Lack of MSH2 involvement differentiates V(D)J recombination from other non-homologous end joining events

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ABSTRACT

V(D)J recombination and class switch recombination are the two DNA rearrangement events used to diversify the mouse and human antibody repertoires. While their double strand breaks (DSBs) are initiated by different mechanisms, both processes use non-homologous end joining (NHEJ) in the repair phase. DNA mismatch repair elements (MSH2/MSH6) have been implicated in the repair of class switch junctions as well as other DNA DSBs that proceed through NHEJ. MSH2 has also been implicated in the regulation of factors such as ATM and the MRN (Mre11, Rad50, Nbs1) complex, which are involved in V(D)J recombination. These findings led us to examine the role of MSH2 in V(D)J repair. Using MSH2+/+ and MSH2−/− mice and cell lines, we show here that all pathways involving MSH2 are dispensable for the generation of an intact pre-immune repertoire by V(D)J recombination. In contrast to switch junctions and other DSBs, the usage of terminal homology in V(D)J junctions is not influenced by MSH2. Thus, whether the repair complex for V(D)J recombination is of a canonical NHEJ type or a separate microhomology-mediated-end joining (MMEJ) type, it does not involve MSH2. This highlights a distinction between the repair of V(D)J recombination and other NHEJ reactions.

INTRODUCTION

Two types of recombination events occur at the Immunoglobulin (Ig) locus of B cells in mice and humans. Initially, combinatorial joining of gene segments that encode either the heavy or the light chain of the Ig receptor by V(D)J recombination generates a diverse nascent repertoire (1). Following an immune response, class switch recombination (CSR) leads to the generation of antibodies of different isotypes (2). At the DNA level, both recombination events consist of a cleavage generating a double strand break (DSB), followed by a joining phase (3–5).

In the case of V(D)J recombination, RAG1 and RAG2 along with other contributing factors such as HMG-1 recognize and bind the 12 or 23 recombination signal sequence (RSS) flanking each recombining V, D or J gene segment (6–8). The RAG complex initiates V(D)J recombination by introducing a nick at the RSS/coding border leaving a 3'-OH coding end. A subsequent inter-strand trans-esterification reaction leads to the generation of a hairpin coding end and a blunt signal end (9,10). In CSR, however, the DSB has been shown to be induced through the action of the enzyme activation-induced cytidine-deaminase (AID) (11–14). Recent work has shown that AID initiates somatic hypermutation (SHM) and CSR by deaminating cytidines in the V-region and switch region of the Ig locus, respectively (15–17). The resulting uridine is then removed by uracil DNA glycosylase (UNG) resulting in an abasic site (18,19). In the case of CSR, the generation of two such abasic sites on opposite strands is thought to result in a DSB in the switch region. Indeed, when AID or UNG are absent, CSR is completely abolished (20,21).

Whether a DSB is generated by V(D)J recombination, CSR or DNA-damaging agents such as ionizing radiation, its repair is essential to the viability and/or progression of the cell. In eukaryotes, DSBs are either repaired by homologous recombination (HR) or non-homologous end joining (NHEJ) (22,23). As HR uses sister chromatids as the source of undamaged template, it functions in late S/G2 phase. NHEJ however is the prominent repair pathway during G0/G1 (24). As such,
CSR and V(D)J recombination both use the NHEJ pathway for the repair of DSBs and all require a set of factors that are essential to NHEJ (25–27). These include ATM, the MRN complex (Mre11, Rad50, Nbs1), Ku70/80 and DNA-PKcs (4,23,28). However, given their distinct DSB-initiating pathways and different cell types in which CSR and V(D)J recombination occur, it is probable that NHEJ repair of each reaction also utilizes a set of other unique factors, though such differences have thus far not been clearly defined.

A possible candidate for a pathway that might be differentially involved in the NHEJ of DSBs that occur during CSR, general DNA damage and V(D)J recombination is the mismatch repair pathway involving MutS homologs MSH2, MSH3 and MSH6 (29). In mice and humans, MSH2 can form homodimers as well as MSH2/MSH3 or MSH2/MSH6 heterodimers (30–32). MSH2/MSH6 heterodimers bind with high affinity to single-base pair mismatches and small 1–2 nt insertion–deletion loops (33–35). Mismatch engagement by MSH2/6 dimers is followed by the recruitment of downstream MMR proteins, including MLH1 and PMS2, in an ATP-dependent manner (36–38).

