B cells undergo Ig class switching, which results in expression of a different heavy chain constant (C\textsubscript{H}) region gene, while maintaining expression of the same variable region gene. Because the C\textsubscript{H} region determines the antibody effector function, class switching allows the humoral immune system to respond adaptively to a variety of different infectious organisms to produce the best antibody for elimination of each pathogen. Class switching is mediated by DNA recombination between switch (S) region sequences residing 5’ to each C\textsubscript{H} gene, except C\textsubscript{H} (1), and is mediated by a type of nonhomologous end-joining (NHEJ; reference 2).

Class switch recombination (CSR) is completely dependent on activation-induced cytidine deaminase (AID; references 3, 4). AID is believed to initiate the formation of single-strand DNA breaks that are converted to the double-strand breaks (DSBs) that are necessary for the intrachromosomal deletions that result in CSR. γH2AX foci, which are indicative of DSBs, have been observed at the Ig locus in cells undergoing CSR, but not in cells deficient in AID (5); AID-dependent DSBs have been detected in Ig S regions by ligation-mediated (LM)-PCR in mice and humans (6, 7). However, the mechanism and site(s) of break formation are unknown. Most data support a model in which AID initiates DNA breaks by directly deaminating cytosines in S region DNA, thus generating uracils (8–12). Uracil DNA glycosylase (UNG), leaving an abasic site (8, 13). Abasic sites can be recognized by an apurinic/apyrimidic (AP) endonuclease (APEX1 or 2) that nicks the DNA backbone (8). CSR is reduced severely in the absence of UNG (13, 14), but the function of UNG has been called into question. A recent study by Begum et al. (15) concluded that although UNG is required for CSR, the glycosylase activity of UNG is not required for γH2AX foci formation. It was suggested that UNG is not required for generating DNA breaks in S regions, but has another function in CSR. This study used UNG mutants that retain low levels of activity (16); thus, it is possible that a low level of uracil glycosylase activity is sufficient for CSR. It has not been reported whether AP endonuclease is involved in CSR.

Although the presence of uracils in Ig S regions has not been demonstrated directly, most data are in agreement with the direct deamination of cytosines in DNA by AID. AID has been shown to deaminate single-stranded (ss), but not double-stranded (ds),
DNA (9–12) in vitro. Transcription of the S region is required for CSR (17, 18), and current results suggest that S region transcription generates an ss DNA substrate for AID (9, 19). Apparently as a result of the G-rich nature of the RNA transcript, RNA transcribed across S regions stably associates with the transcribed (bottom) DNA strand, forming R-loops that leave the nontranscribed (top) strand unpaired and vulnerable to attack by AID (20–22). However, it is not clear how nicks on both strands are made in order to generate DSBs. The bottom strand of the mouse Sµ region has ~2.5-fold more C residues than the top strand, which could increase targeting of AID to the bottom strand if it were ss. Purified recombinant AID can deaminate both DNA strands within a supercoiled plasmid, which suggests that supercoiling might generate DNA with sufficient ss structure to form an AID substrate on both strands (23).

Understanding how the nicks are introduced into S regions and converted into DSBs is essential for understanding the mechanism of CSR. Previous investigators have used LM-PCR to detect S region breaks, but with varying results. Since the discovery of AID, two groups have detected AID-dependent blunt and staggered DSBs in Sµ segments in human peripheral blood B cells (6) and in mouse splenic B cells activated in culture (7). In addition, ss breaks were detected on the transcribed strand of Sµ from the B lymphoma CH12F3-2, a cell line that switches inducibly to IgA (24). However, this group did not detect ss breaks on the nontranscribed strand, nor blunt or staggered DSBs, nor could they demonstrate AID-dependence of the breaks. There is also inconsistency in the sequences at which DSBs were detected among the studies (6, 7, 25), and the ss breaks detected in the B lymphoma occurred preferentially at C residues but not at the known AID hotspot, WRC (26–28).

To test the current model of AID-induced DSBs, we decided to investigate the dependence of S-region DSBs on AID and UNG, and to determine the site and structure of the breaks. We identify DNA breaks induced in Ig S regions during antibody CSR in mouse splenic B cells and show that they are dependent on AID and UNG. We show that the breaks are induced simultaneously with AID protein, and demonstrate that the blunt DSBs occur preferentially at G:C bp in WRC/GYW AID hotspots. The finding that DSBs in WT cells are almost always at G:C bp strongly implicates the glycosylase activity of UNG in DSB formation, whereas the few breaks detected in the absence of UNG do not occur preferentially at G:C bp; this suggests that they are not introduced by way of an alternative uracil glycosylase. The results allow us to conclude that AID-instigated breaks occur on both DNA strands, and then end-processing subsequently generates blunt DSBs at the sites of the ss breaks. We propose a model to explain the types of end-processing events observed, and suggest that certain types of end-processing are preferred.

