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Activation-Induced Cytidine Deaminase-Dependent DNA Breaks in Class Switch Recombination Occur during G\textsubscript{1} Phase of the Cell Cycle and Depend upon Mismatch Repair\textsuperscript{1}

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Ab class switching occurs by an intrachromosomal recombination and requires generation of double-strand breaks (DSBs) in Ig switch (S) regions. Activation-induced cytidine deaminase (AID) converts cytosines in S regions to uracils, which are excised by uracil DNA glycosylase (UNG). Repair of the resulting abasic sites would yield single-strand breaks (SSBs), but how these SSBs are converted to DSBs is unclear. In mouse splenic B cells, we find that AID-dependent DSBs occur in S\textsubscript{\mu} mainly in the G\textsubscript{1} phase of the cell cycle, indicating they are not created by replication across SSBs. Also, G\textsubscript{1} phase cells express AID, UNG, and mismatch repair (MMR) proteins and possess UNG activity. We find fewer S region DSBs in MMR-deficient B cells than in wild-type B cells, and still fewer in MMR-deficient/S\textsubscript{\mu}TR\textsuperscript{−/−} B cells, where targets for AID are sparse. These DSBs occur predominantly at AID targets. We also show that nucleotide excision repair does not contribute to class switching. Our data support the hypothesis that MMR is required to convert SSBs into DSBs when SSBs on opposite strands are too distal to form DSBs spontaneously. The Journal of Immunology, 2007, 179: 6064–6071.

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\textsuperscript{*}Abbreviations used in this paper: CSR, class switch recombination; DSB, double-strand break; AID, activation-induced cytidine deaminase; TR, tandem repeat; UNG, uracil DNA glycosylase; APE, AP endonuclease; SSB, single-strand break; NER, nucleotide excision repair; MMR, mismatch repair; WT, wild type; LM-PCR, ligation-mediated PCR; XPF, Xeroderma pigmentosum F.
DNA duplex, unless the DNA ends are processed. AID is known to deaminate dC nucleotides on both the transcribed and nontranscribed strands (22, 23), and the density of AID hot spots occurring in the S region TRs could lead to nicks on opposite strands in close proximity to each other. However, deletion of the SµTR region results in only a modest (2-fold) reduction in CSR (24). CSR can occur upstream of, as well as within, the SµTR region (25, 26), although recombination outside of the SµTRs requires the DNA mismatch repair (MMR) pathway (26, 27). In fact, CSR is nearly ablated in SµTR−/− B cells that also lack the MMR proteins Msh2 or Mlh1 (28) (J. Eccleston, C. E. Schrader, J. Stavnezer, and E. Selsing, manuscript in preparation). Because MMR is required for recombination outside the SµTR region where AID hot spots are relatively infrequent, we have proposed that MMR is needed to convert SSBs into DSBs when the single-strand nicks are too far apart to form spontaneous DSBs (29). This hypothesis is consistent with the finding that CSR does not require MMR, but is reduced 2- to 5-fold in MMR-deficient B cells, i.e., at least 50% of CSR events require MMR, depending on the isolate (27, 30–34). To test this hypothesis, we analyzed Sµ DSBs in B cells from mice deficient in MMR proteins that have the WT Sµ region or the SµTR deletion. Altogether, the data support the hypothesis that distal SSBs are converted by MMR into DSBs, constituting an important step in CSR.

Materials and Methods

Mice

AID-deficient mice were obtained from T. Honjo (Kyoto University, Kyoto, Japan). Msh2-deficient mice were obtained from T. Mak (University of Toronto, Toronto, Canada). Mlh1- and Pms2-deficient mice were obtained from R. M. Liskay (Oregon Health Sciences University, Portland, OR). XPA-deficient mice were obtained from H. van Steeg (National Institute of Health, Bilthoven, The Netherlands) (35). These lines have all been extensively backcrossed to C57BL/6. sper−/−, uug−/−, and sper−/− uug−/− littermates used for the experiment shown in Fig. 3. Sµ TR−/− B cells that also lack the MMR proteins Msh2 and Mlh1 (28) (J. Eccleston, C. E. Schrader, J. Stavnezer, and E. Selsing, manuscript in preparation). Because MMR is required for recombination outside the SµTR region where AID hot spots are relatively infrequent, we have proposed that MMR is needed to convert SSBs into DSBs when the single-strand nicks are too far apart to form spontaneous DSBs (29). This hypothesis is consistent with the finding that CSR does not require MMR, but is reduced 2- to 5-fold in MMR-deficient B cells, i.e., at least 50% of CSR events require MMR, depending on the isolate (27, 30–34). To test this hypothesis, we analyzed Sµ DSBs in B cells from mice deficient in MMR proteins that have the WT Sµ region or the SµTR deletion. Altogether, the data support the hypothesis that distal SSBs are converted by MMR into DSBs, constituting an important step in CSR.