MSH2 has been demonstrated to play a role in both SHM and CSR (39–44). MSH2+/− mice have reduced A-T mutations during SHM and reduced CSR activity. One study also found altered characteristics of switch junctions, namely a reduction in the usage of terminal microhomologies (45).

A similar phenotype was observed in mice deficient in exonuclease 1, lending support to the proposed role of this enzyme downstream of MSH2 in the mismatch repair of CSR junctions (46). However, mice with a knock-in mutation in MSH2 (MSH2G674A) which is able to recognize mismatches but lacks ATPase activity, exhibited an increased usage in terminal microhomologies in their CSR junctions (47), similar to the phenotype observed in PMS2+/− mice (45). Whatever the exact nature of the role of MSH2 might be, it is clearly involved in the repair of CSR junctions. A recent study has also found reduced usage of terminal microhomologies in the NHEJ repair of a transgenic substrate in a MSH2−/− CHO cell line (48). Other reports implicate MSH2 in signaling as well as cell-cycle regulation of ATM and the MRN complex (49,50) which are not only essential to the repair of CSR DSBs, but also involved in V(D)J recombination (28,51–54). These observations, along with the potential for the generation of single as well as multi-base mismatches in the formation of V(D)J coding joints, prompted us to examine the role of the mismatch repair pathway and in particular its central player MSH2, in V(D)J recombination in vivo in the context of mouse bone marrow and ex vivo, in the context of cell lines differing in the MSH2 status.

**MATERIALS AND METHODS**

**Amplification and sequencing of DJH joints**

Genomic DNA from the bone marrow of two MSH2−/− and wild-type littermates was purified (Qiagen) to a concentration of 100 ng/µl. The mouse line has been described previously (55). Dilutions of 1/4, 1/16 and 1/64 (corresponding to 10000, 2500 and 600 cell equivalents) were used as template. GAPDH amplification primers were GAPDH-F, TCCACACGCTGGTCTCTGTA, and GAPDH-R, GACCACAGTCCATGCCCATCTACT. DJ joints were amplified as described previously (56) by using the DFS primer, AGGGATCCCTGTGAAAGGATCTACTAGTGTG, which hybridizes to the 5′ RSS of all murine DH segments, and JH4-C primer, AAAGACCTTCAGAGGCATTTCTTACC, which hybridizes 50 bp downstream of JH4 in the JH4-C intronic region. PCR was carried out in 25 µl volumes. A cycle of 94°C for 30 s, 50°C for 30 s and 72°C for 2.5 min was repeated 25 times for the GAPDH primer set and 35 times for the DJ primer set. An incubation at 72°C for 10 min followed. PCR product (15 µl) was electrophoresed on 1.5% agarose (BioShop) gels and visualized or transferred to nitrocellulose for Southern analysis to confirm the identity of the bands. Of the same PCR product 4 µl was used in ligation with the PCR2.1 TA cloning vector (Invitrogen), and plated on Kanamycin plates containing X-gal. Blue/white screening was used to identify colonies harboring an insert. Colonies were grown up over night in 96-well plates using the 96-well miniprep kit (Millipore), followed by EcoR1 digest to determine the insert size and identify the JH used. DJH4 inserts were sequenced using the T7 primer. DJH2 and DJH3 inserts were sequenced using the T7 and M13R primers. Sequencing was done by Macrogen, Korea.