RESULTS

To determine when AID-dependent breaks might appear, we examined the time course of induction of AID protein in splenic B cells induced to undergo CSR. B cells from WT and AID-deficient mice were stimulated with LPS plus IL-4 or anti-δ-dextran for various time periods before making cell extracts. BLyS/BAFF was added to all B cell cultures to reduce cell death (29), and to increase cell proliferation and CSR (Schmidt, M.R. and Woodland, R.T., unpublished data). AID protein was not detectable in resting splenic B cells and was barely detectable in the cytoplasm at 24 h, 26 h, and 30 h, but was greatly induced in cytoplasm and nuclei 48 h after activation; levels were even greater at 72 h (Fig. 1 A). AID was induced equally by LPS plus IL-4 or LPS plus anti-δ dextran, less well by LPS alone, and not at all by IL-4 plus anti-δ dextran (Fig. 1 B). These data are consistent with the finding that AID mRNA is not detected until 2 d after splenic B cell activation (reference 30 and unpublished data).

We then used LM-PCR to detect blunt DSBs in Sµ. Resting splenic B cells from WT and AID-deficient mice were activated in culture for various times to determine the onset of

![Figure 1](https://example.com/figure1.png)

**Figure 1.** AID protein is up-regulated 48 h after stimulation of cells to undergo CSR. Western blots of 40 µg cytoplasmic and 40 µg nuclear extracts prepared from WT or aid<sup>−/−</sup> splenic B cells cultured as indicated. Extracts from freshly isolated resting B cells are shown in the left-most lanes. The four blots were incubated with anti-AID antiserum simultaneously, and exposed to film simultaneously. A 42-kD protein in the nuclear extracts that cross-reacts with the AID antibody is shown to demonstrate nearly equivalent loading of protein in the different lanes. The 26-kD protein in cytoplasmic extracts is likely to be a cross-reacting protein, because a band this size also is observed sometimes in aid<sup>−/−</sup> cells (unpublished data).

(A) Time course showing induction of AID in WT cells treated with LPS plus IL-4. (B) Blots of cytoplasmic and nuclear extracts from cells treated under the indicated conditions for 48 h. All conditions included BLyS.
breaks and whether they were dependent on AID. Genomic DNA was isolated, ligated to linker, and LM-PCR was performed using primers specific for Spμ or Cμ. A few breaks were detected at early time points in WT and AID-deficient B cells, but at 48 h, abundant breaks were detected in WT B cells stimulated with LPS plus IL-4 or with LPS plus anti-δ-dextran. This is consistent with the appearance of AID protein at 48 h (Fig. 2 A, 5’ end of Spμ; Fig. S1, 3’ end of Spμ, available at http://www.jem.org/cgi/content/full/jem.20050872/DC1). Very few breaks are observed in B cells from AID-deficient mice at this time point (Fig. 2 A, bottom). The DSBs detected are specific to cells undergoing CSR, because cells stimulated to proliferate with IL-4 plus anti-δ-dextran divide rapidly, but do not induce AID or undergo CSR (Fig. 1 B and not depicted); these cells do not develop AID-dependent DSBs in Spμ (Fig. 2 A). Very few breaks are detected in the Cμ gene, which is not used in CSR, and the few breaks observed are not AID-dependent (Fig. 2 B). AID-dependent DSBs also are introduced into Sy3 segments at 48 h (Fig. 2 C). These data clearly indicate that AID is required for the introduction of blunt DSBs in the Spμ and Sy3 regions during CSR.

B cells from ung−/− mice have been shown to switch very poorly in culture (13, 15); however, whether UNG is required for introduction of S region breaks is controversial (14, 15). To examine this question, we activated ung−/− splenic B cells to undergo CSR, and analyzed surface Ig expression and blunt DSBs as described above. Consistent with the involvement of UNG in excision of uracil bases during CSR, we found a great reduction in CSR (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20050872/DC1) and the numbers of Spμ and Sy3 DNA breaks detected in ung−/− B cells after 48 h of activation (Fig. 2 D).