B cell isolation and culture

Spleen cells were dispersed and RBCs lysed in Gey’s solution followed by T cell depletion with a mixture of anti-T cell Abs, as described previously (30). Cells were cultured at 1 × 10^6/ml in 6-well plates and activated to induce CSR. All cultures contained LPS (50 ng/ml; Sigma-Aldrich) and dextran sulfate (30 μg/ml; Amersham Biosciences) for IgG2b; anti-δ-dextran (0.3 ng/ml) for IgG3, and to induce IgA switching, TGβ-2 (2 ng/ml), IL-4 (800 U/ml), IL-5 (1.5 ng/ml; BD Biosciences), and anti-δ-dextran (0.3 ng/ml) were added. Fixing cells of staining of cell surface Abs were performed as described previously (30), except the results were analyzed by FlowJo software.

Cell cycle sorting

Spleen B cells were cultured for 2 days as described above, except that cell density was adjusted to 1 × 10^6/ml cells/ml and stimulated B cells were incubated for 90 min at 37°C with 5.5 μg/ml Hoechst 33342 (Invitrogen Life Technologies) in HBSS containing 1% FCS. Cells were finally resuspended in 1 ml of HBSS with 1% FCS, 7-aminoactinomycin D was added (0.6 μg/ml) and cells were sorted by flow cytometry based on DNA content using a UV laser-equipped FACSVantage SE (BD Biosciences).

FIGURE 1. AID-dependent Sµ DSBs are detected in G1−, but not in S/G2/M-phase, cells. A, Splenic B cells were activated for 2 days, stained with Hoechst 33342, and sorted into G1 and S/G2/M populations. A representative sorting profile is shown. B, LM-PCR was performed on DNA from viable sorted cells as indicated. Three-fold dilutions of 7200 cell equivalents were amplified. PCR amplification of the GAPDH gene (except for the highest input) is shown below the blots as an internal control for template input. The figure shows representative of two experiments.

Western blotting

To prepare nuclear extracts, cells were resuspended in hypotonic buffer (10 mM HEPES [pH 8], 1 mM EDTA, 10 mM KCl, 0.1 mM EGTA, 1 mM DTT, 2 μM peptatin, and complete protease inhibitor mixture). Protein content of nuclear extracts was determined using the Bradford assay (Bio-Rad). Proteins were electrophoresed on 10% SDS-polyacrylamide gels or 4–20% gradient SDS-polyacrylamide gels (Bio-Rad), and blotted onto Immobilon-P polyvinylidene fluoride membranes (Millipore). Immunoblotting was performed using rabbit polyclonal Abs against AID and UNG (rabbit anti-peptide amino acids 280–295 from mouse UNG), APE1 (36), MSH2 (sc-494), MSH6 (sc-10798), MLH1(sc-582), and GAPDH (Santa Cruz Biotechnology), and monoclonal mouse anti-TBP1 (Abcam) followed by goat-anti-rabbit or donkey anti-mouse-HRP (Santa Cruz Biotechnology) and ECL substrate (Pierce).

