DNA polymerase $\zeta$ generates tandem mutations in immunoglobulin variable regions

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Low-fidelity DNA polymerases introduce nucleotide substitutions in immunoglobulin variable regions during somatic hypermutation. Although DNA polymerase (pol) $\eta$ is the major low-fidelity polymerase, other DNA polymerases may also contribute. Existing data are contradictory as to whether pol $\zeta$ is involved. We reasoned that the presence of pol $\eta$ may mask the contribution of pol $\zeta$, and therefore we generated mice deficient for pol $\eta$ and heterozygous for pol $\zeta$. The frequency and spectra of hypermutation was unaltered between $\text{Pol}\zeta^{-/-}$ and $\text{Pol}\eta^{-/-}$ and $\text{Pol}\zeta^{+/+}$ $\text{Pol}\eta^{-/-}$ clones. However, there was a decrease in tandem double-base substitutions in $\text{Pol}\zeta^{-/-}$ $\text{Pol}\eta^{-/-}$ cells compared with $\text{Pol}\zeta^{+/+}$ $\text{Pol}\eta^{-/-}$ cells, suggesting that pol $\zeta$ generates tandem mutations. Contiguous mutations are consistent with the biochemical property of pol $\zeta$ to extend a mismatch with a second mutation. The presence of this unique signature implies that pol $\zeta$ contributes to mutational synthesis in vivo. Additionally, data on tandem mutations from wild type, $\text{Pol}\zeta^{-/-}$, $\text{Pol}\zeta^{-/-}$, $\text{Ung}^{-/-}$, $\text{Msh}2^{-/-}$, $\text{Msh}6^{-/-}$, and $\text{Ung}^{-/-}$ $\text{Msh}2^{-/-}$ clones suggest that pol $\zeta$ may function in the MSH2–MSH6 pathway.

Somatic hypermutation (SHM) generates nucleotide substitutions in immunoglobulin variable (V) regions at a frequency of $10^{-2}$–$10^{-3}$ mutations per base pair (bp), which far exceeds the frequency of spontaneous mutation. SHM is initiated by activation-induced deaminase (AID), which is targeted to V regions by an unknown mechanism. AID transforms cytosine into mutagenic uracil in DNA (Maul et al., 2011), which can be recognized by DNA repair proteins uracil DNA glycosylase (UNG) to remove the uracil leaving an abasic site, or MSH2–MSH6 to generate a gap in the DNA (Maul and Gearhart, 2010). However, the sheer number of AID-generated uracils appears to overwhelm the error-free base excision and mismatch repair pathways (Saribasak et al., 2011), and the abasic sites and gaps then become substrates for low-fidelity DNA polymerases. Low-fidelity polymerases were originally described for their ability to replicate over DNA lesions, such as base adducts, cyclobutane pyrimidine dimers, and abasic sites. Although these lesions inhibit the replicative and repair polymerases, low-fidelity polymerases have the unique ability to insert nucleotides opposite lesions and to extend from mismatched termini. However, their inherent low fidelity causes them to be remarkably promiscuous when copying undamaged DNA, making them candidates for SHM. It is not fully understood how these polymerases are recruited to the immunoglobulin loci in place of their high-fidelity counterparts, although differential modifications of PCNA have been shown to coordinate some of the events (Langerak et al., 2007; Roa et al., 2008).
Many DNA polymerases have been examined for their role in SHM, with most of them belonging to the Y family of polymerases that exhibit lower fidelity than the other polymerases (Seki et al., 2005). Their involvement is demonstrated by the altered frequency and/or spectra of mutations in mice that are deficient for the polymerases. However, in most cases, the frequency is unchanged because of intense selection in vivo for B cells expressing antibodies with mutations giving high affinity for antigen. Instead, changes in the types of mutations have allowed identification of relevant polymerases based on their intrinsic signature of substitutions, as defined by their enzymatic properties. SHM in wild-type mice is characterized by equal mutations of G:C and A:T bp, with transitions outnumbering transversions. The predominant category of G:C to A:T transitions, which comprises 60% of all G:C mutations, is likely caused by replication past uracil by any of the DNA polymerases (Petersen-Mahrt et al., 2002). Rev1 is the major polymerase that produces G:C to C:G transversions, because its property as a deoxycytidyl transferase is to insert C opposite the abasic site that is produced by removal of uracil by UNG (Jansen et al., 2006). Polymerase (pol) η is the major polymerase that produces mutations of A:T bp, because Polη−/− cells have a 60% decrease in A:T mutations (Zeng et al., 2001). Pol θ can generate half of the residual A:T mutations in the absence of pol η (Faili et al., 2009). Pols λ (McDonald et al., 2003) and the A-family pol θ (Martomo et al., 2008) have also been examined, but there is no clear evidence of a change in spectra in their absence.

