Single-strand DNA breaks in Ig class switch recombination that depend on \textit{UNG} but not \textit{AID}

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Abstract

B lymphocytes switch from secreting IgM to secreting IgG, IgA or IgE through a DNA recombination, class switch recombination (CSR), whose mechanism is incompletely understood. CSR is thought to be triggered by activation-induced deaminase (\textit{AID}), which is believed to deaminate cytosines to uracil in single-strand regions of switch region DNA. Subsequent excision of uracils by uracil DNA glycosylase (\textit{UNG}) (product of the \textit{UNG} gene) generates abasic sites, which are targeted for DNA cleavage, producing DNA breaks that are critical intermediates in CSR. Consistent with this model, CSR-related double-strand breaks (DSBs)—detected by ligation-mediated PCR (LMPCR)—have been reported to be dramatically reduced in B cells from either \textit{AID}^{-/-} or \textit{UNG}^{-/-} mice. Here we examine single-strand breaks (SSBs) using LMPCR and report, surprisingly, that CSR-related anti-sense strand breaks in S\textsubscript{g} regions are dependent only on \textit{UNG}, and not \textit{AID}, suggesting participation of a cytosine deaminase other than \textit{AID}. This conclusion is supported by the sequences at these DNA breaks, which show a bias for a consensus sequence different from that reported for \textit{AID}. The SSBs appear to be part of the normal CSR pathway since in B cells in which CSR is blocked by deletion of S\textsubscript{m}, the content of S\textsubscript{g} SSBs is elevated as though the breaks resolve inefficiently owing to the lack of a recombination partner for completing \textsubscript{m}-to-\textsubscript{g} CSR. These results suggest a narrower role for \textit{AID} in CSR than previously recognized and prompt a search for a putative alternative cytosine deaminase participating in CSR.

Introduction

After activation by antigen binding, B cells expressing IgM may be stimulated by T cell factors—e.g. cytokines and CD40 ligand (collectively known as T cell 'help')—to switch to expression of other Ig isotypes such as IgG, IgA or IgE. The isotype switch resulting from a DNA recombination event known as class switch recombination (CSR) that deletes the S\textsubscript{M} gene from its location downstream of the assembled VDJ region and replaces it with one of the other C genes that lie downstream of S\textsubscript{M} (for a review, see 1). The recombination break points lie within distinct non-coding sequences known as switch regions (S regions). S regions lie upstream of all the CH regions except C\textsubscript{s} and contain multiple tandem repeats of G-rich DNA sequences. The mechanism of CSR is incompletely known, but proposed models must account for three known requirements for normal CSR: (i) transcription across both S\textsubscript{M} and a downstream Sx region (2); (ii) activation-induced deaminase (\textit{AID}), an enzyme that can deaminate cytosines in single-stranded DNA or in transcribed double-stranded DNA (3); and (iii) uracil DNA glycosylase (encoded by the \textit{UNG} gene) which removes uracil bases from DNA, leaving an abasic site (4). According to a widely accepted CSR model, transcription across an S region generates transient single-stranded regions of DNA in which cytosines can be deaminated to uracil by \textit{AID}, and the uracils can then be removed by \textit{UNG}, leaving an abasic site; the DNA backbone at this abasic site is then cleaved by an unknown nuclease, possibly APE1 (apurinic–apyridinic endonuclease 1), creating a single-strand break (SSB) or nick. SSBs produced on opposite DNA strands can lead to staggered-end double-strand breaks (DSBs), which may be converted by trimming (or extension) to blunt ends. According to the model, blunt DSB ends from S\textsubscript{M} and from a downstream Sx are then joined by DNA repair factors present in all cells; these factors include ATM, 53BP1, \gamma H2AX, Ku70, Ku80, DNA–PK and others. The participation of these proteins in CSR is inferred from CSR impairments observed in mouse strains with engineered knock outs in the corresponding genes (reviewed in 5).
Somatic hypermutation (SHM) appears to be initiated by a similar mechanism since AID knockout mice are dramatically impaired in SHM (3), and UNG knock outs show abnormal SHM at C nucleotides (4); however, SHM does not require the DNA repair factors noted above.

Although the mechanism for CSR described above is widely accepted, some reported evidence conflicts with it. Thus, it has been suggested that the enzymatic activity of uracil DNA glycosylase is not required for CSR (6) or for internal deletions in S\(_{\mu}\) that depend on AID-induced recombination (7). Moreover, DSBs that occur during SHM have been reported to occur independently of AID, suggesting that AID may have a role in SHM after DNA cleavage (8–10). A post-cleavage role for AID in CSR has also been proposed (11).

An early and obligatory intermediate in all models of CSR is a DNA break in at least one strand of DNA. To probe the mechanism of CSR, we have used ligation-mediated PCR (LMPCR) to study single-strand DNA ends in S regions of B cells undergoing CSR in culture. In our previous investigation (12), we studied the murine B cell line CH12F3-2, which switches to IgA expression at high efficiency when stimulated in culture with anti-CD40 and the cytokines IL-4 plus transforming growth factor \(\beta\). We found that we could detect LMPCR bands corresponding to SSBs in the lower (antisense) strand of S\(\alpha\) from CH12F3-2 cells that were stimulated to switch, but not from control unstimulated cells. Our findings on SSBs differed in some respects from those of several other laboratories that have used a modified LMPCR protocol to examine blunt DSBs in S regions during CSR (13–16). The SSB LMPCR bands we detected were most intense at 24 h of stimulation and generally diminished by 48 h, whereas the stimulation-dependent DSBs reported from cultured splenic B cells were extremely infrequent until 48 h. By cloning and sequencing LMPCR-amplified fragments, we found the sequence C\(^{\ast}\)AG at the S\(\alpha\) breaks in 16 of 21 clones (where \(\ast\) represents the cleavage site); but the consensus reported for DSB ends (in S\(\mu\) and S\(\gamma/3\)) was the \(\text{in vitro} AID\) deamination hot spot WRC\(^{\ast}\), where \(W = A\) or \(T\) and \(R = \text{purine}\) (16). Finally, our evidence was consistent with the conclusion that most of our lower strand SSBs derived from nicks rather than blunt or staggered-end DSBs; indeed, we could not detect any upper strand breaks in DNA from 24- or 48-h stimulated CH12F3-2 cells using several primer strategies that easily detected upper strand cuts in purified genomic DNA cleaved by a restriction enzyme. We concluded that upper strand cuts must be processed differently from those on the lower strand, perhaps rapidly converted to a form that is not amplified by our LMPCR protocol.