UNG assay

Whole cell extracts were prepared from splenic B cells activated for 2 days, washed in ice-cold PBS, and lysed in buffer I (10 mM Tris-HCl [pH 7.8], 200 mM KCl with Complete protease inhibitors [Roche]). An equal volume of buffer II was added (buffer I with 2 mM EDTA, 40% glycerol, 0.2% Nonidet P-40, and 2 mM DTT) added before tumbling for 1 h at 4°C. Supernatants were stored at −80°C. Nuclear extracts were made from sorted cells by lysis in buffer A (10 mM HEPES [pH 8], 10 mM KCl, 0.1 molar TEA, 20 μM DTT, 2 μM peptatin, and complete protease inhibitor mixture). Protein content of nuclear extracts was determined using the Bradford assay (Bio-Rad). Proteins were electrophoresed on 10% SDS-polyacrylamide gels or 4–20% gradient SDS-polyacrylamide gels (Bio-Rad), and blotted onto Immobilon-P polyvinylidene fluoride membranes (Millipore). Immunoblotting was performed using rabbit polyclonal Abs against AID and UNG (rabbit anti-peptide amino acids 280–295 from mouse UNG), APE1 (36), MSH2 (sc-494), MSH6 (sc-10798), MLH1(sc-582), and GAPDH (Santa Cruz Biotechnology), and monoclonal mouse anti-TBP1 (Abcam) followed by goat-anti-rabbit or donkey anti-mouse-HRP (Santa Cruz Biotechnology) and ECL substrate (Pierce).
mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2 μM pepstatin, and Complete protease inhibitor) with 0.625% Nonidet P-40. Nuclei were pelleted and extracted with buffer C (20 mM HEPES (pH 8), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 40% glycerol, 0.5 μM pepstatin, and Complete protease inhibitor). Extracts were incubated with a [32P]end-labeled uracil-containing oligonucleotide (5'-GATTCCCATCTCTCGGATTTCACT/ideoxyU/CTGCACCGCATG-3') and [32P]ATP and washed at 55°C with 2 M urea, 15% polyacrylamide bis (19:1) gel.

FIGURE 2. The proteins important for CSR are present in all phases of the cell cycle in activated B cells. A, Splenic B cells were activated for 2 days, stained with Hoechst 33342, and sorted into G1, S, and G2/M populations. B–D, Western blots of extracts from sorted cells, B and C, A total of 35 μg of nuclear or cytoplasmic extract, as indicated, electrophoresed on a gradient gel. ung-/- and aid-/- control extracts are from unsorted B cells. D, Five micrograms of nuclear extract electrophoresed on a 10% polyacrylamide gel. E, UNG assay. Five-fold dilutions (10, 50, and 250 ng) of whole cell extracts from unsorted cells or nuclear extracts from cells sorted as in A were added to a [32P]end-labeled uracil-containing oligonucleotide. Upper band, Unlabeled oligo; lower band, cleaved product.

Cloning, identification, and sequence analysis of PCR products
LM-PCR products were cloned into the vector pCR4-TOPO (Invitrogen Life Technologies) and sequenced by Macrogen using T3 and T7 primers. Cloned breaks in Sμ were aligned with germine Sμ sequenced from C57BL/6 chromosome 12 (GenBank Accession number AC073553) with numbering starting at nt 136,645. This is the 5′ Sμ primer-binding site and the average + SEM is shown.

FIGURE 3. CSR is not reduced in XPA-deficient B cells. A and B, Cells from spo−/− mice were cultured for 4 days with LPS and cytokines to induce switching to the indicated isotypes and cells were analyzed by flow cytometry for surface Ig. Data from four experiments were normalized to the percent of switching by B cells from WT littermates (shown as 100%) and the average ± SEM is shown. B and C, Examples of flow cytometric analyses for CSR to IgG1 (B) and IgG2a (C). Switching to all four IgG isotypes and IgA were also analyzed with similar results. All mice used were littermates with the exception of aid−/−.