The B family DNA pol ζ is up-regulated in replicating cells, including germinal center B cells (Zeng et al., 2001), but its role in SHM has been difficult to analyze because mice deficient for the enzyme are not viable (Bemark et al., 2000; Esposito et al., 2000; Wittschieben et al., 2000). Several studies in cell lines and transgenic mice showed that reduction of pol ζ by knockdown or antisense techniques lowered the frequency of SHM but did not change the spectra (Diaz et al., 2001; Zan et al., 2001). Recently, Schenten et al. (2009) generated mice that conditionally knocked out Rev3, the catalytic subunit of pol ζ, in B cells. In these mice, half of the B cells were deficient for pol ζ, and they had a lower frequency of SHM but no change in the types of substitutions. The authors concluded that the lower frequency was simply caused by a decreased rate of cell division and reduced germinal center formation, suggesting that pol ζ did not play a direct role in SHM.

These previous studies indicate that either pol ζ is not used during SHM, or it has only a modest role that may be masked by other low-fidelity polymerases. Therefore, we examined mice deficient for the dominant pol η that contained one or two alleles encoding pol ζ, to see if pol ζ plays a more visible role in the absence of pol η. Because pol ζ is comprised of two subunits, the catalytic Rev3 protein and the noncatalytic Rev7 protein, we used mice with a targeted allele of Rev3 (Wittschieben et al., 2010) and crossed them with pol η-deficient mice.

RESULTS AND DISCUSSION
Combined deficiencies of pols ζ and η did not alter cell division, class switch recombination (CSR), and SHM
We have previously shown that Polη−/− mice had a normal frequency of germinal center B cells and CSR (Martomo et al., 2005). In this study, we tested if Polη−/− cells with one allele of pol ζ behaved differently than cells with two alleles. To confirm that pol ζ is down-regulated in the heterozygous cells, mRNA levels were measured by quantitative PCR from B cells. Primers were used that were located within the two deleted exons, 26 and 27, encoding Rev3 on the targeted allele to ensure (Diaz et al., 2001; Zan et al., 2001). Recently, Schenten et al. (2009) generated mice that conditionally knocked out Rev3, the catalytic subunit of pol ζ, in B cells. In these mice, half of the B cells were deficient for pol ζ, and they had a lower frequency of SHM but no change in the types of substitutions. The authors concluded that the lower frequency was simply caused by a decreased rate of cell division and reduced germinal center formation, suggesting that pol ζ did not play a direct role in SHM.

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that we did not detect any mRNA from potential alternatively spliced transcripts. Compared with Polξ+/− Polη−/− cells, Rev3 transcripts were significantly decreased (25–30%) in Polξ+/− Polη−/− cells that were either resting (Fig. 1A; P = 0.001) or stimulated in vivo with 4-hydroxy-3-nitrophenyl-acetyl conjugated to chicken γ globulin (NP35-CCG) for 14 d (Fig. 1B; P = 0.016). In contrast, there was no change in mRNA encoding Rev7, or in Rev1, a binding partner of pol ζ, showing that lower Rev3 levels did not affect these transcripts. There was also no significant change in the levels of pol η and κ, indicating that they did not overcompensate for the reduction in Rev3.

