Corrections

APPLIED BIOLOGICAL SCIENCES

The authors note that the following statement should be added to the Acknowledgments: “This work was supported in part by NIH Grant R01 GM089652 (A.E.C.).”

www.pnas.org/cgi/doi/10.1073/pnas.1302919110

IMMUNOLOGY
Correction for “Alternative end-joining catalyzes robust IgH locus deletions and translocations in the combined absence of ligase 4 and Ku70,” by Cristian Boboila, Mila Jankovic, Catherine T. Yan, Jing H. Wang, Duane R. Wesemann, Tingting Zhang, Alex Fazeli, Lauren Feldman, Andre Nussenzweig, Michel Nussenzweig, and Frederick W. Alt, which appeared in issue 7, February 16, 2010, of Proc Natl Acad Sci USA (107:3034–3039; first published January 25, 2010; 10.1073/pnas.0915067107). The authors note that the National Institutes of Health Grant AI031541 should instead appear as AI077595.

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www.pnas.org/cgi/doi/10.1073/pnas.1303075110

IMMUNOLOGY

www.pnas.org/cgi/doi/10.1073/pnas.1303078110

EVOLUTION
Correction for “Diversification of rhacophorid frogs provides evidence for accelerated faunal exchange between India and Eurasia during the Oligocene,” by Jia-Tang Li, Yang Li, Sebastian Klaus, Ding-Qi Rao, David M. Hillis, and Ya-Ping Zhang, which appeared in issue 9, February 26, 2013, of Proc Natl Acad Sci USA (110:3441–3446; first published February 11, 2013; 10.1073/pnas.1300881110).

The authors note that, within the author line, “Yang Lia,c” should instead appear as “Yang Lib,c.” The corrected author line appears below. The online version has been corrected.

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www.pnas.org/cgi/doi/10.1073/pnas.1304031110

IMMUNOLOGY
Correction for “Integrity of the AID serine-38 phosphorylation site is critical for class switch recombination and somatic hypermutation in mice,” by Hwei-Ling Cheng, Bao Q. Vuong, Uttiya Basu, Andrew Franklin, Bjoern Schwer, Jillian Astarita, Ryan T. Phan, Abhishek Datta, John Manis, Frederick W. Alt, and Jayanta Chaudhuri, which appeared in issue 8, February 24, 2009, of Proc Natl Acad Sci USA (106:2717–2722; first published February 5, 2009; 10.1073/pnas.0812304106). The authors note that the National Institutes of Health Grant AI31541 should instead appear as AI077595.

www.pnas.org/cgi/doi/10.1073/pnas.1303069110

IMMUNOLOGY
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www.pnas.org/cgi/doi/10.1073/pnas.1303075110

IMMUNOLOGY

www.pnas.org/cgi/doi/10.1073/pnas.1303078110
Downstream class switching leads to IgE antibody production by B lymphocytes lacking IgM switch regions

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Contributed by Frederick W. Alt, December 30, 2009 (sent for review December 22, 2009)

Ig heavy chain (Igh) class-switch recombination (CSR) replaces the Igh constant region exons with one of several sets of downstream Igh constant region exons (e.g., Cγ, Cε, or Cμ), which affects switching from IgM to another Igh class (e.g., IgG, IgE, or IgA). Activation-induced cytidine deaminase (AID) initiates CSR by promoting DNA double-strand breaks (DSBs) within switch (S) regions flanking the donor Cμ (Sμ) and a downstream acceptor Cε (e.g., Sγ, Sμ, Sτ, Sτ′) that are then joined to complete CSR. DSBs generated in Sμ frequently are joined within Sμ to form internal switch region deletions (ISD). AID-induced ISD and mutations have been considered rare in downstream S regions, suggesting that AID targeting to these S regions requires its prior recruitment to Sμ. We have now assayed for CSR and ISD in B cells lacking Sμ (Sμ−/− B cells). In Sμ−/− B cells activated for CSR to IgG1 and IgE, CSR to IgG1 was greatly reduced; but, surprisingly, CSR to IgE occurred at nearly normal levels. Moreover, normal B cells had substantial Sγ1 ISD and increased mutations in and near Sγ1, and levels of both were greatly increased in Sμ−/− B cells. Finally, Sμ−/− B cells underwent downstream CSR between Sγ1 and Sε on alleles that lacked Sμ CSR to these sequences. Our findings show that AID targets downstream S regions independently of CSR with Sμ and implicate an alternative pathway for Igh class switching that involves generation and joining of DSBs within two different downstream S regions before Sμ joining.