Results
AID-dependent DSBs are predominantly detected in the G1 phase of the cell cycle
A possible mechanism for conversion of SSBs into DSBs is DNA replication, and many of the proteins involved in CSR are associated with DNA surveillance and repair during replication, e.g., MMR proteins and UNG (37). To determine when AID-dependent DSBs are made, we examined the cell cycle regulation of Sμ DSBs. DSBs dependent on AID and UNG are induced in the S regions of B cells activated in vitro to undergo CSR and can be detected by linker LM-PCR (15). We activated splenic B cells for 2 days with LPS and IL-4 to induce IgG1 CSR or LPS and anti-δ-dextran (a-δ-dex) to induce IgG3 CSR, stained with Hoechst 33342, and then sorted cells into G1 and S/G2/M fractions on the basis of DNA content (Fig. 1A). Dead cells were excluded by 7-aminoactinomycin D staining. At 48 h, there are no undivided cells in these cultures, as determined by CFSE staining (our unpublished data). As shown in Fig. 1B, AID-dependent...
DSBs are detected almost exclusively in the G1 fraction in cells activated under either condition. There are a few breaks detected in S/G2/M phase, but there are about as many in AID-deficient B cells. These data are therefore inconsistent with the hypothesis that DSBs detected in S/M in switching B cells are due to replication across SSBs. Although a few DSBs are detected in aid/−/− cells, they do not specifically occur at the G:C base pair in the AID target hot spots, unlike DSBs in WT cells (15). They might be due to mechanically induced breaks or apoptosis occurring during the procedure, or perhaps due to replication.

Proteins important for CSR are expressed in G1-phase cells

We then asked whether the levels of AID, UNG, APE1, and MMR proteins are regulated by the cell cycle. B cells activated with LPS and α−/−-dex were sorted into G1, S, and G2/M fractions (Fig. 2A) and extracts were prepared for Western blotting (Fig. 2, B–D). AID protein is difficult to detect in nuclear extracts, but is expressed in the cytosol in all phases of the cell cycle (Fig. 2C). UNG is abundant both in the G/C base pair in the AID target hot spots, unlike DSBs in WT cells (15). They might be due to mechanically induced breaks or apoptosis occurring during the procedure, or perhaps due to replication.

FIGURE 4. DSBs in Sμ are reduced in MMR-deficient splenic B cells. A, A representative Southern blot of Sμ LM-PCR products from B cells lacking the indicated protein, induced with LPS and IL-4. PCR amplification of the GAPDH gene is shown below the blots as an internal control for template input: 3-fold dilutions of 2400 cell equivalents. B, Quantification of DSBs in B cells lacking MMR or AID relative to DSBs in WT cells.Autoradiographs were scanned to measure total density in all three lanes for each sample, and results were normalized relative to density of WT bands in each experimental set. Error bars indicate SEM. The apparent discrepancy between the numbers and intensities of breaks in the pms2−/− and aid−/− samples between the blot shown in A and the compiled results in B is because in the other pms2−/− experiment there were more DSBs (data not shown).

FIGURE 5. Sμ DSBs are greatly reduced in B cells lacking both SμTRs and Msh2. Sμ LM-PCR products (A and B) amplified as in Fig. 4 from cells with the indicated deficiencies activated as indicated. C, Quantification of DSBs by densitometry (as in Fig. 4B) of the indicated number of experiments. Error bars represent SEM. The aid−/− histogram presents the same data shown in Fig. 4B.

The role of Ercc1 in CSR does not involve the NER pathway

We next asked whether NER might contribute to the generation of DSBs during CSR. We have previously shown a minor role for

G1-phase cells have UNG activity

UNG is not present in resting (G0) cells (14) and has been proposed to act in S phase at replication forks during CSR and somatic hypermutation (40). G1-phase B cells have not been examined for UNG activity. We prepared nuclear extracts from G1- and S-phase B cells activated for 2 days and assayed UNG activity by an oligonucleotide cleavage assay (Fig. 2E). Activity is clearly detectable in as little as 10 ng of nuclear extract from G1-phase cells, which has as much activity as extracts from S-phase cells. As this substrate is single stranded, the predominant activity detected here is likely to be due to UNG and not SMUG-1, as SMUG-1 has 800-fold lower activity on ssDNA than UNG (kcat/Km) (41). From these combined results, we conclude that AID-dependent DSBs in Sμ are made and resolved in S/M in G1 phase and are not due to replication across SSBs.