To test whether our findings with CH12F3-2 cells are representative of CSR in normal cells, we have recently applied our LMPCR strategy to examine SSBs in S regions of murine splenic B cells undergoing CSR in culture. This system allowed us to ask whether the S region SSBs are abolished in AID and UNG knock outs, as reported for DSBs. We report here that the S region SSBs are almost completely dependent on UNG, but that, surprisingly, the pattern of LMPCR bands from the AID knockout B cells is almost identical to the wild-type (WT) pattern, and the bands are at most only modestly diminished in intensity, suggesting that most of the lower strand SSBs occur by an AID-independent mechanism. In contrast, we confirm that DSB formation requires AID as previously reported (13, 14, 16).

**Methods**

**Animals and cells**

For experiments on normal splenic B cells, we used normal 6- to 12-week-old C57BL/6 from a colony in our institution; strain 129 mice were graciously provided by R. Siegel. AID\(^{-/-}\) B cells were obtained from AID\(^{-/-}\) spleens (3) kindly provided by A. Nussenzweig. UNG\(^{-/-}\) B cells were obtained from UNG\(^{-/-}\) spleens (17) kindly provided by M. Nussenzweig. The B cells with a homozygous large deletion of \(S\mu\) and surrounding repeat sequences were derived from spleens of the mouse strain constructed by Khamlichi \textit{et al.} (18) kindly provided by David Schatz and Shyam Unniraman. As controls for mice with genetic deletions, littermates (where available) or genetically closely matched mice were used. Mice were used according to a protocol approved by our institutional Animal Care and Use Committee.

**B cell preparation and culture**

B cells were isolated from spleen cell suspensions by red blood cell depletion followed by cell purification with anti-mouse-CD19 magnetic microbeads (Miltenyi Biotech, Auburn, CA, USA). Purified B cells were washed with PBS and incubated in RPMI medium at an initial cell density of 0.5 \times 10^6 cells ml\(^{-1}\). Purity of the B cell population was routinely assessed by flow cytometry using anti-mouse CD45R/B220 antibody (BD Biosciences Pharmingen, San Diego, CA, USA) and was >95%. To stimulate CSR to \(\gamma/3\), the purified B cells were incubated with either 500 ng ml\(^{-1}\) of anti-CD40 (Pharmingen) or 25 \(\mu\)g ml\(^{-1}\) of LPS (catalog #L-7895, Sigma, St Louis, MO, USA) in RPMI medium. To stimulate CSR to \(\gamma/1\), the cells were cultured with anti-CD40 plus 25 \(ng\) ml\(^{-1}\) of IL-4 (R&D Systems, Minneapolis, MN, USA). Unstimulated cells were grown as controls.

Cells were harvested after 24-h incubation (with or without stimulation) or 48 h (only with stimulation) and stained with Trypan blue solution (Media Tech, Manassas, VA, USA) to detect the percentage of viability, typically >97%. In one experiment, live cells were further purified by centrifugation on Lympholyte (Cedarlane Laboratories, Burlington, NC, USA), removal of any residual apoptosing cells by staining with Annexin V microbeads (Miltenyi) and passage through a MACS column.

**Ligation-mediated PCR**

We followed the protocols of Mueller \textit{et al.} (19) for DNA preparation and LMPCR. Briefly, after culture, the splenic B cells (20–30 million from a single spleen) were recovered by centrifugation, washed once in 1× PBS, re-suspended in 2-ml lysis solution (0.2 mg ml\(^{-1}\) protease K, 0.2% SDS) and incubated at 37°C overnight. The lysis mixture was then gently extracted with phenol (by inverting 50 times in a 15-ml Falcon tube), followed by similar extractions with...
phenol:chloroform and then chloroform alone. The DNA was then precipitated by ethanol, centrifuged and re-suspended in 200–300 μl of water by gentle pipetting. The DNA concentration was estimated by OD260 (usually 400–1500 μg ml⁻¹).

Two micrograms of genomic DNA was denatured at 95°C and annealed with primer P1 as diagrammed in the right panel of Fig. 1, extended with Vent Polymerase (New England Biolabs) and then ligated overnight at 16°C to the linker according to the published protocol (19). Oligonucleotides used in these experiments are shown in Table 1. In some experiments (as indicated in the text), blunt ends in the genomic DNA were directly ligated without denaturation and extension (Fig. 1, left panel).

After ligation, the DNA was subjected to PCR using primer P2 and the Linker Primer, and 23 amplification cycles using the protocol of Mueller et al. (19) and the following annealing temperatures: 59.6°C for 5μ, 61.8°C for 3μ1 and 58.4°C for 3μ3.

The amplified products were analyzed by several techniques. For our routine experiments, the products were displayed by denaturation, followed by extension of radiolabeled P3 oligonucleotide (19), electrophoresis on a denaturing acrylamide (sequencing) gel and imaging on a Fuji FLA-3000 digital scanner.

**Reverse transcription–PCR**

After incubation, cells were harvested and total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. RNA was treated with DNaseI (Invitrogen) and cDNA was synthesized from 2 μg of total RNA with the First-Strand cDNA Synthesis Kit (Roche). (Invitrogen) and cDNA concentrations were adjusted to give equal intensities after PCR of dilutions with GAPDH primers (Table 1). The IgH primer pairs used are shown in Table 1. Reverse transcription (RT)–PCR products were analyzed on 10% polyacrylamide gels and stained with SYBR Green I stain (Invitrogen/Molecular Probes).