Antibodies are comprised of Igh heavy (Igh) and light (Igl) chains. The N-terminal portion of Igh and Igl chains, termed the variable region, binds antigens, whereas the C-terminal portion of the Igh chain, termed the constant region, determines antibody class and effector functions. The Igh variable region is encoded in a distinct exon from those that encode the constant region. The variable region exon is assembled early in B-cell development from VH, D, and JH segments through V(D)J recombination (1). The Igh V(D)J exon is first expressed with proximal downstream exons that encode the Cμ constant region, leading to expression of μ Igh chains and IgM antibody. Newly generated B cells express surface IgM and migrate to the periphery where they can be induced to express a different Igh class (e.g., IgG, IgE, or IgA). The mouse Igh locus hosts eight sets of Cμ exons, referred to as Cμ1 genes, arranged as S−V(D)J-Cμ-C6-C7-Cy1-Cy2b-Cy2a-Cε-Cα3 over a 200-kb region (2). Igh class switching involves a recombination/deletion event, termed class-switch recombination (CSR), in which the Cμ gene is replaced with a downstream Cε gene (2, 3). Both CSR and the related somatic hypermutation (SHM) process, which introduces mutations into variable region exons to allow production of higher affinity antibodies, are initiated by activation-induced cytidine deaminase (AID) (4, 5).

Each Cμ gene that undergoes CSR is organized into a unit from S′ to 3′ that includes a transcriptional promoter followed by a noncoding exon (termed I1 exon), a switch (S) region, and a set of Cμ exons (6). CSR involves the introduction of a DNA double-strand break (DSB) in the donor S region flanking the Cμ gene (Sμ) and into an acceptor S region flanking a downstream Cε gene; this is followed by the joining of the breaks by general DSB end-joining pathways (7). S regions are long (1–10 kb) intronic sequences containing characteristic repeated motifs that include AID target motifs, and the Sμ is the most repetitive and harbors the highest numbers of AID targets (6, 8). Although there is some homology between Sμ, Sε, and Sτ, there is little or no homology between Sμ and Sγ regions (8). Transcription through S regions targets AID activity, which generates primary lesions that are processed into DSBs in Sμ and the downstream acceptor S region required to initiate CSR (7, 9). Thereby, specific induction of transcription from the I-region promoter flanking a particular acceptor S region targets that region for CSR. AID also introduces lesions into Igh and Igl variable region exons through a transcription dependent process, and they are converted into SHMs (9, 10). During CSR, AID also generates SHMs in S regions and immediate flanking sequences (11).

S regions serve primarily as specialized DNA structures that target the AID DSB-inducing activity. Thus, most CSR junctions occur within or occasionally just outside of S regions (12). In addition, B cells that lack the donor Sμ are greatly impaired for CSR to all tested Cμ genes (13, 14). Correspondingly, deletion of Sγ1 abrogates CSR only to Cγ1 (15). Finally, recombinational Igh CSR from Igm to IgG1 can be achieved in cells lacking those S regions when DSBs are introduced into their former sites by a yeast endonuclease (16). There are several mechanisms by which transcribed S regions may become AID targets. First, mammalian S regions form R loops that provide substrates for the single-stranded DNA-specific activity of AID (17–19). Second, AID seems capable of gaining access to sequences, such as S regions rich in AID target motifs, in a phosphorylation and Replication Protein A (RPA)-dependent fashion (20).