**Nucleotide sites of DNA breaks**

The AID–UNG–APEX model for introduction of DNA breaks into S regions predicts that the initiating ss breaks will occur at dC residues in the AID hotspot motif WRC (where W = A or T, and R = purine). However, because blunt DSBs require end-processing from the ss nicks, it is unclear whether DSBs would occur at one of the original ss break sites. To address this question, we cloned the blunt DSB fragments, and analyzed the nucleotides at which they occurred. The base indicated in the first column of Table I refers to the site of the break (i.e., the nucleotide that is deleted and replaced by the linker [reading the top strand]). As shown in Table I, 89% of the breaks in the Spμ region from WT cells occurred at G:C bp—mostly at G residues; this is a highly significant preference in comparison with the sequence itself. Similarly, 88% of Sy3 breaks occurred at G residues. By contrast, 67% of the Spμ breaks cloned from aid−/− cells were at G:C bp; this is not different from random, and is similar to the few Cμ breaks cloned from WT cells.

Additionally, 41 and 40% of the Spμ and Sy3 breaks, respectively, from WT cells occurred at the underlined C or G in WRC/GYW AID hotspots; this is a significant increase relative to random expectations (23.2 and 14.8%, respectively;
Table I. Blunt DSBs in S\(\mu\) and S\(\gamma3\) from WT splenic B cells occur preferentially at G:C bp and at GYW/WRC AID hotspots

| nt at break | WT | aid\(^{--}\) | ung\(^{--}\) | Sequence\(^{a}\) | S\(\mu\) | S\(\gamma3\) | C\(\mu\) |
|-------------|-----|-------------|-------------|-------------|--------|--------|--------|
| % (nbr) | % (nbr) | % (nbr) | % | % (nbr) | % (nbr) | % |
| G | 83.9 (47) | 62.5 (15) | 53 (9) | 40.7 | 84 (21) | 48.7 | 57 (4) |
| C | 5.4 (3) | 4.2 (1) | 5.8 (1) | 16.1 | 4.0 (1) | 14.7 | 0 |
| A | 7.1 (4) | 20.8 (5) | 35.3 (6) | 21.4 | 8.0 (2) | 18.3 | 43 (3) |
| T | 3.6 (2) | 12.5 (3) | 5.8 (1) | 21.9 | 4.0 (1) | 18.4 | 0 |
| Total | 56 breaks | 24 breaks | 17 breaks | 2,000 nt | 25 breaks | 1,460 nt | 7 breaks |
| G+C | 89.3 | 66.7 | 58.8 | 56.8 | 88.8 | 63.4 | 57 |
| p-value\(^{b}\) | <0.001 | NS | NS | 0.039 | NS |
| Hotspots | 41.1 | 4.2 | 11.8 | 23.2\(^{c}\) | 40 | 14.8 | 14.3 |
| p-value\(^{b}\) | 0.049 | NS | NS | <0.001 | NS |

\(^{a}\)The distribution of nucleotides in the genomic sequence analyzed.

\(^{b}\)Significance of difference from random.

\(^{c}\)Percentage of nucleotides in genomic sequence that are in G:C bp at the underlined nucleotide in WRC/GYW hotspots.

Table I). These data are consistent with the level of preference for hotspots by AID in vitro and in vivo during somatic hypermutation of antibody variable region genes (26–28, 31). We also determined the sequences of 26 breaks by amplifying with a primer from the 3' side of S\(\mu\). Consistent with the break sites identified with the 5' primer, 77% occurred at G:C bp, and 53.9% occurred in AID hotspots. The S\(\mu\) breaks cloned from aid\(^{--}\) cells and C\(\mu\) breaks from WT cells did not occur at WR\(\mu\)/GYW motifs (Table I). These results are entirely consistent with the introduction of breaks into S regions by the AID-UNG-APEX pathway, and also suggest that the blunt DSBs are located at sites of ss nicks instigated by AID.

The S\(\mu\) break sites amplified with the 5'S\(\mu\) primer from WT cells are indicated on the S\(\mu\) sequence shown in Fig. 3. They are found at sites across the S\(\mu\) region, including the region 5’ to the tandem repeats, although most are found within the tandem repeat region. Breaks occurred at the same site very rarely, and because of the possibility of PCR contamination from previously cloned breaks, they were eliminated from our analysis.