**Results**

Our principal conclusion is that the SSBs we detect by LMPCR in IgS regions early in CSR do not depend on AID. This conclusion would contradict the widely accepted view that AID is required to initiate S region DNA breaks in CSR, and it also contrasts with (but does not contradict) reports from several laboratories that double-strand S region breaks—detected by a somewhat different LMPCR technique—are strongly AID dependent. The two LMPCR techniques are compared in Fig. 1. In view of these apparent conflicts, and because LMPCR is a technique sometimes plagued by artifactual results, we have used several strategies to characterize our LMPCR products and to explore how they relate to CSR.

**SSBs in 5μ from splenic B cells**

In initial experiments, we designed primers to amplify lower strand breaks in the 5′ region of 5μ. Splenic B cells were prepared by MACS selection for CD19+ cells and were

Table 1. Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>5μ</th>
<th>Sμ upper strand</th>
<th>Sμ lower strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1-TGACCCAGACAGAGAAAGGCAG</td>
<td>P1-GCTCAGCTTCCATCATAATTC</td>
<td>P1-CTTGGAATCTTCCATCATAATTC</td>
</tr>
<tr>
<td>P2-GAAGTCGATCCAGACAGAGAA</td>
<td>P2-GACATCGATCCAGACAGAGAA</td>
<td>P2-GACATCGATCCAGACAGAGAA</td>
</tr>
<tr>
<td>P3-CCAGTTGAGCCAAAATGAACTGAC</td>
<td>P3-CCAGTTGAGCCAAAATGAACTGAC</td>
<td>P3-CCAGTTGAGCCAAAATGAACTGAC</td>
</tr>
<tr>
<td>P4-GTGGTGTCATCCAGACAGAGAA</td>
<td>P4-GTGGTGTCATCCAGACAGAGAA</td>
<td>P4-GTGGTGTCATCCAGACAGAGAA</td>
</tr>
<tr>
<td>P5-GTACCCCTTCCATCATAATTC</td>
<td>P5-GTACCCCTTCCATCATAATTC</td>
<td>P5-GTACCCCTTCCATCATAATTC</td>
</tr>
<tr>
<td>5μ upper strand</td>
<td>5μ lower strand</td>
<td></td>
</tr>
<tr>
<td>P1-CTGCAGCTGTTTCCATCATAATTC</td>
<td>P1-CTGCAGCTGTTTCCATCATAATTC</td>
<td></td>
</tr>
<tr>
<td>P2-GACTCAGCTGTTTCCATCATAATTC</td>
<td>P2-GACTCAGCTGTTTCCATCATAATTC</td>
<td></td>
</tr>
<tr>
<td>P3-TACCTCAGCTGTTTCCATCATAATTC</td>
<td>P3-TACCTCAGCTGTTTCCATCATAATTC</td>
<td></td>
</tr>
<tr>
<td>P4-CAAGCTGTTTCCATCATAATTC</td>
<td>P4-CAAGCTGTTTCCATCATAATTC</td>
<td></td>
</tr>
<tr>
<td>P5-GTACCCCTTCCATCATAATTC</td>
<td>P5-GTACCCCTTCCATCATAATTC</td>
<td></td>
</tr>
</tbody>
</table>

Linker oligonucleotides

| Linker primer | GCGGGTACCCGCGGGGAGATCTGAAT |
| LMPCR1-GAATTCAGATC |
| DNA loading control primer |
| 37.7A-AAAAAGGAGTGTGGAGCAGTCGCTACC |
| 37.7B-GTTAAGGTGATACTGCAGAACCCG |

RT-PCR primers

| 37.7F-GCAGGATCTTTTCCAGAGACGATCCAGATTT |
| CHAB3F-GGCGCCCTTCCCATGATGATCTTTT |
| C3FR-CTTAGGGGCCAGAAGTTCGCTGAG |
| GAPDH-A-CACACGTTCATTCCGACATGGCACC |
| GAPDH-B-CCACGGACGGAGACATTTGGGCTAGG |
incubated for 24 h in either medium alone or medium supplemented with anti-CD40, which induces CSR to γ3-δ. DNA prepared from the stimulated cells and subjected to our LMPCR protocol for SSBs (Fig. 1, right panel) using primers from upstream of Sµ (shown in Fig. 2A) produced a reproducible ladder of weak bands as displayed by the sequencing gel shown in Fig. 2B; the unstimulated cells produced no detectable bands.

To verify that our LMPCR procedure was amplifying products from the Sµ region as intended, and to see whether breaks in Sµ occurred within a C*AG consensus, we TOPO cloned our amplified products for sequence analysis. Roughly two-thirds of all clones passing colony PCR screening for well-defined inserts contained the expected Sµ sequence ends (while about one-third were a variety of artifacts, including spurious inserts bounded by two Linker Primers). Several hundred clones were obtained from stimulated cell DNA, while only 17 clones were obtained from unstimulated cell DNA. As judged from the boundary between the linker and genomic IgH sequence in each clone,
single-strand DNA breaks occurred both upstream of the S\textsubscript{\textit{\textmu}} tandem repeat regions and within the repeats. Among SSBs ends within the tandem repeat region, C*AG was the most frequent trinucleotide at the break (Fig. 2C and D and Table 2). In clones from normal cells stimulated by anti-CD40, this trinucleotide occurred at 8 of 14 sequences analyzed (57.1%) compared with a 12% occurrence frequency of this trinucleotide in the same region. Cytosine was present immediately 5' of the break in 86% of the sequences, but this represents <2-fold preference since the lower strand of the S\textsubscript{\textmu} sequence is highly enriched in cytosines, which represent 48% of the nucleotides over the region of S\textsubscript{\textmu} where our breaks were detected. Among breaks from the region 5' of the S\textsubscript{\textmu} tandem repeats, C*AG occurred in 8 of 15 clones (53% versus an occurrence frequency of 5.3%) and cytosines occurred in 60% of clones (occurrence frequency of 21%).

An interesting feature of these sequences is that the nucleotide 3' of the break shows a strong preference for A, which occurs at all 14 independent ends from stimulated cells in the S\textsubscript{\textmu} tandem repeats (compared with a 21.9% frequency of A in the same region) and 13 of 15 independent ends in the 5' region (compared with 25.5%). Thus, the bias for A 3' of the break is stronger than the bias for C* 5' of the break. Adenosines at the 3' end of the break were also observed in 100% of the breaks we previously reported in Sx (12).