Because complete loss of pol ζ in conditional knockout mice caused slower B cell growth (Schenten et al., 2009), we examined Polξ+/− Polη−/− cells after stimulation with LPS and IL-4 for cell proliferation by CFSE. As shown in Fig. 1C, haploinsufficiency of pol ζ did not affect cell division after 3 d. Pol ζ is also recruited to sites of double-strand DNA breaks (Hirano and Sugimoto, 2006), which are key intermediates in CSR. To see if a deficiency of pol ζ cripples recombination, Polξ+/− Polη−/− and Polξ−/− Polη−/− naïve B cells were stimulated for 4 d to induce switching from IgM to different IgG isotypes. Compared with Polξ+/− Polη−/− cells, Polξ−/− Polη−/− cells had a slight decrease in recombination to IgG1, IgG2a, and IgG2b, although the numbers were not significantly different (Fig. 1D). Thus, in contrast to the conditional knockout mouse, which showed a 50% decrease in switching caused by defective nonhomologous end joining (Schenten et al., 2009), the residual pol ζ protein in the heterozygous cells was sufficient to permit CSR at normal levels.

To analyze the contribution of pol ζ to SHM, we immunized mice with NP35-CCG, and isolated germinal center cells 14 d later. SHM was analyzed in the JH4 intron downstream of rearranged VH-J558 genes, and Polξ−/− Polη−/− clones had a similar mutational frequency and mutations per clone compared with Polξ+/− Polη−/− clones (Fig. 2, A–C). Analysis of the types of mutations showed no difference between Polξ+/− Polη−/− and Polξ−/− Polη−/− clones (Fig. 2D).

Identification of a new signature for pol ζ in SHM: tandem mutations

The ascribed role of pol ζ is to extend mismatched primer termini (Lawrence and Maher, 2001) produced by other low-fidelity polymerases when copying past lesions, by adding the correct base (Johnson et al., 2000). However, recent evidence indicates that pol ζ, which has an error rate of ~10−3, can also extend mismatches produced by itself or other polymerases with a second mutation, generating tandem double-base mutations (Zhong et al., 2006; Sakamoto et al., 2007). The second mutation occurs at a rate of 10−2, indicating that pol ζ frequently inserts incorrect bases when confronted with mismatched termini. Therefore, we examined DNA sequences to see if we could find this signature of pol ζ (Fig. S1).

First, we looked at published wild-type sequences (Rada et al., 2002; Martomo et al., 2008; Schenten et al., 2009; Saribasak et al., 2011) to establish the location and spectra of tandem mutations. The aligned mutational data in 492 bp encoding the JH4 intron in Fig. 3 A shows the distribution of 1,811 single mutations and their location relative to the 6 WG CW (W = A/T) hotspots in the sequence. As seen in Fig. 3 B, the distribution of 50 pairs of tandem mutations resembled the location of single mutations, and only 18% of them occurred in WG CW motifs.

Second, to see if the distribution of tandem pairs was different in the absence of the dominant pol η, we examined sequences from Polξ+/− Polη−/− clones. Data were combined from approximately two-thirds immunized spleen cells (Fig. 2 A) and one-third Peyer’s patch cells generated in this study, because there was no difference in SHM between the two populations. The location of 33 pairs of tandem substitutions and 1,060 single mutations were similar, and just 21% tandems occurred in WG CW hotspots (Fig. 3, C and D). Furthermore, only 40% tandems were located in 27 of the less stringent DGYW motifs (D = A/G/T; Y = C/T; Rogozin and Diaz, 2004). Thus, the majority of tandem pairs in wild-type and
pol η–deficient mice were found outside of the hotspots, suggesting that they did not arise from multiple events targeting only these motifs.