**Extra-chromosomal recombination assay**

The Plasmid pmlDJ+ was generated from the pV81x-D-J microloci described (57). PCR on the pV81-D-J was carried out using a DFLS5′RSS primer with an engineered BamHI site GGATCCGGTTTTTGGTCATGGATATAGCACTGTG and an anti-sense primer specific for the polyoma region CAAC-GAAGG TTCCTACT. After a hot start at 85°C for 5 min, 30 cycles of a 3 step PCR (94°C for 30 s, 55°C for 1 min and 72°C for 1 min plus 5 s per cycle) was followed by 72°C for 10 min. The PCR product of 650 kb was cloned into the PCR II vector (TA cloning kit, Invitrogen), digested and sequenced from each end. The verified products were cloned into the backbone of pJC119 via the flanking BamHI sites (Figure 4). The orientation of the inserts in the final constructs were determined by restriction digestion and sequencing. Two Abelson murine leukemia virus (A-MuLV) transformed pre-B cell lines (58) were transfected with the pmlDJ+ microlocus recombination substrate. These included 15–63 (MSH2−/−) and 8–58 (MSH2+/−), the kind gift of Dr N. Rosenberg (Tufts University School of Medicine, Boston). Cells were cultured in RPMI supplemented with 10% FBS, penicillin and streptomycin. The transfection assay has been described previously (57,59). Briefly, 1 µg DNA was used to transfect 2 × 10⁶ cells by the DEAE-Dextran method. DNA recovered from transfections was treated with DpnI to digest non-replicated plasmid DNA. Southern analysis confirmed that recombination had indeed taken place on the microlocus. The DpnI digested transformation DNA was transformed into ElectroMax DH10B competent bacteria (Invitrogen) by electroporation with a GenePulsar (BioRad). Transformants were amplified for 16 h in an additional 4 ml Luria–Bertani containing 100 μg/ml ampicillin. Plasmids were recovered by alkaline lysis and digested sequentially with BamHI to release the insert from the vector. The resultant DNA fragments were fractionated by gel electrophoresis and analyzed on Southern blots with oligomer probes according to
the manufacturer’s suggestions (Hybond-N; Amersham). The micrococcal nuclease and its various rearrangement products differed sufficiently in size to identify all recombination generated by deletion with probe 3′J1CY, CCAGTCGACCCTGAGGAAACGGTGACC complementary to JH1. Bands were visualized PhosphorImager (Molecular Dynamics).

Isolation and sequencing of microhomology directed V(D)J joints

To isolate DFL16.1/JH1 recombinants from transfected cell lines, PCR was performed on 1 μl of DpnI-digested transfection DNA using the DFS primer described above and the JH1 specific primer 3′ J1CY which hybridizes to a sequence 40 bp downstream of JH1 located in the JH1.2 intronic region, which is included in the JH1 cassette of pmlDJ+. PCR conditions were described as above. PCR products were electrophoresed on a 2.5% agarose gel and the region of the gel in the 100–200 bp range (corresponding to the size of the recombiant DJ PCR product) was cut out and DNA purified using Qiagen gel extraction kit. A second round of PCR using same primers yielded a 150 bp product, which was cloned into the PCR2.1 TA vector and sequenced using the T7 primer (Macrogen).

To specifically isolate endogenous DH/JH1 joints from the bone marrow DNA, 1 μl of the DFS/JH4-C PCR described above was used as a template for a nested PCR using the DFS/JH1 primer pair. A 150 bp product was obtained which was cloned into the PCR2.1 TA vector and sequenced using the T7 primer.

Western blot analysis and antibodies

Whole cell extracts were prepared from 2 × 10^6 cells. Proteins were separated on 12% SDS–PAGE at 100V for 10 min followed by 200V for 30 min. Proteins were transferred to nitrocellulose membrane (Pall Gelman Laboratories) at 50V for ~4 h. Membrane was blocked in 3% BSA 1× TBS-T, washed in 1× TBS-T and probed with anti-MSH2 or anti-β-Actin antibodies for 1 h followed by secondary antibodies for 1 h. All antibodies were diluted in 1% BSA 1× TBS-T. For MSH2, mouse anti-MSH2 antibody (Zymed) was used at a 1:5000 dilution. Secondary antibody used was goat anti-mouse IgG-HRP (Jackson) at a 1:5000 dilution. For β-actin, rabbit anti-mouse β-actin antibody (Abcam) was used at a 1:10 000 dilution. Secondary antibody used was goat anti-rabbit IgG-HRP (Southern Biotech) at a 1:5000 dilution. Membranes were exposed to ECL for 1 min and image was obtained using VersaDoc.