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Ercc1 in CSR (20). The heterodimer Ercc1-XPF is an essential component of the NER pathway, but Ercc1-XPF can also act as a structure-specific endonuclease in the absence of the rest of the NER pathway, cutting at the junction of ssDNA and dsDNA (19). Here, we analyzed CSR in vitro in B cells from mice lacking XPA, a protein essential for NER. We found no reduction in CSR in xpa−/− mice relative to cells from WT littermates (Fig. 3A). In the absence of UNG, it seemed possible that the accumulation of uracils might result in distortion of the DNA helix sufficient to create a substrate for NER, so we also analyzed CSR in ung−/− xpa−/− B cells. CSR is severely reduced in B cells deficient in UNG, as reported by others (13, 14), but still detectable relative to AID-deficient B cells. However, CSR is not further reduced in ung−/− xpa−/− B cells relative to B cells from ung−/− littermates (Fig. 3A). In this experiment, there was a reduction in IgG1 and IgG2a CSR in xpa−/− cells, but this was not typical as can be seen from the compiled data in Fig. 3A. We conclude that the minor role played by Ercc1-XPF in CSR most likely involves its ability to cut at the junction of ssDNA and dsDNA (discussed below and in Ref. 29), and does not involve other components of the NER pathway.

S-region DSBs are reduced in MMR-deficient B cells

The hypothesis that SSBs are converted to DSBs in S regions by MMR predicts fewer DSBs in Sμ in MMR-deficient B cells relative to WT cells. To test this prediction, LM-PCR was performed on genomic DNA isolated from B cells 2 days after induction of CSR. A representative Southern blot of LM-PCR fragments amplified with a 5′ Sμ-specific primer from WT, msh2−/−, mlh1−/−, and pms2−/− B cells is shown in Fig. 4A. Blunt DSBs in MMR-deficient cells are reduced compared with WT cells analyzed in parallel. Quantitation by densitometry scanning of the autoradiographs from several experiments demonstrates that MMR deficiency results in 20–50% of the number of Sμ DSBs in WT cells (Fig. 4B). Thus, blunt DSBs induced in Sμ during CSR are reduced in MMR-deficient cells to a degree consistent with the reduction observed in CSR.

Sites of DSBs in SμTR-deficient cells

Relative to the WT Sμ region, the SμTR−/− intron has a 2-fold lower incidence of AID hot spots (WRC/GYW, where the underlined nucleotide is the target site), and the strongly preferred hot-spot GCT (11, 33) is 3.6-fold less frequent than in WT Sμ (Fig. 6).
hot spots (GYW), and at the four different GYW motifs (GCT, GCA, GTT, and GTA), as well as the frequency of occurrence of these motifs in the Sμ-region sequence in WT and SμTR−/− cells. The percentage of DSBs located at GYW sequences is increased in SμTR−/− B cells (51.6% compared with 40.5% in WT), supporting the hypothesis that distal AID hot spots, which is consistent with the location of DSBs in S phase, but not during G2/M phase in splenic B cells activated to undergo CSR (45). We also found that AID, UNG, and MMR proteins are present in the G1 phase of the cell cycle, and that extracts from G1-phase B cells possess UNG activity.

MMR proteins could convert SSBs into DSBs in the course of normal MMR activity if nicks are present on both strands. Both AID and UNG prefer ssDNA substrates and appear to act on both DNA strands during transcription (1, 12, 22, 46). We hypothesize that the DNA duplex reforms before all of the dUs can be removed by UNG, and MMR would then compete more effectively than UNG for repair of dUs in duplex DNA. AID is a highly processive enzyme and although UNG has a very high catalytic rate, it appears to be unable to remove all the dUs introduced by AID into S regions (6, 12, 22, 23, 41). UNG is likely, however, to create numerous abasic sites, which could then be nicked by APE to generate entry sites for excision by Exo1 in the 5′ to 3′ direction. If excision continued until a SSB on the opposite strand is reached, a DSB would be formed. A similar model has been proposed for Escherichia coli dam cells exposed to the methylating agent MNG, in which DSBs arise when MMR excision on one strand encounters a nick generated by APE on the other (47). Depending on the orientation of the SSBs relative to each other, some DSBs created by MMR activity will be blunt and some will have 5′ or 3′ overhangs. 5′ overhangs can be filled in by polymerase to become blunt, and we have proposed that Ercc1-XPF can remove 3′ overhangs (20, 29). Ercc1-XPF cuts 3′ overhangs at the junction of ssDNA and dsDNA without requiring additional NER proteins (48). Consistent with Ercc1-XPF acting independently of NER in CSR, we found that XPA, an essential NER protein, is not involved in CSR even in the absence of UNG, where helix-distorting lesions recognizable by NER might form.