The high yield of clones with the characteristics expected for SSBs in or near S\textsubscript{\textmu}, and the dependency of these bands on B cell stimulation by anti-CD40, are both consistent with the interpretation that the LMPCR bands we detect are related to CSR rather than artifacts.

**Isotype specificity of SSBs in cells switching to \(\gamma\text{1}\) versus \(\gamma\text{3}\)**

Because we could achieve most efficient in vitro switching of splenic B cells to \(\gamma\text{1}\) and \(\gamma\text{3}\), we developed LMPCR primers specific for these isotypes (Fig. 3A and B). To verify that our incubation conditions induced the previously observed isotype-specific sterile transcripts, RT-PCR for \(\gamma\text{1}\)-C\textsuperscript{\gamma}\textsuperscript{1} sterile transcripts was carried out on RNA extracted from cells incubated for 24 h with anti-CD40, anti-CD40 plus IL-4 or neither stimulator. As expected, robust \(\gamma\text{1}\)-C\textsuperscript{\gamma}\textsuperscript{1} transcription was observed with anti-CD40 stimulation, but this was inhibited with addition of IL-4 (shown in Fig. 3C), whereas robust \(\gamma\text{1}\)-C\textsuperscript{\gamma}\textsuperscript{1} transcription required both anti-CD40 and IL-4.

We consistently found that the incubation conditions favoring CSR to each \(\gamma\) subtype led preferentially to SSBs in the corresponding S region. Incubation with anti-CD40 alone (or LPS alone) leads to efficient switching to S\textsubscript{\textmu} and also caused robust S\textsubscript{\textmu} LMPCR bands after 24 h of stimulation (Fig., 3D lanes 2 and 3). IL-4 is known to inhibit switching to S\textsubscript{\gamma}, and we found that it significantly reduced the intensity of S\textsubscript{\gamma} SSBs (Fig. 3D, lane 7 versus lane 3). Conversely, IL-4 is required for efficient switching to S\textsubscript{\gamma}, and this cytokine was also necessary to generate detectable S\textsubscript{\gamma} SSBs (Fig. 3D, lane 10 versus lane 9; see also lane 11, from cells treated with IL-4 + anti-CD40 and then processed with Annexin V to remove any apoptotic cells).

To verify that the bands in an LMPCR ladder from an S\textsubscript{\gamma} amplification actually reflected S\textsubscript{\gamma} products, individual bands were excised from the gel, extracted DNA was amplified with P3 and the Linker Primer and the products were cloned and sequenced. All clones contained S\textsubscript{\gamma} products of approximately the expected size (data not shown). The correlation between culture conditions favoring SSBs in a particular S\textsubscript{\gamma} region and those favoring CSR to the corresponding isotype supports the relationship of our LMPCR bands to CSR and suggests that the isotype specificity of the corresponding isotype supports the relationship of our LMPCR bands to CSR and suggests that the isotype specificity of the CSR switching event acts on a step at or prior to DNA cleavage. This correlation also weakens the likelihood of alternative interpretations attributing the LMPCR bands to apoptotic DNA degradation or to Okazaki fragment ends, which would not be expected to show this isotype specificity. Similarly, the diminution of S\textsubscript{\gamma} LMPCR bands in Fig. 3D, lane 7 versus lane 3 cannot be attributed to IL-4 induction of PCR inhibitors because the same DNA gave robust LMPCR bands when amplified for S\textsubscript{\gamma} SSBs in lane 9. A formal test comparing amplifiability of the different DNA samples using an irrelevant oligo pair (37.7A and 37.7B in Table 1) showed that these samples all amplified with essentially equal efficiency; see Supplementary Figure S.0, available at International Immunology Online.)

Sequence analysis of amplified S\textsubscript{\gamma} breaks cloned from genomic DNA of stimulated B cells (Fig. 3E and Table 2) revealed a relatively weak consensus at the breaks: 5 of 21 sequences (24%) ending within the tandem repeats contained C*AG, but this is still elevated compared with the low occurrence frequency of 4% in the same sequence segment. Among the S\textsubscript{\gamma} SSBs, no cytosine preference was found: cytosines occurred just 5' of the break in 48% (10/21).

**Table 2. Frequencies of C* or C*AG at the ends of SSB clones**

<table>
<thead>
<tr>
<th>Isotype</th>
<th>LMPCR</th>
<th>Cells</th>
<th>(\alpha\text{CD40} ) stimulation</th>
<th>DNA region</th>
<th>C*/total clones (%)</th>
<th>C/bp (%)</th>
<th>C*fold increase</th>
<th>C*AG/total clones (%)</th>
<th>CAG/bp (%)</th>
<th>C*AG fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\gamma\text{3})</td>
<td>WT</td>
<td>+</td>
<td>S\textsubscript{\gamma3 TR}</td>
<td>5' of S\textsubscript{\gamma3 TR}</td>
<td>26/47 (33)</td>
<td>11/47 (23)</td>
<td>1.5</td>
<td>1/6 (17)</td>
<td>2/46 (4.3)</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>S\textsubscript{\gamma3 TR}</td>
<td>2/6 (33)</td>
<td>11/47 (23)</td>
<td>1.5</td>
<td>1/6 (17)</td>
<td>2/46 (4.3)</td>
<td>3.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Within S\textsubscript{\gamma3 TR}</td>
<td>10/21 (48)</td>
<td>187/363 (52)</td>
<td>0.9</td>
<td>5/21 (24)</td>
<td>16/363 (4.4)</td>
<td>5.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\)Some sequence ends appeared in more than one clone. Such sequence repeats were counted as separate clones in the frequency calculations only when the clones were clearly independent (i.e. from different amplifications).

\(^{b}\)Clones with SSBs falling in the tandem repeats of S\textsubscript{\gamma} (156–625 bp from the end of P3) or S\textsubscript{\gamma3} (163–526 from P3).