RESULTS

Levels of DH to JH joining in the bone marrow of MSH2+/− and MSH2−/− mice

Aside from susceptibility to lymphoid tumors and microsatellite instability, MSH2−/− mice have been shown previously to have normal B and T cell development at the gross level (60). Although B cells in MSH2−/− mice can also undergo SHM and CSR, they differ in the pattern of SHM mutations and the sequence of CSR joints from their wild-type counterparts (44,45,61). Since the same DNA repair machinery (NHEJ) that is involved in CSR is also utilized in V(D)J recombination, we sought to examine whether the loss of MSH2 has any influence on the fine details of V(D)J joints.

To assess whether the frequency of V(D)J rearrangements were affected in MSH2−/− mice compared with littermate controls, we performed a semi-quantitative PCR analysis of DH-JH joints. The DJH junctions were examined since their frequency and sequence are not affected by cellular selection of pre-B cells harboring them. The schematic representation of the PCR assay is shown in Figure 1A. As described previously (56), the DFS primer hybridizes with the 5′ RSS of all 15 DH segments in the mouse and the JH4-C primer hybridizes with a sequence in the intron between JH4 and Cα. Thus, this primer pair amplifies all 60 possible DJH recombinations in mice. The amount of template DNA for the DJH PCR was normalized using a PCR for GAPDH, shown in Figure 1B (lower panel). Starting with ~10000 cell equivalents, bone marrow DNA from the MSH2+/− and MSH2−/− littersmates was serially diluted and used as template for PCR using the DFS/JH4-C primer sets. As shown in Figure 1B (upper panel), products of expected sizes were amplified from all four mice. Using PCR conditions and template dilutions in the linear range of PCR amplification, comparable total number of DJH joints between MSH2+/− and MSH2−/− littersmates were amplified (Figure 1B, upper panel). These results indicate that the frequency of V(D)J recombination initiation is comparable in MSH2+/− and MSH2−/− mice.

Sequences of the DJH joints in the bone marrow of MSH2+/− and MSH2−/− mice

Although the frequency of DJH joints was not affected in MSH2−/− mice, it was possible that the V(D)J joints differed in their sequence, as was the case in the NHEJ-repaired switch and other DSB junctions in MSH2−/− mice. To examine the sequence of the DJH joints, PCR-amplified products shown in Figure 1B were sequenced and the data is shown in Figure 2. We observed a similar profile of utilized DH and JH segments between MSH2+/− and MSH2−/− littersmates. All sequences contained deletions, N-additions and P-additions. Quantitative analysis of end processing is shown in Figure 3. A comparison of the distribution pattern as well as the location of the horizontal bars which represent the average number of nucleotides added or deleted for each mouse shows no appreciable differences between the MSH2+/− and MSH2−/− mice. That is, the lengths of deletions or additions as well as the number of joints that had undergone each type of modification were comparable. Of particular interest, the relative number of joints which exhibited P-nucleotides were also not significantly different among all littersmates, indicating that MSH2 does not play a role at the hairpin-opening stage of coding end processing, since it is the asymmetric opening of the hairpin coding end that leads to the addition of such palindromes (62). Based on these data, we conclude that the mismatch repair machinery does not influence the efficiency of the processing of V(D)J recombination in mouse bone marrow.

The influence of MSH2 on joining by homology during V(D)J recombination

MSH2 has been shown to influence the usage of terminal microhomologies in the formation of switch junctions during CSR (45,47) and in the repair of a transgenic substrate in CHO
cells (48). This suggests a role for MSH2 in the usage of short homologous sequences during NHEJ. Thus, we examined whether MSH2 serves a role in microhomology mediated joining during NHEJ repair of coding ends generated by V(D)J recombination. The most abundant and notable example of ‘joining by homology’ occurs between the two segments DFL16.1 and JH1, which end and begin respectively in the same 4 nt: CTAC (63). This phenomenon has been the subject of multiple studies (64,65) since DFL16.1 is the most commonly used DH segment in early mouse B cell development and since the DJH junction encodes for amino acids within the CDR3 region which is the most diverse region of the Ig molecule.