AID activity can generate multiple DSBs within Sμ (21, 22). Such DSBs within a given S region can be religated, joined to another DSB in the same S region, or joined to a DSB in a downstream S region to affect CSR. Religation of a resected DSB within a given S region or ligation of two intra-S region DSBs will generate internal S-region deletions (ISD). ISD are usually assayed by be viewed by Southern blotting. These large ISD occur frequently within the Sμ region in B cells or B-cell lines activated for CSR (23–26). They are AID-initiated (24) and mostly seem to occur through end joining (27). However, prior studies have found ISD in downstream S regions to be quite rare (24–26, 28), leading to suggestions that Sμ drives CSR by providing excess DSBs to ensure


The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/ 0915072107/DCSupplemental.
joining to less frequently occurring DSBs in downstream S regions (6, 16, 29). AID activity during CSR also generates SHMs in Sμ and adjacent sequences, including the upstream I region (29, 30). However, such mutations again were rarely found in downstream Cμ genes in wild-type (WT) B cells (29). The lack of SHM or ISD in downstream S regions also led to suggestions that AID activity on these sequences may only occur after AID has acted on Sμ, perhaps because of induction of factors or formation of complexes required for AID access to downstream S regions (25, 29). However, AID-initiated mutations in downstream S regions were observed in the context of a DNA-repair-deficient background (30).

IgH class switching to certain downstream Cμ genes (e.g., Cε) often occurs in humans and mice through sequential CSR involving two downstream acceptor S regions (31–34). Among the lines of evidence for sequential CSR are findings of remnants of an additional intervening S region in CSR junctions between Sμ and a downstream S region. In particular, such findings suggest that most switching to IgE may involve a pathway in which Sμ first joins to Sy1 to form a Sμ/Sy1 fusion S region that subsequently switches to Se (31–34). Although it is assumed that such junctions occurred by the pathway outlined above, it was not ruled out that some might occur through a pathway in which two downstream S regions are joined before joining with Sμ. However, such a pathway would require simultaneous DSBs in two downstream S regions, which might be considered unlikely given the apparently low frequency of DSBs in downstream S regions found in prior studies.

Deletion of sequences upstream of Sμ, including the IgH Jμ segments and the intronic enhancer element, inactivated CSR and led to increased Sy1 ISD in appropriately activated B cells (35). Although there may be several explanations for this finding, we considered the possibility that inactivation of Sμ might lead to increased AID targeting of downstream S regions. To test this possibility and also better define potential CSR pathways, we assayed for IgH class switching, ISD and SHM of downstream S regions, and downstream CSR between the Sy1 and Se in B cells stimulated for CSR to IgG1 and IgE.

**Results**

**IgH CSR to IgE Occurs at Nearly Normal Levels in B Cells Lacking Sμ.** To further elucidate mechanisms that target AID during IgH CSR, we induced mice generated previously by gene-targeted mutation that harbor an essentially complete deletion of the entire 4.6-kb Sμ region in their germline (13). Mice homozygous for this Sμ deletion mutation (Sμ−/− mice) show a great reduction in IgH CSR to all tested antibody classes, including IgG3, IgG2b, IgG1, IgG2a, and IgA (13). However, CSR to IgE was not previously studied in these mice. To further characterize potential IgH CSR defects in Sμ−/− mice, we purified splenic B cells from WT and Sμ−/− spleens and stimulated them in vitro with anti-CD40 and IL-4 to stimulate CSR from IgM to IgG1 and IgE. We assayed for CSR to IgG1 by surface staining and flow cytometry at days 2, 3, and 4 of stimulation. Consistent with previous studies (13), we observed greatly decreased (between 5- and 10-fold) IgH CSR in Sμ−/− B cells (Fig. 1, A and B).

We confirmed this finding by hybridoma analyses that showed increased levels of IgM-expressing hybridomas and greatly decreased levels of IgG1-expressing hybridomas from Sμ−/− versus WT B cells (Fig. 1C and Table S1).