\(^{c}\)Clones with SSBs falling 5' of the S\textsubscript{\gamma} (or S\textsubscript{\gamma3}) tandem repeats.
of the breaks within the tandem repeats, almost exactly the cytosine frequency in this C-rich region (52%). The lower preference for C+C at the breaks in S\textsubscript{c3} versus S\textsubscript{l} suggests that S\textsubscript{c3} and S\textsubscript{l} CSR events may differ either in their preferred cleavage sites or in post-cleavage processing of the SSBs. Such differences would not be completely unexpected, given that CSR events at S\textsubscript{c3} differ from those at S\textsubscript{l} in other respects, including their lower frequency of CSR-associated mutations (20) and the reported presence of NF\textsubscript{kb} at S\textsubscript{c3} during CSR (21).

As in our earlier studies of S\textsubscript{a} breaks in CH12F3-2 cells, we designed several sets of primers in an attempt to detect CSR-associated SSBs in the upper strand of S\textsubscript{c3} as well as S\textsubscript{c1}. Extracted DNA was amplified for SSBs in either S\textsubscript{c3} or S\textsubscript{c1} using strategies shown in (A) and (B) and the primer pairs are shown in Table 1. (Primers sets designed to yield bands in the S\textsubscript{c1} tandem repeats were unsuccessful. With the S\textsubscript{c1} primer set finally chosen, bands represent SSBs in direct repeat I and II.) (C) RNA was extracted and evaluated for \textgamma-C\gamma transcripts by RT-PCR using primers shown in Table 1; RT-PCR for GAPDH served as a loading control. (D) LMPCR band patterns under various conditions. Lane 11 shows the LMPCR pattern from cells incubated 24 h with anti-CD40 and then purified to remove cells undergoing apoptosis (see Methods); the pattern was similar to that of the unpurified population (lane 9). As loading controls, dilutions of all DNA samples were subjected to amplification with the primer pair designated 37.7A and 37.7B in Table 1, which amplifies an arbitrary 94-bp sequence downstream of the murine Ca gene; for each dilution, all samples produced bands of essentially equal intensity (shown in Supplementary Figure S.0, available at International Immunology Online). (E) Sequences from LMPCR of S\textsubscript{c3} SSBs, displayed as in Fig. 2.

CH12F3-2 cell line, that breaks in the two strands are processed differently so that the steady-state level of lower strand SSBs detectable by LMPCR is higher than that of upper strand SSBs.

Furthermore, to examine whether the SSBs were specific to the S\textsubscript{c3} region or might be generated by any transcription in the stimulated B cells, we designed primers to detect lower strand SSBs within the CH2 exon of C\gamma\textsubscript{3}. Although these primers successfully detected restriction enzyme-induced breaks in C\gamma\textsubscript{3} DNA, they detected no SSBs in our single attempt using DNA from anti-CD40-stimulated B cells (see Supplementary Figure S.2, available at International Immunology Online), despite the fact that abundant transcripts through the same region of C\gamma\textsubscript{3} could be detected in the same experiment (Fig. 3C).

**Influence of S\textsubscript{c3} deletion on S\textsubscript{c3} SSBs**

If the LMPCR products from S\textsubscript{c3} reflect genuine CSR-related SSBs, these breaks might be perturbed in mice whose CSR is impaired because of a deletion of S\textsubscript{c3}. To test this...
possibility, we obtained splenic B cells from mice with a ∼5-kb deletion that removes the Sμ tandem repeats as well as all flanking instances of the repeat pentamers (GGGGT, GGGCT, GAGCT), leading to a profound impairment in CSR (18). We found higher levels of SSBs at S3 in the Sμ-deleted versus normal B cells, as shown by increased band intensities on our usual LMPCR ladders (Fig. 4A). To estimate a quantitative difference in the total amplified products, the LMPCR products from each P2–Linker Primer amplification—which includes fragments largely between ∼150 and 800 bp—were denatured and extended with a radiolabeled anti-sense primer P5, designed to extend to the 5’ end of the P2 primer to yield a 141-bp product from all amplified products larger than 141 bp (as shown in Fig. 4B and described in Methods). The P5 experiments showed that the intensity of the amplified band from the homozygous Sμ-deleted cells was several-fold higher than that from WT cells (Fig. 4C). These results suggest that CSR-associated SSBs in S3 can be made independently of breaks in Sμ; current models of the CSR mechanism make no clear prediction on this point, though it may be noted that for V(D)J recombination it has been proposed that breaks do not occur efficiently unless both recombination partners are brought together in synopsis (22). One plausible though speculative explanation for the increased density of the S3 P5 band from Sμ-deleted B cells is that lower strand SSBs persist longer in these cells because the cells cannot complete later steps of CSR in the absence of Sμ.

In light of this interpretation, we reasoned that a longer half-life might also apply to breaks in the S3 upper strand, which we have been consistently unable to detect from normal cells, as described above. Indeed, we were able to detect faint LMPCR bands from the S3 upper strand in the Sμ-deleted cells stimulated with anti-CD40 (but not in unstimulated cells or in stimulated normal cells; see Supplementary Figure S.3, available at International Immunology Online) and have verified by sequence analysis that LMPCR products expected for upper strand breaks occur after stimulation of mutant, but not normal, B cells.

S region SSBs depend on UNG

Previous reports have indicated that an intact UNG gene is required for the CSR-associated generation of S region DSBs (16) as well as for CSR itself (4). To test whether SSBs were similarly UNG dependent, we compared UNG+/− B cells to normal splenic B cells in the generation of S3 lower strand SSBs. Our usual LMPCR assay (with products displayed on a denaturing acrylamide gel) showed that the UNG deletion completely eliminated the S3 SSBs bands in our LMPCR assay (Fig. 5A, left panel, lane 4 versus lane 2). WT mice of strain 129 are known to be somewhat inefficient in CSR compared with C57BL6 (23), so to check whether this inefficiency might be related to the absence of SSBs in the B cells from the UNG−/− mice—which were created on a 129 background and which had been incompletely back-crossed to C57BL6—we compared WT 129 and C57BL6 B cells in our CSR assay; the two B cell cultures yielded LMPCR band ladders that were indistinguishable in pattern and intensity (see Supplementary Figure S.4, available at International Immunology Online); thus, the absence of LMPCR bands in the UNG−/− B cells appears to be directly related to the absence of a functioning UNG gene.