Third, we analyzed sequences from pol η–deficient mice with one or two alleles of pol ζ to quantify the contribution of pol ζ to the tandem signature. Adjacent mutations could be produced by polymerases in a single event or sequentially after cell division. Using a calculation based on frequency and random distribution of mutations (Winter et al., 1998), the expected number of tandems increases as the number of mutations per clone increases, indicating that they occur in multiple events (Michael et al., 2002). Table 1 lists the observed and expected number of adjacent mutations in clones from spleen and Peyer’s patch cells. Notably, the majority of tandem mutations occurred in clones which had very few mutations (Table 1), further supporting the hypothesis that tandems occur in clones which had very few mutations. The total observed and expected number of adjacent mutations in clones from spleen and Peyer’s patch cells.

To further confirm that pol ζ generated the tandem signature, sequences from Polζ+/– and Polζ−/− single cells from conditional knockout mice were analyzed (Schenten et al., 2009). Although there were fewer mutations in the single-cell analyses, the formula used to calculate the expected number of tandems takes into account the overall frequency of mutations per clone. The observed number of tandems was significantly higher than the expected number in Polζ+/– cells (P = 0.03), but not in Polζ−/− cells (P = 0.35). The Polζ−/− cells had a ratio of observed to expected tandems of 2.1, with Polζ+/– Polη−/− clones at 2.5 (Fig. 4 A).

The increase in contiguous mutations from clones with two copies of pol ζ compared with clones with one copy suggests that pol ζ is important for introducing tandem mutations into the Ig loci. Furthermore, the ratio in wild-type mice expressing the low-fidelity polymerases was lower at 2.2 compared with pol η–deficient clones. Because both pol η (Matsuda et al., 2001) and pol ζ have the capability to extend mismatches in vitro, and therefore potentially introduce contiguous mutations, the observation that the ratio in Polζ+/– Polη−/− clones was higher than in Polζ−/– Polη−/− clones suggests that most of these tandems are not introduced by pol η. Furthermore, the spectra of tandem mutations were similar to the single mutations (Fig. 3), which suggest that pol ζ can extend mismatched termini produced by other polymerases. In wild-type mice, pol ζ could extend mismatched termini produced by pols η (A/T bias), θ (A/T bias), Rev1 (C bias), and itself (no bias). In pol η–deficient mice, the overall single mutation spectra and the tandem spectra are decreased for A/T mutations because there are no pol η–generated mismatches. Thus, the first mutation that the signature of whatever polymerase is being used, and the second mutation produced by pol ζ will be random, because pol ζ does not have a strong substitution bias (Zhong et al., 2006).

To further confirm that pol ζ generated the tandem signature, sequences from Polζ+/+ and Polζ−/− single cells from conditional knockout mice were analyzed (Schenten et al., 2009). Although there were fewer mutations in the single-cell analyses, the formula used to calculate the expected number of tandems takes into account the overall frequency of mutations per clone. The observed number of tandems was significantly higher than the expected number in Polζ+/+ cells (P = 0.03), but not in Polζ−/− cells (P = 0.35). The Polζ−/− cells had a ratio of observed to expected tandems of 2.1, with Polζ+/– Polη−/− clones at 2.5 (Fig. 4 A).
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whereas Pol$\zeta^{-/-}$ cells had a lower ratio of 1.4. Therefore, in the complete absence of pol $\zeta$, the observed number of tandems was similar to the expected number, indicating that they arose by two separate events. This data, summarized in Fig. 4 B, supports the hypothesis that pol $\zeta$ generates tandem mutations during SHM by extending mismatches produced by other polymerases. Slippage of polymerases on DNA templates also has the potential to produce multiple substitutions (Wilson et al., 1998), but the frequency of insertions and deletions in V genes is low, suggesting that this is not a major mechanism.