IgE class switching cannot be readily analyzed by flow cytometry, because anti-CD40 treatment induces up-regulation of CD23 (FcεRII), the Fc receptor for IgE, on activated B cells (36), which leads to binding of soluble IgE antibodies to non-IgE-producing B cells and causes false-positive staining. Therefore, we analyzed IgE switching by assaying the frequency of IgE-producing hybridomas generated from day 4 anti-CD40 plus IL-4 stimulated B cells. Under our current stimulation conditions, activated WT B cells switch to IgE at quite substantial levels; indeed, ~40% of the recovered hybridomas were IgE producers (Fig. 1C and Table S1) (27). In this regard, our stimulation conditions also seem to give very high levels of IgG1 class switching (Fig. 1C) (27, 37). Unexpectedly, Sμ−/− B cells also displayed very high levels of IgE switching; we recovered IgE-expressing Sμ−/− hybridomas at nearly 70% the frequency of WT IgE-producing hybridomas. Indeed, the frequency of IgE-expressing Sμ−/− B hybridomas was more than 3-fold greater than that of IgG1-expressing Sμ−/− hybridomas (Fig. 1C and Table S1).

To further elucidate the nature of the apparently high level of IgE class switching in Sμ−/− B cells and to determine if it truly represented CSR, we employed a PCR approach, using primers from Iμ and just downstream of Se, to isolate potential CSR junctions from a series of WT and Sμ−/− IgE-expressing hybridomas.
B-cell hybridomas, con and moreover, that AID-initiated mutations are greatly as those recovered B cells.

Fig. S2

hybridomas

Table S2

to S probe to detect potential S B cells. To assay for *Fig. S4 and but that had

Zhang et al. | (portion 10 end of the I γ B cells had an even higher level of regions. CSR junctions from the S−/- region utilize sequences in the more 5′ portion of S with sequences in the more 3′ portion of S (Fig. S1), perhaps reflecting PCR biases of the assay, AID targeting hotspots, or continued AID activity and ISD within fused Sε/S regions. CSR junctions from the S−/− B-cell hybridomas occurred in a similar region of Sε as those recovered from WT. As expected (13), the junctions mostly occurred in the region just upstream of Sε, but one junction occurred in a plasmid sequence inserted through gene targeting and the other occurred in the 5′ portion of Cμ (Fig. S1). Based on these findings, we conclude that B cells lacking the donor Sγ region utilize sequences outside of Sγ to undergo substantial levels of class switching to IgE.

Frequent Rearrangement of Sγ1 in Normal and Sγ-Deleted B Cells in the Absence of CSR to Sμ. To further analyze factors that may regulate AID targeting on downstream S regions, we assayed for ISD junctions of IgM-secreting hybridomas generated from day 4 anti-CD40 plus IL-4 stimulated WT and S−/− B cells. To assay for potential rearrangements of Sγ1 that did not involve bona fide CSR, we digested genomic hybridoma DNA with EcoRI and then used Southern blotting to assay for non-germline fragments that hybridized to an Iγ1 probe. The Iγ1 probe derives from sequences just upstream of the Sγ1 region and hybridizes to an ~17-kb EcoRI fragment that encompasses the Iγ1 exon and Sγ1 region; therefore, any rearrangement within this region (except for CSR, which deletes the Iγ1 exon) will alter the size of the EcoRI fragment that hybridizes to the Iγ1 probe (Fig. 2A Upper; Fig. S2). In marked contrast to other studies, we found, in three independent sets of IγM-secreting WT hybridomas, that nearly 20% of the IγH alleles had undergone non−CSR-associated Sγ1 rearrangements (Fig. 2A and B; Table S2). Thus, we identified Sγ1 rearrangement events at Sγ1 in the absence of an upstream donor Sμ. These results are indicated that, under our anti-CD40 plus IL-4 induction conditions, AID targets the downstream Sγ1 region independently of Sγ to Sμ CSR on that IγH allele. Strikingly, we found that nearly 80% of IγM-producing hybridomas isolated from three sets of anti-CD40 plus IL-4–stimulated S−/− B cells contained Sγ1 rearrangements on alleles in which Sγ1 had not undergone bona fide CSR with Sμ (Fig. 2A and B; Table S2). Thus, there is a substantial accumulation of apparent AID targeting events at Sγ1 in the absence of an upstream donor Sμ.