The low frequency of CSR in ung\(^{--}\) B cells suggests that a few breaks can be introduced into S regions independently of UNG (13). To examine how these breaks are introduced, we determined the sites of S\(\mu\) DSBs from ung\(^{--}\) cells, and found that they do not occur at G:C bp in WRC/GYW hotspots (Table I). This suggests that these few breaks are not introduced by an alternative uracil DNA glycosylase, followed by APEX action, because this would introduce DNA breaks at G:C bp in hotspots. Instead, it seems to be more likely that they are introduced by a pathway that introduces ss breaks at sites away from the initial lesion (e.g., nucleotide excision repair).

Most breaks are staggered

The AID-UNG-APEX pathway would be predicted to introduce ss breaks and staggered DSBs, rather than blunt DSBs. We determined whether treatment of DNA with T4 DNA polymerase to fill-in or excise ss overhangs before ligation of the linker-primer would increase the number of DNA breaks detected; we found that, indeed, it did increase the number of breaks detected (Fig. 4 A). Thus, similar to previous results (7), it seems that most S\(\mu\) breaks are staggered. Similar to the sites of blunt breaks, we found that 85% of the 26 cloned staggered breaks occurred at G:C bp.

DISCUSSION

The finding that the blunt DSBs are almost all at G:C bp was surprising. Staggered ss nicks resulting from AID deamination of C residues on opposite strands would require processing of DNA ends to generate blunt DSBs, and it seemed likely that end-processing activity would move the site of the break away from the original ss lesion. The additional finding that breaks occur preferentially at AID hotspots further demonstrates that the initial AID-dependent ss breaks become the site of blunt DSBs. These blunt DSBs would provide a favorable substrate for NHEJ. The lack of preference for S–S junctions using microhomologies is consistent with the use of blunt DSBs in recombination (32). Although some S–S junctions do show short microhomologies, they do not seem to be favored (i.e., their occurrence does not seem to be increased significantly relative to random expectations).

Fig. 4 B presents a model for how ss breaks and staggered DSBs that are introduced by the AID-UNG-APEX pathway could be converted to blunt DSBs. Because the blunt DSBs are detected at G residues on the nontranscribed strand, and AID attacks C residues, our data suggest that end-processing of the ss breaks could occur by fill-in DNA synthesis of 5’ overhangs (left-side pathway) and by removal of 3’ overhangs by exonuclease I and the structure-specific endonuclease ERCC1-XPF (right-side pathway). The lack of blunt DSBs at top-strand C residues suggests that 5’ overhangs are converted...
infrequently to DSBs by exonuclease or endonuclease activity (middle pathway). Exo1 and ERCC1-XPF are known to be involved in CSR (33, 34).

Our data also clearly indicate that C residues on the transcribed strand are targeted by AID. Although it has been shown that AID preferentially deaminates cytosines on the top, nontranscribed strand of plasmid DNA in vitro or in *Escherichia coli* (9, 19, 28), it also was shown that cytosines on both DNA strands of a nontranscribed supercoiled plasmid can be deaminated equally in vitro (23). Because of the apparent bias in types of processing events observed, the relative frequency of targeting the transcribed and nontranscribed strands cannot be determined from the sites of the blunt breaks. Consistent with this bias, breaks at C residues are distributed more equally on the transcribed and nontranscribed strands after T4 DNA polymerase treatment (*Sμ*/*H9262* breaks: G:C ratio is 3.4 for staggered breaks and 15.7 for blunt breaks).
breaks detected after T4 DNA polymerase treatment include blunt breaks and those that require T4 DNA polymerase processing to become ligatable. Thus, the finding that the G:C ratio of these breaks approaches the ratio in the sequence itself (which is 2.5) suggests that breaks occur approximately equally on both strands. This further suggests that the bottom transcribed strand exists as ss DNA—accessible to AID—at least transiently during CSR. Yu et al. (22) suggested that this might occur when R-loops collapse out-of-register because of the presence of tandem repeats in S regions.

The finding that the breaks in ung−/− cells do not occur preferentially at G:C bp in AID hotspots is consistent with recent results which showed that overexpression or deletion of the uracil DNA glycosylases, SMUG-1 or MBD4, respectively, have no effect on CSR (35, 36). Although it has been shown that a double-deficiency of UNG and the mismatch repair protein Msh2 completely eliminates CSR (36), it is not clear how mismatch repair could introduce the initiating DNA breaks. ERCC1-XPF is the only endonuclease that is known to be recruited by Msh2 (as a heterodimer with Msh3) (37). ERCC1-XPF does not seem to function with Msh2 in CSR (34), and Msh3 does not seem to be involved in CSR (38, 39). Although two endonucleases, ERCC1-XPF and XPG, participate in the nucleotide excision pathway in mammals—each of which introduce ss DNA breaks at sites distal to the lesion itself—this pathway is not known to recognize dU residues or abasic sites in vivo (40). However, nucleotide excision repair might recognize DNA that is distorted by multiple lesions that might accumulate in the absence of UNG.