Mismatch repair promotes pol $\zeta$ access to DNA during SHM

There are two pathways that could allow synthesis by pol $\zeta$ during SHM. In the UNG pathway, the polymerase could synthesize across abasic sites introduced by UNG removal of uracils. In the MSH2–MSH6 pathway, pol $\zeta$ could synthesize in the gap produced by exonuclease 1. To determine which pathway is used by pol $\zeta$, we analyzed previously published sequences of the J$\lambda$4 intron from Ung$^{-/-}$ (Rada et al., 2002), Msh2$^{-/-}$ (Frey et al., 1998), Msh6$^{-/-}$ (with new sequences added for this study; Martomo et al., 2004), and Ung$^{-/-}$ Msh2$^{-/-}$ clones (Rada et al., 2004). The observed number of tandems was significantly higher than the expected number in Ung$^{-/-}$ clones (P < 10$^{-3}$), and the ratio of observed to expected tandems was 2.2, which was identical to the ratio seen in wild-type clones (Fig. 4, A and B). This indicates that pol $\zeta$ is not introducing tandem substitutions in the UNG pathway. However, disruption of the mismatch repair proteins MSH2 and MSH6 decreased the observed number of tandem pairs, so there was no difference between observed and expected values (P = 0.19). Mice deficient for both UNG and MSH2 also had no significant difference between observed and expected values (P = 0.12). Thus, in the absence of MSH2 and MSH6, the ratio of observed to expected tandems was lower at 1.4, and in the combined absence of MSH2 and UNG, the

Table 1. Observed and expected tandem mutations in pol $\eta$-deficient cells from spleen and Peyer’s patches

| Mutations per clone | No. of clones | No. of mutations | Tandems observed | Tandems expected$^a$ | No. of clones | No. of mutations | Tandems observed | Tandems expected$^a$ |
|--------------------|---------------|------------------|------------------|----------------------|---------------|------------------|------------------|----------------------|
| 1                  | 157           | 157              | 0                | 0.00                 | 140           | 140              | 0                | 0.00                 |
| 2                  | 76            | 152              | 3                | 0.31                 | 77            | 154              | 1                | 0.30                 |
| 3                  | 34            | 102              | 5                | 0.41                 | 33            | 99               | 0                | 0.40                 |
| 4                  | 19            | 76               | 3                | 0.46                 | 13            | 52               | 4                | 0.32                 |
| 5                  | 19            | 95               | 5                | 0.77                 | 11            | 55               | 0                | 0.45                 |
| 6                  | 9             | 54               | 3                | 0.55                 | 4             | 24               | 0                | 0.24                 |
| 7                  | 9             | 63               | 1                | 0.77                 | 4             | 28               | 2                | 0.34                 |
| 8                  | 11            | 88               | 4                | 1.25                 | 4             | 32               | 1                | 0.46                 |
| 9                  | 5             | 45               | 0                | 0.73                 | 1             | 9                | 0                | 0.15                 |
| 10                 | 3             | 30               | 1                | 0.55                 | 3             | 30               | 1                | 0.55                 |
| 11                 | 3             | 33               | 1                | 0.67                 | 0             | 0                | 0                | 0.00                 |
| 12                 | 2             | 24               | 0                | 0.54                 | 0             | 0                | 0                | 0.00                 |
| 13                 | 3             | 39               | 3                | 0.95                 | 0             | 0                | 0                | 0.00                 |
| 14                 | 3             | 42               | 0                | 1.11                 | 1             | 14               | 0                | 0.37                 |
| 15                 | 4             | 60               | 4                | 1.71                 | 0             | 0                | 0                | 0.00                 |
| Total:             | 357           | 1,060            | 33               | 10.78                | 291           | 637              | 9                | 3.58                 |

$^a$Expected tandems were calculated by the formula n(n − 1)/k in a sequence of k nucleotides long containing n mutations (Winter et al., 1998).