Frequent Mutation of the Iγ1/Sγ1 Region in the Absence of Rearrangement to Sμ. To further test whether or not AID can target downstream S regions independently of initiating a bona fide CSR event, we assayed for SHM of sequences within the region covering the 3′ portion of Iγ1 through the 5′ portion of Sγ1 after anti-CD40 plus IL-4 stimulation of WT and S−/− B cells for 7 days. This ~1.6-kb region, which contains about 500 bp from the 3′ end of the Iγ1 exon and then extends ~1.1 kb into the 5′ portion of Sγ1, has been assayed similarly for AID-initiated hypermutations by other groups (29, 30). Analyses of mutations within the Iγ1/Sγ1 region from three sets of activated WT and control AID−/− B cells revealed a significantly higher mutation level in the WT B cells (~4 × 10−4 bp versus 3 × 10−5 bp) (Fig. 3A; Table S3). Moreover, activated S−/− B cells had an even higher level of mutations within the Iγ1/Sγ1 region (~1.4 × 10−3 bp) (Fig. 3A). In addition, there was a higher level of mutations within this given sequence from the Sμ−/− samples than in WT samples (Fig. 3B). Among the scored mutations, which include point mutations, insertions, and deletions, there seemed to be an increased frequency of deletions in the Iγ1/Sγ1 sequences from the Sμ−/− samples (Table S3), potentially reflecting increased AID targeting.

However, the overall spectrum of point mutations was similar between WT and Sμ−/− sequences, consistent with the same intact mutation mechanism in both (Fig. S3). These results provide further evidence that AID targets downstream S regions independently of Sμ and moreover, that AID-initiated mutations are greatly increased in downstream S regions on IgH alleles that lack Sμ.

Frequent Recombination Events Between Sγ1 and Sε in the Absence of Recombination with Sμ. To investigate AID targeting efficiency at downstream S regions, we assayed for Sε rearrangements in the same sets of IgM-producing hybridomas in which we assayed for Sγ1 rearrangements. We first employed Southern blotting to assay genomic DNA from these hybridomas for rearrangements within Sε. For this purpose, we assayed BamH1- or EcoRI-digested DNA for hybridization to an Iε probe and separately, for hybridization to a Cε probe to detect potential Sε ISD (Fig. 4A Upper; Fig. S4). We noted that most of these hybridomas clearly arose from activated B cells that had not undergone CSR to Sγ1 involving Sμ but that had undergone some type of Sγ1 rearrangement, because they contained two distinct, rearranged Iγ1-hybridizing alleles (Fig. 2A). Despite the apparently high level of IgE switching and particularly high frequency of Sγ1 rearrangements that did not involve CSR.

Fig. 2.

Sγ1 rearrangements on unswitched alleles in WT and Sμ−/− IgM-producing hybridomas. (A Top) Map of the Cγ1 gene. The EcoRI sites are indicated (RI), and the Iγ1 probe is indicated. (Middle and Bottom) Southern blotting analysis of EcoRIdigested genomic DNA extracted from IgM-producing hybridomas generated from day 4 anti-CD40IL-4--stimulated WT (Middle) and Sμ−/− (Bottom) B cells for hybridization to an Iγ1 probe (1B). Iγ1-hybridizing germline bands of EcoRIdigested genomic DNA from WT (129SvJ background) mouse kidneys are indicated with an arrow head and labeled as GL. Lanes with − on top show no bands hybridizing to the Iγ1 probe or a Jγ4 or Cμ probe (used to show that assay hybridized) had rearranged Sγ1 alleles and to confirm their genotype as WT or mutant, respectively (Fig. S2). These lanes were not counted in the calculations shown in Fig. S2B, whereas lanes with * on top contain one or two unswitched alleles that have undergone rearrangements of the Iγ1-hybridizing fragment. (B) Percentage of total unswitched Sγ1 alleles in WT and Sμ−/− IgM-producing hybridomas that contain rearrangements of the Iγ1-hybridizing fragment (presumed Sγ1 rearrangements). SDs calculated from the three experiments are shown. Detailed numbers are listed in Table S1.
with Sμ in Sμ−/− B cells, we detected only one potential Se ISD with the 1e probe in 20 Sμ−/− IgM-producing hybridoma clones (Fig. S4). However, when DNA from the same set of Sμ−/− IgM-producing hybridomas was assayed by hybridization to a Cε probe, we observed a substantial number of rearrangements (Fig. 4B Upper). Because many of these Sμ−/− IgM-producing hybridomas contained two rearranged Iγ1-hybridizing alleles (Fig. 2A), the rearrangements detected with the Cε probe cannot all be explained by the recombination events on the Sμ-deleted allele that lead to class switching (Fig. S1). Additionally, as outlined above, they are not Se ISD. Therefore, we considered the possibility that they represent downstream CSR events between Sγ1 and Se. In support of this notion, stripping and reprobing these blots with an Iγ1 probe revealed that a substantial proportion of the Se rearrangements detected with the Cε probe are exactly the same size as the Iγ1-hybridizing rearrangements (Fig. 4A and B; common bands are indicated with asterisks).