Our data support a model in which most CSR is initiated by introduction of ss DNA breaks into the transcribed and nontranscribed strands of the S region by AID and UNG, likely followed by APEX activity. Single-strand breaks (SSBs) are converted into blunt DSBs that end at the site of one of the original ss lesions. If the SSBs on opposite strands are near each other, they could form staggered DSBs spontaneously that could be recombined by NHEJ. However, if the SSBs are more distal than a few bp, they are not likely to form a DSB. We propose that end-processing sometimes converts these distal SSBs to the blunt DSBs detected by LM-PCR.

MATERIALS AND METHODS

Mice. Mice deficient in AID were obtained from T. Honjo (Kyoto University, Kyoto, Japan) and were backcrossed to C57BL/6 mice for four generations. They were housed in the Institutional Animal Care and Use Committee–approved specific pathogen-free facility at the University of Massachusetts Medical School. WT littermates were used as controls for all experiments shown. Some WT S region DSB sequences were obtained from (C57BL/6 × FVB) F1 mice. There was no difference in the break sites between these F1 mice and C57BL/6 mice. UNG-deficient mice were obtained from T. Lindahl and D. Barnes (London Research Institute, London, England). The mice were bred and used under guidelines formulated by the University of Massachusetts Animal Care and Use Committee.

Antibodies. Antibody to AID was induced in rabbits by immunization with the COOH-terminal peptide (with an added NH2-terminal C) CEVDDLRDARMLGF-OH. Antibody was purified by ammonium per sulfate precipitation of IgG, followed by immunoadsorption to the AID COOH-terminal peptide coupled to a Pierce Sulfo-link column, following the manufacturer’s specifications. Isotype-specific antibodies, conjugated to FITC or PE, were purchased from Southern Biotechnology Associates, Inc.

Western blotting. Cells were lysed in 0.6% NP-40, pelleted, and supernatants were taken as cytoplasmic extract. The nuclear pellet was washed once with low-salt buffer and extracted in high-salt buffer (41). 40 μg of extracts were electrophoresed on 12% polyacrylamide gels. The four blots shown were incubated with anti-AID antisera simultaneously, followed by goat anti–rabbit horseradish peroxidase (Southern Biotechnology Associates, Inc.) and Western Lightning PLUS chemiluminescence reagent (PerkinElmer), and then were autoradiographed simultaneously.

B cell isolation and culture. Single cell suspensions from spleens of 2–5-mo-old mice were subject to Percoll gradient centrifugation. Small cells from the 60/70% interface were collected and depleted of CD43+ cells by MACS (Miltenyi Biotech GmbH). CD43+ small B lymphocytes were cultured at 4 × 10^6/ml in six-well plates in RPMI 1640 (BioWhittaker), with 10% FCS (HyClone), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Gibco BRL), and 5 × 10^-5 M 2-mercaptoethanol (Sigma–Aldrich). All cultures contained LPS (50 μg/ml, Sigma–Aldrich)—except where indicated in Fig. 2—and BlyS (recombinant, 100 ng/ml; Human Genome Sciences). IL-4 (800 U/ml; provided by W. Paul, National Institutes of Health, Bethesda MD) and anti-6-dextran (0.3 ng/ml; gift from C. Snapper, Unified Services University of Health Sciences, Bethesda MD) were added where indicated.

BlyS was added to increase cell viability; it also increased proliferation as assayed by CFSE monitoring (unpublished data) and by thymidine uptake (Schmidt, M.R., personal communication). The frequency of CSR in the cultures also increased (e.g., CSR to IgG3 after 4 d of culture increased from 10.1% in its absence to 23.5% in its presence; the average increase in five experiments was 1.9-fold). BlyS, in the absence of LPS, did not induce AID (Fig. 1, C and D), DNA breaks (Fig. 2 A), or CSR in splenic B cells (not depicted).