As an alternative method for visualizing the S3 SSBs, products of a 23-cycle P2–Linker Primer amplification were visualized on a Southern blot hybridized to a short probe between P3 and the P5 anti-sense primer at the boundary of the tandem repeat region of S3. Because the many different-sized amplification products were poorly resolved on the agarose gel, they appear as a smear in the lane. Although some 200- to 300-bp background products appeared in all lanes, the deletion of UNG virtually abolished the SSB products produced in response to anti-CD40 treatment of the cells (Fig. 5A, lane 8 versus lane 6) corroborating the results of the higher resolution acrylamide gel assay.

When we applied our cloning protocol to the DNA amplified for Sμ SSBs from the cytokine-treated WT and UNG−/− samples, the WT sample yielded roughly 600 clones containing Sμ ends (based on extrapolations from the volumes plated and the percentage of clones containing sequence-validated Sμ ends); the UNG−/− sample yielded only three...
**Comparison between SSBs versus DSBs in CSR**

Because of the contrast between our LMPCR assay for SSBs versus previously published LMPCR assays for DSBs—respect to both time course and AID dependency—we attempted a side-by-side comparison of the two techniques. Normal splenic B cells were incubated with anti-CD40 (to induce switching to \( \gamma_3 \)) or with medium alone for 24 h, and the anti-CD40-stimulated cells were incubated for another 24 h. (Unstimulated cells showed poor viability at 48 h and were not tested by LMPCR.) DNA was then extracted from the cells and samples (2 mg) were either ligated directly to the Linker Primer (to initiate detection of blunt DSBs by PCR) or denatured and extended with our \( \gamma_3 \) P1 primer to create blunt ends and then ligated to the Linker Primer (to detect SSBs). The ligated samples were then amplified with our P2 primer and the Linker Primer for 23 cycles. In all, 10% of each amplification mix was removed and subjected to further amplification for 10 cycles with a fresh mix containing the same primers, and amplification products were detected by Southern blotting.

As shown in Fig. 7 (lane 8), samples from WT cells stimulated with anti-CD40 for 24 h and amplified using the blunt DSB protocol for \( \gamma_3 \) produced weak LMPCR bands; after 48 h of stimulation, more intense bands in the same pattern were produced (lane 9). (No bands were produced from unstimulated cells.) These results are similar to the previously reported time course of DSB production, requiring 48 h of stimulation for the greatest response (16). Significantly, the DNA samples from \( \text{AID}^{+/+} \) cells showed essentially no blunt DSBs even after 48 h of stimulation (lane 12), consistent with previously published observations. In contrast, the

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**Table: S\(_3\) amplification**

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<th>*P3 ext’n</th>
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**Fig. 5.** S region SSBs depend on UNG. Splenic B cells from mice with a homozygous deletion of UNG or from control WT mice were incubated for 24 h with anti-CD40 to induce CSR to \( \gamma_3 \) or with medium alone as a negative control. S\(_3\) amplification products were displayed either by extension with radiolabeled P3 (*P3 ext’n*) or by Southern blotting and hybridization with a probe generated by P5–P5 amplification. Loading controls of the same DNA samples are shown as in Fig. 4. Lane 9 shows hybridization with PvuII-digested genomic DNA, a control for transfer and hybridization.

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**Fig. 6.** AID-independent DNA breaks in CSR

- **A**. Lane 9 shows hybridization with PvuII-digested genomic DNA, a control for transfer and hybridization.

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**Fig. 7.** S region SSBs are largely independent of AID

According to the currently accepted model for the mechanism of CSR, DSBs in S regions should be dependent on AID, and this prediction was supported by the observation that AID is required for the formation of microscopic foci of H2AX (a histone variant known to accumulate at DSBs) in Ig loci undergoing CSR (24). More recent studies have used LMPCR to examine the effects of AID deficiency on production of DSBs in S regions and have found dramatic decreases, but not complete elimination of these breaks (13, 14, 16). We set out to test whether the SSBs we detect are also dependent on AID. To our surprise, we found only minimal AID dependency. In multiple experiments, cytokine-treated \( \text{AID}^{+/+} \) B cells showed LMPCR ladders with nearly identical banding pattern and only modest diminution of intensity compared with WT. Fig. 6A (left panel) shows LMPCR bands derived from splenic B cells from mice with homozygous knockouts for AID, treated with anti-CD40 and amplified for S\(_3\) SSBs; while the AID\(^{+/+}\) ladder is less intense than WT, most band positions are nearly identical in the two samples. Similar results were obtained in experiments with anti-CD40 + IL-4 to amplify SSBs in S\(_1\) (data not shown).

The modest effect of AID deletion was confirmed with a Southern blot of products amplified for SSBs (Fig. 6A, right panel). Our P5 technique for quantitating the total amount of LMPCR product confirmed the very similar response to anti-CD40 in WT and AID\(^{+/+}\) (see Fig. 6B). All DNA samples designated AID\(^{+/+}\) were verified to have the AID\(^{+/+}\) genotype by PCR analysis (data not shown).

Because of the low intensity of S\(_3\) LMPCR electrophoresis bands from DNA of normal B cells, the effect of AID knock out on S\(_3\) SSBs was assessed by cloning LMPCR products. A roughly equivalent yield of clones was obtained from DNA extracted from WT and AID\(^{+/+}\) B cells, suggesting that substantial cleavage of the lower strand in S\(_3\) occurs in the absence of AID. The sequences of the SSB ends from AID\(^{+/+}\) DNA were similar to that of from WT, though the frequencies of \( *^\text{AG} \) and of \( C^* \) at the breaks were somewhat lower in the AID\(^{+/+}\) clones (Fig. 6C and Table 2).