To directly test for downstream CSR between Sγ1 and Se in activated Sμ−/− cells, we assayed for Sγ1–Se junctions in genomic DNA from a set of IgM-producing Sμ−/−-activated B-cell hybridomas through PCR with a forward primer derived from the 3′Iγ1 region and reverse primer derived from the region immediately downstream of Se (Fig. 4A Lower). In unrearranged genomic DNA, the two PCR primers are derived from regions more than 60 kb apart, and therefore, they would only be amplified if they underwent a rearrangement, such as an Sγ1 to Se recombination, that placed them more proximal to each other. Although we did not amplify bands by this approach from the WT IgM-secreting B-cell hybridoma DNA, we amplified bands of distinct sizes from 15 of 42 Sμ−/− IgM-secreting B-cell hybridomas (Fig. 4C). DNA of cloned Iγ1/downstream Se PCR fragments from six Sμ−/− IgM-producing hybridomas confirmed that each harbored a unique Sγ1 to Se junction (Fig. S5). These findings show that Sγ1 and Se can harbor AID-initiated breaks simultaneously and undergo a form of downstream CSR in the absence of bona fide CSR with Sμ.

Discussion

Mechanisms that coordinate action of AID on donor and acceptor S regions are not fully understood. One hypothesis, based on prior findings that AID seemed to have little or no activity on downstream S regions other than in the context of CSR, was that AID might have to be recruited to Sμ and/or introduce lesions into Sμ to gain downstream access (21, 25, 26). However, we now show that WT IgM-producing B cells activated for CSR to IgG1 accumulate ISD events within Sγ1 and mutations within Iγ1/Sε, both hallmarks of AID activity, on alleles that have not undergone CSR with Sμ. One difference between our studies and most earlier studies is that our current anti-CD40 plus IL-4-activation conditions allow much higher levels of IgG1 and IgE switching (e.g., refs. 27 and 37 and this study). Thus, these potentially increased-activation conditions may facilitate observation of AID targeting events in downstream Sγ1 or Iγ1
regions. Moreover, we find that $S_\mu^{-/}$ B cells activated for CSR to IgG1 and IgE accumulate greatly increased levels of $S\gamma1$ ISD, downstream CSR events involving $S\gamma1$ and $Se$, and mutations in $I\gamma1/S\gamma1$. These findings clearly show that AID can access downstream $S$ regions without first interacting with $S\gamma$. Finally, the downstream CSR observed between $S\gamma1$ and $Se$, in the absence of CSR with $S\gamma$, implies that AID activity can lead to simultaneous DSBs in these two downstream $S$ regions, thereby suggesting an alternative pathway for downstream CSR (see below). In this regard, whereas the dependence of $I$-region promoter transcription and CSR to most downstream $S$ regions on the $3’$ IGH regulatory region may preclude activating more than one of these promoters at a time (39, 40), the $I\gamma1$ promoter functions relatively independently from known IgH $3’$ regulatory region elements and is activated by the same pathways as the $I\varepsilon$ promoter, which could allow simultaneous activation and facilitate downstream $S\gamma1$ to $Se$ CSR.