Figure 4. More tandem mutations are introduced by pol $\zeta$ in the absence of pol $\eta$. (A) Tandem mutations in different strains. All data are from the J$\lambda$4 intron, except Pol$\zeta^{-/-}$ and Pol$\zeta^{-/-}$ are from the J$\lambda$1–4 introns. P values are based on Poisson calculations comparing the statistical difference between total observed and expected tandem mutations. (B) Tandem ratios in different strains. Dotted line indicates a ratio of 1 where observed and expected numbers are the same. *, Observed tandems compared with expected tandems were significantly different (P ≤ 0.03).
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ratio was 1.2. These results indicate that tandem mutations are produced more frequently in the MSH2–MSH6 pathway. The notion that pol ζ might be used during mismatch repair is consistent with reports from yeast, where pol ζ synthesis is reduced in mismatch repair-deficient strains (Lehner and Jinks-Robertson, 2009). One potential way pol ζ could function during gap synthesis in SHM would be if another polymerase stalls after replicating over AID-induced uracils on the template strand to produce a A:U mismatch. Pol ζ could then be recruited to extend the mispair and generate a second mispair. Additionally, pol ζ could extend mismatched termini produced by other error-prone polymerases, i.e., pol η, κ, or Rev1, and generate a second mispair. Because pol ζ has no preferential mutational signature, both mechanisms will produce a spectra of tandems that matches the overall single mutation spectra as shown in Fig. 3.

A potential advantage of using pol ζ to introduce contiguous mutations could be to more efficiently change amino acids in V genes to produce higher affinity. Codons are degenerate in the third base, and if two adjacent bases are changed simultaneously, the chance for an amino acid substitution is greater than with a single substitution. Interestingly, tandem mutations are found at extraordinary levels in nurse shark Ig genes (Zhu and Hsu, 2010), suggesting that nurse sharks may use pol ζ more frequently than mice. Thus, our data support the participation of pol ζ in SHM, and suggest that it has an increased role in the absence of the dominant pol η protein.

MATERIALS AND METHODS

Mice. Pol ζ+/− mice on a mixed C57BL/6 × 129 background were obtained from J. Wittschieben and R. Wood (University of Texas, MD Anderson Cancer Center, Smithville, TX; Wittschieben et al., 2010) and bred to Pol ζ−/− mice on a C57BL/6 background (Martomo et al., 2008). We have previously reported that SHM is identical in both the C57BL/6 and 129 strains of mice (McDonald et al., 2003). Genotypes were confirmed by PCR of tail DNA using Rev3L+ and Rev3L− primer sets (Wittschieben et al., 2010) and Pol ζ primer sets (Martomo et al., 2008). Pol ζ+/− Pol ζ−/− mice were then bred to each other, which generated Pol ζ+/+ mice and Pol ζ+/− Pol ζ−/− mice. Littermate mice were used at 3–8 mo of age. All animal procedures were reviewed and approved by the Animal Care and Use Committee of the National Institutes of Health, National Institute on Aging. Mice were immunized with intraperitoneal injection of 100 µg NPα-CGG (Biosearch Technologies) in adjuvant complex (Sigma-Aldrich). On day 14, splenic B cells were stained with FITC anti-B220 and Alexa Fluor anti-GL7. In nonimmunized mice, Peyer’s patch B cells were also isolated and stained. B220+ GL7+ cells were isolated by flow cytometry, and DNA was prepared. The 492-bp intronic region downstream of JH4 from rearranged VjH558 genes was amplified by nested PCR. The first round used forward primer J558 5′-AGGTCATCA-3′ and reverse primer J92906 5′-GTGTTCTTCTTGAAGCTGGAC-3′, and the second round used forward primer J558Eco 5′-CAGGAATTCCTGACATCT-3′ and reverse primer JH2827 Bam 5′-CCGCGATCCATCTTCCTGACTC-3′. The amplified DNA was then digested with EcoRI and BamHI restriction enzymes, cloned into pBS-SK vector, and sequenced. Only clones with unique VDJ joins were scored.

Online supplemental material. Fig. S1 lists 101 sequences with tandem mutations in the JH4 intron and shows where they occur. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20112234/DC1.

We thank Richard Wood and John Wittschieben for Pol ζ−/−; Klaus Rajewsky for sequence data; R. Wersto, C. Morris, J. Scheers, C. Nguyen, and T. Wolf for flow cytometry analyses; and Ranjan Sen and Kimberly Zanotti for valuable comments. This research was supported entirely by the Intramural Research Program of the National Institutes of Health, National Institute on Aging. The authors declare no competing financial interests.

Submitted: 21 October 2012
Accepted: 3 May 2012

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