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<td>αCD40</td>
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**Fig. 8.** AID-independent DNA breaks in CSR

Comparison between SSBs versus DSBs in CSR

As shown in Fig. 7 (lane 8), samples from WT cells stimulated with anti-CD40 for 24 h and amplified using the blunt DSB protocol for S\(_3\) produced weak LMPCR bands; after 48 h of stimulation, more intense bands in the same pattern were produced (lane 9). (No bands were produced from unstimulated cells.) These results are similar to the previously reported time course of DSB production, requiring 48 h of stimulation for the greatest response (16). Significantly, the DNA samples from AID\(^{+/+}\) cells showed essentially no blunt DSBs even after 48 h of stimulation (lane 12), consistent with previously published observations. In contrast, the
amplifications for SSBs showed abundant products at 24 h, and the same pattern of bands in AID−/−/C0/C0 cells, with no diminution in intensity (actually an increase in this experiment, though more typically we see a modest decrease). Thus, the same AID−/−/C0/C0 DNA samples yielded essentially no LMPCR products with the blunt DSB protocol (confirming earlier published reports) but plentiful products with our SSB protocol. (Amplifications of AID−/−/C0/C0 DNA for breaks in Sl also yielded SSB bands but no DSB bands; data not shown.)

To judge the relative amplification efficiency of the DSB versus the SSB protocols, we amplified (in parallel with the above experiments) two identical 1-μl samples of genomic DNA digested with EcoRI (a blunt cutter) with the SSB and DSB techniques; the amplifications yielded bands of the expected size (130 bp) that were of roughly equal intensity (lanes 13 and 14), indicating similar amplification efficiency of the two techniques. Given this similar efficiency, the much greater intensity of LMPCR products amplified from DNA of stimulated B cells by the SSB protocol (versus DSB protocol) probably reflects a higher frequency of SSBs versus blunt DSBs in the stimulated B cells.

Discussion

Nature of DNA breaks detected by LMPCR

Breaks in both strands of DNA must occur during the course of a DNA recombination event, and several laboratories...
using an LMPCR protocol have reported detection of DSBs as intermediates in CSR (13–16). However, the fact that both strands are cleaved during CSR does not imply that upper and lower strands are cut simultaneously, or in equal numbers, or by precisely the same mechanism. Indeed, the currently accepted model of an R-loop in CSR suggests a mechanistic distinction between the C-rich lower strand, which forms a stable hybrid with the RNA transcript, and the G-rich upper strand, which is displaced by the RNA to form an R-loop that should be susceptible to deamination by AID, according to the demonstrated in vitro properties of this deaminase. The only way to assess potentially different fates of the two DNA strands is to study SSBs in the two strands individually as we have done.

Our ready detection of lower strand ends and our failure to detect upper strand ends from WT B cells undergoing CSR is consistent with mechanistic asymmetry between the two strands. Since our protocol detects upper strand cuts generated in vitro by a restriction enzyme, the absence of LMPCR products from the upper strand of B cell DNA does not appear to be due a complete technical failure, but likely reflects a lower steady-state level of amplifiable upper strand DNA ends, perhaps below our detection limit. The low level of upper strand cuts might be explained by several non-exclusive mechanisms. We have suggested that, in comparison to the lower strand, upper strand nicks may have a shorter half-life, perhaps due to rapid conversion of the upper strand breaks into a form not detectable by our LMPCR protocol, perhaps a ligated CSR product. This explanation would be consistent with our detection of some upper strand cuts in DNA from B cells with an $\text{S}_\mu$ deletion, as previously discussed.

Alternatively, the low level of upper strand cuts may reflect infrequent nicking of the upper strand or a cleavage mechanism that does not generate a ligatable 5’ end. The breaks detected by the LMPCR protocol depend on ligation of the 3’-OH end of the Linker Primer to a phosphate at the 5’ terminus of the in vivo cut DNA strand in the context of a blunt double-stranded DNA end. However, in vivo cytosine deamination followed by UNG-catalyzed uracil removal and by APE1-catalyzed cleavage of the abasic site would not immediately yield such a 5’ phosphate end since APE1 cleaves 5’ to the abasic site and would leave a 5’ deoxyribose phosphate (dRP) group. The dRP group might be removed in vivo by the DNA lyase activity of DNA polymerase β (Pol β), which cleaves at the appropriate position 3’ to an abasic site and is much more active if APE1 has already cut the DNA 5’ to the abasic site (25). Pol β may actually be recruited by APE1 (26), perhaps as part of a preformed complex (including Ung2, APE1 and pol β) that carries out base excision repair of uracil residues (27, 28). Other mechanisms for in vivo generation of the 5’ terminus are also possible and may differ between the upper and lower strands of DNA, perhaps creating some ends that are not ligatable by our LMPCR protocol.

Relationship of the LMPCR products to CSR

Our results suggest that B cells stimulated to undergo CSR use an UNG-dependent but AID-independent mechanism to generate lower strand SSBs in the S regions that are targeted for CSR. The possibility that the LMPCR bands we detect in B cell DNA are artifacts unrelated to CSR is rendered unlikely by the following observations:

1. The SSB LMPCR bands were detected in S regions of B cells stimulated to undergo CSR, but not unstimulated cells.
2. The LMPCR products are not simply an artifact of transcription since no LMPCR bands were detected from the $\text{C}_{4\gamma2}$ exon of $\gamma_3$, an exon that we have shown is included the $\text{I}_{2\gamma3}$ transcripts induced by anti-CD40.
3. Cloned amplification products showed structures expected for S region DNA breaks ligated to our Linker Primer.
4. LMPCR products from $\gamma_3$ were produced efficiently by incubation conditions favoring CSR to $\gamma_3$, but not by conditions favoring CSR to $\gamma_1$; while LMPCR products from $\gamma_1$ only appeared with stimuli for CSR to $\gamma_1$.
5. The amount of $\gamma_3$ LMPCR product detected was enhanced in B cells from mice carrying a deletion of $\text{S}_\mu$.
6. The LMPCR products could be detected by several different methods: by extension of a radiolabeled P3 primer, by Southern blotting or by cloning. Therefore, the results appear to be robust.
7. The LMPCR products were almost entirely dependent on UNG, as expected if they were triggered by deamination of cytosines, a step believed to initiate CSR.