We now show that downstream $S$ regions can be targeted for AID-induced mutations and DSBs in the absence of AID activity on $S\gamma$ and in the absence of CSR with $S\gamma$. In this context, independent recent studies from our lab have confirmed this finding and have also confirmed previous observations that normal B cells activated for CSR to IgG1, under our current conditions, show more ISD within $S\mu$ compared with $S\gamma1$ (24–28). Factors that lead to large ISD detectable by Southern blotting likely include DSB frequency within an $S$ region and ISD within $S$-region junction sequences that might influence end-joining events with respect to rejoining DSBs within an $S$ region or to a DSB within a separate $S$ region—potentially by using different end-joining pathways (27). Assuming that DSB frequency is a major factor, the higher level of detectable ISD at $S\mu$ may reflect greater AID activity at $S\mu$ than other $S$ regions because of its transcription, sequence, or other factors (29). In this context, a high frequency of DSBs in $S\mu$ may well drive CSR to ensure that DSBs in downstream $S$ regions, which we now show can be independently introduced, find a partner $S\mu$ DSB for CSR (7). This general model also has been supported by our findings that independently introduced IScel-endonuclease DSBs within the IgH locus can be joined at long range by general cellular repair processes to promote recombination of IGH class switching (16). Finally, the finding of greatly increased levels of AID-initiated events on downstream $S$ regions in activated $S_\mu^{-/}$ B cells is intriguing. One explanation for this finding is that events simply are more efficient in cells that have undergone efficient CSR in the absence of $S\gamma$. A more interesting possibility is that deletion of $S\gamma$, which is such an efficient AID target, allows AID to act more efficiently on the downstream $S$ regions. Although only speculative, such a model could suggest functional roles for ISD in CSR by promoting increased AID targeting to downstream $S$ regions.

The $S\mu$ deletion we have studied removes most $S\mu$ AID target sites (13), removes the major region of $S\mu$ involved in R-loop initiation (18), and greatly decreases the size of the CSR target region. Therefore, this deletion should vastly diminish the level of DSBs that could serve as upstream CSR donors, and residual DSBs presumably should occur through low-level AID targeting by R-loop (41, 42) or non–R-loop mechanisms (7). Thus, given the apparent role for $S\mu$ DSBs in driving CSR, the dramatic decrease in CSR to most IgH isotypes in $S_\mu^{-/}$ B cells was expected (13). However, our finding that IgE CSR in $S_\mu^{-/}$ B cells is relatively unimpaired was indeed unexpected, especially considering that CSR to IgG1 in the same cell population was severely decreased. One can conceive of several possible explanations for this finding, but we propose a general model that we consider attractive (Fig. S6). Se CSR junctions in $S_\mu^{-/}$ cells must involve direct or indirect joining of $S\gamma$ to a presumably rare DSB in the remaining region upstream of $C\mu$, suggesting that increased $S\gamma$ DSBs might compensate for loss of $S\mu$ DSBs in $S_\mu^{-/}$ B cells and thereby, drive CSR. In this regard, $S_\mu^{-/}$ B cells showed increased ISD and SHM in $S\gamma1$ along with substantial levels of downstream CSR between $S\gamma1$ and $Se$. Thus, in the absence of $S\mu$, frequent $S\gamma$ breaks may drive downstream CSR with $Se$, potentially then leaving the fused $S\gamma1/Se$ region as a major driver for CSR with infrequent breaks upstream of $C\mu$. If this model is correct, we must explain why $Se$ CSR junctions isolated from $S_\mu^{-/}$ cells lacked intervening $S\gamma1$ region sequences. Although such intermediate sequences might be eliminated by ongoing ISD in the hybrid $S\gamma1/Se$ region, a more intriguing possibility is that the $I\gamma1$ promoter is more effective than the $I\varepsilon$ promoter in driving AID access to $Se$ and that, based on its $S\mu$ homology, $Se$ might serve as a better AID target than $S\gamma1$ (Fig. S6). Finally, our current work raises the possibility that some observed sequential CSR (31–34) might involve an alternative pathway in which the downstream CSR provides the first intermediate sequence.