Genomic DNA preparation. After culture for the indicated time periods, viable cells were isolated by flotation on Ficoll/Hypaque gradients (6 = 1.09). 10^6 cells in 25 μl PBS were mixed with an equal volume of 1% low-melt agarose in 40 mM EDTA in 1.5 ml Eppendorf tubes and put on ice. Agarose plugs were overlaid with proteinase K (1 ng/ml; Sigma–Aldrich) in 50 mM Tris-Cl, pH 8.2, 20 mM EDTA, and 1% SDS and incubated overnight at 55°C. Supernatant was removed and plugs were washed with 1 ml TE (50 mM Tris, 20 mM EDTA) rotating at room temperature for 1 h and the proteinase K treatment was repeated. Plugs were then washed three times in TE, treated with 2 mM PMSF for 30 min at 37°C, washed three more times in TE, and then overlaid with and stored in 1× ligase buffer (New England Biolabs, Inc.).

Linker LM-PCR. Supernatant was removed from agarose plugs, replaced with 50 μl fresh 1× ligase buffer, and plugs were heated to 62°C to melt agarose. 20 μl DNA (~10,000 cell equivalents) was added to 2 μl T4 DNA ligase (2 Weiss units, MBI Fermentas), 10 μl ds annealed linker in 1× ligase buffer, 3 μl 10× ligase buffer, and 30 μl dH2O, and incubated overnight at 18°C. Linker was prepared by annealing 5 nmol each of LMPCR-1 (5'-GCCGTGACCCCGGGGATCTGAAATC-3') and LMPCR-2 (5'-GAA-TTCCGATGC-3') in 300 μl 1× ligase buffer, which results in a ds oligo with a 14-nt ss overhang that can only ligate unidirectionally. Ligated DNA samples were heated to 70°C for 10 min, diluted fivefold in distilled H2O, and assayed for GAPDH by PCR to adjust DNA input before LM-PCR. The following primers (Integrated DNA Technologies) were used in conjunction with linker primer (LMPCR-1) to amplify DNA breaks: 5'-CAAGCTTCCGATGC-3', 3'-CCGGAATCTGAAATC-5'.

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GCAGAAAATTTTGATAAAATGATACCTCGAGG-3′ (used for all experiments except for data shown in Fig. S1); 3′Sa: 5′-GGCTCATC-CCGAGGATCTCAGAGG-3′ (used only for Fig. S1); 5′GACATTTCGAGGAGCCCGGGAGG-3′ (25); and CmuL2: 5′-CTG- CGAGAGCCCCCGTCTGATAAG-3′ (42).

Three-fold dilutions of input DNA (0.5, 1.5, and 4.5 μL) were amplified by Hotstar Taq (QIAGEN) using a touchdown PCR program. PCR products were run on 1.25% agarose gels and vacuum blotted (VacuGene XL, Pharmacia) onto nylon membranes (GeneScreen Plus, PerkinElmer). Blots were hybridized with oligonucleotide probes end-labeled with γ32P-ATP at 37°C overnight and washed at 55°C with 2X SSC/0.1%SDS. The following probes were used: μ probe: 5′-AGGAGACGGCCTTTAATGAG-3′; Sa probe: 5′-GTTGAGAGCCCCCTAGTAAGGAGG-3′ (7); Sa3 probe: 5′-GGGCTGGCT-GATGGGATGCCC-3′ (used only for Fig. S1); Sy3-LP: 5′-GAGC CGGAGGGTTTCAGATCCTGGG-3′ (25); and Cμ: 5′-TGG-CGATGCGCTTGGGACCGGATTCTC-3′ (42).

Cloning, identification, and sequence analysis of PCR products. PCR products from cells cultured 24–48 h were cloned into the vector pCR4-TOPO (Invitrogen) and sequenced by Macrogen using T3 and T7 primers. Cloned breaks in Sμ were aligned with germline Sμ sequences from CS7BL/6/12 chromosome (GenBank Accession #AC073553) with numbering starting at nt 136,645. This is the 5′Sa primer binding site and ~800 nt upstream of the beginning of the tandem repeats. Cloned breaks in Sγ3 were aligned with germline Sγ3 sequences from BALB/c (MUSIGCD18), and cloned breaks in Cμ were aligned with germline Cμ from BALB/c (MUSIGCD10).

Statistics. Fisher’s exact test was used to determine the difference between the occurrence of breaks at hotspots (G+C residues) and the occurrence of hotspots (G+C residues) in the genomic sequence. The genomic sequence analyzed begins at the 5′ LM-PCR primer and extends to the most 3′ break cloned.

Online supplemental material. Fig. S1 shows DSBs detected using the 3′S′Sa primer from WT and aid−/− cells. Fig. S2 shows flow cytometric analysis of surface Ig isotype expression in splenic B cells from WT, aid−/− and ung−/− mice induced to undergo CSR. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20050872/DC1.

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