The consilience of these observations suggests an association between the LMPCR bands and CSR; indeed, beyond mere association, the apparent accumulation of excess SSSs in CSR-defective B cells lacking $\text{S}_\mu$ suggests that the $\gamma_3$ SSSs are bona fide intermediates in CSR to that isotype. If this is the case, our data imply the surprising result that CSR-related lower strand SSBs can occur in the absence of AID, even though DSBs require AID.

Alternative deaminase candidates

How could the SSBs we see in the lower strand of S regions be generated with a similar pattern in $\text{AID}^{-/-}$ and WT cells? Since no bands are generated with DNA from $\text{UNG}^{-/-}$ B cells, it is likely (barring a non-enzymatic role for UNG) that all the SSSs depend on generation of uracil residues by cytosine deaminase action; since the SSS bands are produced in the absence of AID, perhaps another cytosine deaminase generates the uracils.

At least four other cytosine deaminases homologous to AID are known in mice: the four members of the APOBEC family. APOBEC1, the originally discovered member of the family, is thought to deaminate exclusively RNA in its physiological role, though it can act on DNA in vivo (29). APOBEC2 is a phylogenetically old gene expressed in muscle- (30) and cytokine-activated hepatocytes (31) but for which no function or enzymatic activity has been demonstrated. APOBEC4 is a homolog identified by a database search which appears to be expressed (as an RNA at least) in testis and which has no known function or enzymatic activity (32). Thus, none of these three APOBEC family members seems a strong candidate to generate SSBs in B cells.

In contrast, murine APOBEC3 is primarily expressed in lymphoid tissues, especially spleen (30), and we have found that APOBEC3 transcripts are present in splenic B cells after...
cleavage at cytosine residues with minimal post-cleavage DNA trimming. While C*AG was the most frequent trinucleotide at the S\*u breaks from splenic B cells that we studied in the present work, it was present at a much lower frequency than we previously detected at Sx: only ~55% of all the breaks in stimulated WT cells and only at 43% of the breaks in AID\(^{-/-}\) cells. However, these lower frequencies still represent substantial enrichment over the frequency of the CAG trinucleotide occurrence in the sequence under study (~11%). The lower frequency of C*AG at the S\*u break position was also seen in S\*u SSBs recovered from stimulated CH12F3-2 cells (40% of 10 recovered ends in the tandem repeats; data not shown), suggesting that this lower frequency of C*AG may reflect an isotype-specific difference (S\*u versus Sx) rather than a difference between CH12F3-2 and normal spleen cells. (Such isotype specificity in preferred break points may also be relevant to breaks in Sy3, as discussed below.)

Remarkably, the highest frequency of C* (86%) and of C*AG (79%) was found in 14 clones with SSBs in the S\*u tandem repeats that we recovered from unstimulated B cells. Indeed, these clones seemed to have a very extended consensus sequence that was quite precisely adhered to. As shown in Fig. 2, apart from two clones that lacked a C* at the break (possible victims of trimming), all the other 12 clones had either an exact match to the extended sequence (44). However, an argument against trimming as an explanation for all clones lacking a C* at the break is that among the seven such S\*u clones that we obtained after B cell stimulation with anti-CD40 (Fig. 2D), four still show a consensus *AG after the apparent nick (and five show *AX). These numbers are higher than would be expected by chance and raise the possibility that some nicks may be initiated at an *AG consensus independently of cytosine deamination; alternatively, *AG might represent a consensus trimming stop site.

AID has been reported to preferentially deaminate cytosines in the sequence WRC [where W is A or T and R (purine) is A or G] (45, 46), and WRC was reported to be enriched at the ends of DSBs in S\(\mu\) (16). WRC* appears at only one of the S\*u SSBs we detected (in two clones) from WT cells: at position 151. Absence of the WRC motif at most SSB cut ends is consistent with a non-AID-related cleavage mechanism in the generation of these cuts. We note that the extended consensus in the rare S\*u SSBs from unstimulated cells conforms to WRCTC\*WRC with the break occurring at the C outside the consensus WRC. The significance of this is not clear. Possible sequence preferences of murine APOBEC3 have not been well characterized, but the preferences for several of the human homologs have been studied and differ between certain family members (47).

Compared with our S\*u SSBs, the SSBs we recovered from Sy3 showed a weaker C*AG consensus—3 of 16 sequences, i.e. 19%—but still representing a preferential cleavage...
site compared with the 4% occurrence frequency in the sequence under study. The lack of an apparent preference for C at the break point, compared with a roughly 2-fold C preference at Sy SSBs in the same cells, might result from greater trimming at Sy3 versus Sy ends. If we assume that a break after A, T or G results from trimming, and if we focus on the 10 clones with SSBs occurring after C nucleotides, we can see exact matches to a CCC*A sequence in 8 of the 10 clones. It may be significant that CCCA is the preferred sequence for human APOBEC3G cleavage (48).

In conclusion, our evidence suggests that most SSBs in the lower strand of Sy, Sy1 and Sy3 sequences from B cells undergoing CSR are UNG dependent but AID independent. We speculate that, at least in the absence of AID, these SSBs may be triggered by cytosine deamination carried out by an alternative deaminase, possibly APOBEC3. The earlier appearance of these SSBs—24 h after stimulation, compared with 48 h for DSBs—suggests that these lower strand breaks may be followed by AID-dependent upper strand breaks, leading to DSBs. These DSBs are eventually resolved by ligation to DSBs from a partner S region to complete CSR, as suggested by accumulation of higher levels of Sy3 breaks when no partner Sy is available.

Supplementary data
Supplementary Figures S.0–S.4 are available at International Immunology Online.

Acknowledgements
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Abbreviations
AID: activation-induced deaminase
CSR: class switch recombination
dRP: deoxyribose phosphate
dSB: double-strand break
LMPCR: ligation-mediated PCR
Pol β: polymerase β
RT: reverse transcription
SHM: somatic hypermutation
SSB: single-strand break
S region: switch region
UNG: uracil DNA glycosylase
WT: wild type

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