**Materials and Methods**

**Mice.** $S_\mu^{-/}$ mice were generated previously (13) and were maintained with a homozygous 129Sv IgH locus background. The genotype of each experimental mouse was confirmed by Southern blotting analysis. $S_\varepsilon^{-/}$ mice were analyzed at 8–16 weeks of age with age-matched controls that included WT mice on 129Sv background and AID–/– mice (provided by Dr. Taku Honjo). All experiments with mice followed the protocols approved by Boston Animal Care Facility of the Children’s Hospital, Boston, MA 02115.

**CSR Assay and FACS Analysis.** B cells were harvested from spleens of age-matched mutant mice and controls and purified by selection of the CD43− population through a mouse B-cell enrichment kit (STEMCELL Technologies). Purified B cells were cultured in RPMI medium containing 50 μM 6-MP and 15% FCs (as described in ref. 37) in the presence of anti-CD40 antibodies (eBioscience) and IL-4 (20 ng/ml; eBioscience) to stimulate CSR to IgG1 and IgE. Cells were kept under 0.5 million/ml and assayed by surface staining and flow cytometry on days 2, 3, and 4 with anti-IgG1-FITC and anti-B220-PE-Cy5 antibodies as previously described (38).

**Hybridoma Analysis for CSR.** Five to ten million anti-CD40/I–L−4–stimulated B cells from each mouse were fused with NS-1 fusion partner myeloma cells on day 4 and recovered after 7 days selection with 1x Hypoxanthine Aminopterin Thymidine (HAT) medium. Single clones from each well were picked and screened by ELISA on their supernatants with IgM, IgG1, and IgE capture antibodies (Southern Biotech). Only clones that were single positive for one of the three antibody classes were counted. Clones that were negative for all IgH isotypes or positive for more than one IgH isotype were found at very low levels (below 5% of total clones) and not taken into the calculations.

**Somatic Hypermutation Assay.** Five to 10 million anti-CD40/I–L−4–stimulated B cells were harvested on day 7 for genomic DNA extraction with DNeasy Blood and Tissue Kit (QIAGEN). iProof High-Fidelity DNA polymerase (Bio-Rad Life Sciences) was used to amplify the $I\gamma1/S\gamma1$ region with forward primer $Sg1.2F$ (TGTCATCTGGTCTTACGACATCA) and reverse primer $Sg1.2R$ (CCATCGCTC-TAGCCATTGATT). PCR products were purified and cloned into vectors as previously described (37). Plasmids with proper inserts were sequenced, and a 1,640-bp region from the 3′ end of Ig1 to 5′ $S\gamma1$ was analyzed.

**Southern Blotting Analysis.** At least 5 μg genomic DNA isolated from each hybridoma clone was digested with EcoRI or BamHI overnight and run on a 0.7% agarose gel. DNA was transferred from the gels to membranes that were hybridized with corresponding probes (Figs. 2 and 4 and Figs. 52 and 54), washed, and put on XAR film (Kodak Biomax) for exposure.

**Switch-Region Junction Cloning.** $S\gamma1/Se$ and $S\gamma1/Se$–1 junctions were amplified by Advantage cDNA polymerase Mix and PCR Kits (Clontech) following their protocols. For Sm–Se junction cloning, we used forward primer imF1 (ACTAGTCTAGT-CAGTGGGCTAAGGCGCT) and reverse primer Se−1.Rv (CATACGGCTTGGTCACCTA). For $S\gamma1/Se$–1 junction cloning, we used forward primer $Sg1.2F$ (TGTCATCTGGTCTTACGACATCA) and reverse primer Se−1.Rv (CCATCGCTC-TAGCCATTGATT). PCR products were purified and cloned into vectors as previously described (37).

**Acknowledgments.** We thank Jing H. Wang, Duane Wesemann, Yu Nee Lee, FeiLong Meng, and Michael G. Khara for stimulating discussions and Michael Lieber and Barry Sleckman for critical review of the manuscript. This work was supported by National Institutes of Health Grants AI031541 and CA092625 (to F.W.A.). C.B. is supported by a Cancer Research Institute training grant. F.W.A. is an investigator of the Howard Hughes Medical Institute.

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