In order to examine the role of MSH2 in microhomology mediated joining in V(D)J recombination, three separate approaches were used. First, existing sequence data shown in Figure 2 was analyzed, because the majority of DH segments terminate in the di-nucleotide ‘AC’ and JH2 begins with this di-nucleotide sequence. As we observed no difference among the DJH2 joints between MSH2+/+ and MSH2−/− littermates, we set out to analyze DJH1 joints using two experimental approaches. We analyzed bone marrow derived DJH1 joints which include DFL16.1-JH1 among other DH segments joined to JH1. This analysis was done using a nested PCR approach, with the primary reaction identical to that shown in Figure 1 and the secondary reaction employing a JH1–JH2 intronic primer (J1CY). In the third approach, we utilized an extra-chromosomal recombination substrate shown in Figure 4, which contains only two gene segments, DFL16.1 and JH1, and has been shown to undergo recombination and

Figure 1. Semi-quantitative PCR analysis of V(D)J recombination. (A) Schematic representation of the PCR assay to amplify DH–JH joints from the mouse bone marrow. The DFS primer hybridizes to the 5′ RSS of all 15 DH segments in mice and the JH4-C primer hybridizes downstream of the JH4 segment. PCR products corresponding to each JH segment used have a different size as shown. (B) Upper panel shows an Ethidium-bromide stained agarose gel of semi-quantitative PCR using the DFS/JH4-C primers with serial 4-fold dilutions, starting with 10^6 total bone marrow cell equivalents. Lower panel shows PCR of GAPDH from the same template dilution corresponding to the lanes in the top panel.
end processing when transfected in A-MuLV pre-B cell lines expressing RAG1/2 and terminal dideoxynucleotidyl transferase (TDT) (57). The DJ construct was transfected into two cell lines differing in their MSH2 expression status. The 15–63 line is MSH2+/− and the 8–58 line is MSH2+/−. RAG and TDT expression in these cell lines was confirmed by RT–PCR analysis (data not shown). Western analysis showing the MSH2 expression status of these cell lines is shown in Figure 2. DJH joint sequences from MSH2+/+ and MSH2+/− littermates. DJH PCR products from the reaction shown in Figure 1B were cloned into the PCR2.1 vector and sequenced. (A) Upper panel shows sequences from littermate WT-1 (MSH2+/+) and lower panel shows sequences from littermate WT-2 (MSH2+/−). (B) Upper panel shows sequences from littermate MSH2−/− and lower panel shows sequences from littermate MSH2−/−. P-nucleotides are indicated in bold.
DJH1 sequences were obtained by PCR using the DFS and J1CY primer pairs. DJH1 sequences from mouse bone marrow are shown in Figure 5 and DFL16.1/JH1 sequences from the transfection assay are shown in Figure 6B. The frequency of microhomology mediated joining is shown in Table 1. The combined frequency of joining by homology among the DFL16.1-JH1 joints in MSH2⁺/⁺ and MSH2⁻/⁻ mice was 11 and 7.1%, respectively. The frequency of joining by homology events in the MSH2⁺/⁺ and MSH2⁻/⁻ cell lines was identical (24%). We conclude that while the lack of MSH2 reduces the usage of terminal microhomologies in NHEJ repair of CSR and other DSBs, it does not have a significant effect in the usage of microhomologies in NHEJ repair of V(D)J joints.

**DISCUSSION**

It has been known that ablation of MSH2 diminishes levels of CSR and influences usage of microhomology directed switch junctions (43–45,47). MSH2 has been shown to associate with transcribed S-regions in primary murine B cells activated for switch recombination and promoting synapsis between S-regions (66). The same report also raises the possibility that the ATPase-independent component of MSH2 may function in CSR, thereby providing an explanation for the differences between the MSH2⁻/⁻ and MSH2G674A phenotypes. In a non-Ig transgene in CHO cell lines lacking MSH2, DSBs were repaired with a lower frequency of microhomology usage as compared with their wild-type counterpart, though in this case the absolute number of repaired junctions was not affected (48). Thus, as general DSB repair and CSR repair both proceed through the NHEJ pathway, it appears that the mismatch repair machinery is clearly tied to this mode of DSB repair.

MSH2 has also been shown to be an important regulator of the DNA-damage-response signaling molecule ATM and the MRN complex (49,50), both of which have been demonstrated to be involved in the NHEJ repair of V(D)J junctions (4,28,54). These findings led us to hypothesize that MSH2 may play a direct or indirect role in the outcome of V(D)J joints. However, our results obtained from mouse bone marrow sequences indicated that the loss of MSH2 does not influence the frequency or the end processing of V(D)J recombination joints.