We asked whether the DSBs in SμTR−/− cells occur preferentially at these remaining hot spots by cloning and sequencing LM-PCR products from WT and SμTR−/− B cells. Table I presents the percent of DSBs occurring at the G-C or A:T base pair, at all AID hot spots (GYW), and at the four different GYW motifs (GCT, GCA, GTT, and GTA), as well as the frequency of occurrence of these motifs in the Sμ-region sequence in WT and SμTR−/− cells. The percentage of DSBs located at GYW sequences is increased in SμTR−/− cells (51.6% compared with 40.5% in WT), supporting the importance of this sequence for AID targeting.

Further analysis of the breakpoints used in the SμTR−/− intron support previous conclusions that GCT/AGC is the strongest AID hot spot. The preference for breaks at GCT in WT Sμ is 3.3-fold over random (35.4% of DSBs at GCT vs 10.6% frequency of GCT in the sequence, p ≤ 0.001). However, GCT occurs in the WT Sμ sequence at a much higher frequency than the other GYW hot spots (Table I). In the SμTR−/− intron, the frequency at which GCT occurs relative to the other GYW motifs is lower; interestingly, DSBs at GCT occur 10-fold more often than predicted by the sequence (29% of DSBs are at GCT compared with 2.9% frequency in the sequence, p ≤ 0.001). These results confirm that the preference of AID for GCT hot spots previously found in vitro with purified AID and from analyses of somatic hypermutation (6, 11, 12, 42, 43) is also true during CSR, and is even more apparent when these motifs are less abundant. We hypothesize that the reduction in AID hot spots in SμTR−/− B cells results in SSBS that are farther apart, and thus less likely to be sufficiently close to form a DSB spontaneously. DSB formation and CSR are therefore more dependent on MMR in these mice.

**Discussion**

The results from our studies support the hypothesis that distal SSBS are converted into DSBs by MMR recognition and repair of dU:dG mismatches introduced by AID. We obtained data arguing against an alternative model for conversion of SSBS to DSBs, i.e., replication across a SSB, as we detected very few DSBs in S/G2/M phase cells. Instead, the AID-dependent SSBS occur in the G1 phase. These results are in agreement with previous reports showing that AID-dependent yH2AX/Nbs1 foci colocalize with IgH loci during the G1/early S phase in splenic B cells activated to switch (18). They are also consistent with the finding that switch recombination is not accompanied by sister-chromatid exchange (44), and that a specific protein complex binds near the transcriptional initiation site for germline Ig transcription during G1/early S phase, but not during G2/M phase in splenic B cells activated to undergo CSR (45). We also found that AID, UNG, and MMR proteins are present in the G1 phase of the cell cycle, and that extracts from G1-phase B cells possess UNG activity.

MMR proteins could convert SSBs into DSBs in the course of normal MMR activity if nicks are present on both strands. Both AID and UNG prefer ssDNA substrates and appear to act on both DNA strands during transcription (1, 12, 22, 46). We hypothesize that the DNA duplex reforms before all of the dUs can be removed by UNG, and MMR would then compete more effectively than UNG for repair of dUs in duplex DNA. AID is a highly processive enzyme and although UNG has a very high catalytic rate, it appears to be unable to remove all the dUs introduced by AID into S regions (6, 12, 22, 23, 41). UNG is likely, however, to create numerous abasic sites, which could then be nicked by APE to generate entry sites for excision by Exo1 in the 5′ to 3′ direction. If excision continued until a SSB on the opposite strand is reached, a DSB would be formed. A similar model has been proposed for Escherichia coli dam cells exposed to the methylating agent MNG, in which DSBs arise when MMR excision on one strand encounters a nick generated by APE on the other (47). Depending on the orientation of the SSBs relative to each other, some DSBs created by MMR activity will be blunt and some will have 5′ or 3′ overhangs. 5′ overhangs can be filled in by polymerase to become blunt, and we have proposed that Ercc1-XPF can remove 3′ overhangs (20, 29). Ercc1-XPF cuts 3′ overhangs at the junction of ssDNA and dsDNA without requiring additional NER proteins (48). Consistent with Ercc1-XPF acting independently of NER in CSR, we found that XPA, an essential NER protein, is not involved in CSR even in the absence of UNG, where helix-distorting lesions recognizable by NER might form.