To analyze a specific phenotype in which MSH2 has been shown to play a role in CSR as well as repair of other DSBs...
in non-lymphocytes, we examined the frequency of 'joining by homology' in V(D)J recombination in the bone marrow of MSH2+/+ and MSH2−/− littersates, as well as MSH2+/+ and MSH2−/− A-MuLV pre-B cell lines. Several studies have shown that when the two gene segments to be recombined have a homology of at least 2 but up to 4 nt at the respective 3′ and 5′ ends, the two segments are often joined without further processing of the ends, with one copy of the homologous sequence remaining, which could be assigned to either segment (64, 67). We analyzed joining by homology among all JH2-containing joints, since the JH2 segment shares a di-nucleotide microhomology with most of the DH segments and all the JH1-containing joints, since the JH1 segment shares a 4 nt microhomology with most DH segments, including the often used DFL16.1. In addition to the analysis of joints in the mouse bone marrow, we analyzed DFL16.1-JH1 joints from an extra-chromosomal recombination substrate transfected into MSH2−/− and MSH2−/− cell lines. Several studies have shown that when the two gene segments to be recombined have a homology of at least 2 but up to 4 nt at the respective 3′ and 5′ ends, the two segments are often joined without further processing of the ends, with one copy of the homologous sequence remaining, which could be assigned to either segment (64, 67). We analyzed joining by homology among all JH2-containing joints, since the JH2 segment shares a di-nucleotide microhomology with most of the DH segments and all the JH1-containing joints, since the JH1 segment shares a 4 nt microhomology with most DH segments, including the often used DFL16.1. In addition to the analysis of joints in the mouse bone marrow, we analyzed DFL16.1-JH1 joints from an extra-chromosomal recombination substrate transfected into MSH2−/− and MSH2−/− cell lines. We reasoned that this approach would not only allow for the analysis of a large pool of joints between the two specific gene segments most implicated in joining by homology, it would also shed light on whether MSH2 plays a differential role in V(D)J recombination in mouse bone marrow as compared with V(D)J recombination in A-MuLV cell lines which represent an immortalized window of B cell development. Results from both the bone marrow and cell line generated DJH joints indicate that joining by homology occurs with equal frequency in the presence of absence of MSH2.

Microhomology mediated joining in V(D)J recombination may proceed through the same generic NHEJ pathway as all other V(D)J recombination events. Alternatively, it may proceed through a microhomology-mediated-end joining (MMEJ) pathway that has been shown to be independent of some of the components that are essential to NHEJ, while still requiring others such as the MRN complex (68–70). Whatever the case might be, mismatches generated in the joining of coding ends are either not recognized by MSH2 or are resolved via an alternative pathway.

While it is of very high likelihood that single or multiple base pair mismatches are encountered during the joining of the two gene segments in V(D)J recombination, we show here that they are not resolved via a MSH2-dependent mismatch repair pathway.

We conclude that while most factors involved in NHEJ (e.g. ATM, DNA-PK, MRN) are involved in all NHEJ processes including CSR and V(D)J recombination, DSBs formed by...
these two processes also utilize a set of unique factors that distinguishes them from each other. An interesting explanation for the differential role of MSH2 in the repair of CSR and V(D)J breaks may be that the coding ends formed in V(D)J recombination are sequestered and 'protected' by the RAG proteins as supported by experimental evidence (71–73). Coding ends in V(D)J recombination are also unique in their hairpin structure. Thus, it is equally possible that the mismatch repair machinery is excluded from their repair by factors involved in hairpin processing such as Artemis (74).

Based on the data presented here, we speculate that the lack of MSH2 involvement in the NHEJ repair of V(D)J recombination may lead to a lower degree of restriction in the resolving of V(D)J joints, thereby contributing to further diversity in the Ig repertoire. In contrast, the repair of other genomic DSBs including switch junctions may require a higher degree of fidelity. Further biochemical studies could shed light on whether this is indeed the case.

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Conflict of interest statement. None declared.

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