If the SSBs are the results of AID-instigated lesions, the resulting blunt DSBs would occur preferentially at the G-C base pair in AID hot spots, which is consistent with the location of DSBs in Sμ determined by LM-PCR (Table I) (15, 29). No significant differences were observed between the sites of Sμ DSBs in WT and MMR-deficient B cells (Table II). In the absence of MMR, we predict that the only DSBs formed would do so spontaneously from closely spaced nicks. The staggered DSBs produced in the absence of MMR could then be end processed as described above to form the blunt DSBs at the G-C base pair in AID hot spots detected by LM-PCR. The staggered ends can be detected by LM-PCR by pretreatment with T4 DNA polymerase before ligation (15, 17). We find a 3-fold increase in DSBs after T4 treatment of DNA from both WT and MMR-deficient cells (data not shown).

It has been reported that 94% of Sμ-Sy3 junctions in Msh2-deficient cells occur within the Sμ TRs, which contain numerous closely spaced GAGCT sequences, and only 5% occur 5′ to the TRs, whereas in WT cells a greater proportion (17%) of the junctions occur 5′ to the repeats (26, 27). These results predict that blunt DSBs in MMR-deficient B cells would localize preferentially to the Sμ TRs. However, we found a similar fraction of DSBs occur 5′ to the Sμ TRs in WT and msh2−/− cells, 42 and 40%, respectively. This difference from the previous reports might be due to differences in the analysis methods. We used a PCR primer located at the 5′ side of Sμ, and extension would terminate at the first break, favoring detection of breaks located upstream of Sμ.

We consistently find fewer blunt DSBs in B cells from mice lacking Mlh1 or Pms2 than in those lacking Msh2. This might be explained by the recent finding that Mlh1 decreases Exo1 processivity (49). If Exo1 excises farther in the absence of Mlh1-Pms2, perhaps past the nick on the opposite strand, long single-strand

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**Table 1. DSBs occur preferentially at AID hot spots in WT and SμTR−/− B cells**

| Motif | WT Frequency | Sμ TR−/− Frequency |
|-------|--------------|---------------------|
| G:C   | 83.5%        | 83.9%               |
| A:T   | 16.5%        | 16.1%               |
| GYW   | 40.5%        | 51.6%               |
| GCT   | 35.4%        | 29.0%               |
| GCA   | 2.5%         | 3.2%                |
| GTT   | 1.3%         | 9.7%                |
| GTA   | 1.3%         | 9.7%                |
| Total | 79 breaks nt 1–2000 | 31 breaks nt 1–1439 |

*The frequency at which the nucleotide or sequence motif occurs in the sequence analyzed (Sμ or SμTR−/−). *

*Percent of DSBs located at the indicated base pair or sequence motif.

*Significantly targeted relative to the DNA sequence, p ≤ 0.012 (Fisher’s exact test).

*nt 1, Nucleotide 136,645 in the chromosome 12 sequence from C57BL/6 (GenBank Accession AC073553).

*The underlined G, G:C bp at which the DSB occurs within the hot spots, reading the top strand sequence.
more dependent on MMR. which can lead to translocation and tumor development, might be targets in these genes are infrequent and therefore, DSB formation, significant role in DSB formation at other loci at which AID might contribute significantly to S-region breaks.

Our results are consistent with the model that MMR is more important for CSR in situations when SBSs are limiting, as when AID targets are scarce. Thus, loss of both MMR and the SpA TR region results in a dramatic reduction in switching and a reduction in SpA DSBs. Although we cannot determine the sites of the SBSs that gave rise to any given DSB, we were able to confirm that DSBs occur preferentially at AID hot spots even when the frequency of hot spots is low. We propose that only SSBs on opposite strands within a few nucleotides of each other can form a DSB in the absence of MMR. It is possible that MMR may play a significant role in DSB formation at other loci at which AID might initiate DSB formation, such as bc6l and c-myc (51, 52). AID targets in these genes are infrequent and therefore, DSB formation, which can lead to translocation and tumor development, might be more dependent on MMR.

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