DNA polymerases \( \nu \) and \( \theta \) are required for efficient immunoglobulin V gene diversification in chicken

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The chicken DT40 B lymphocyte line diversifies its immunoglobulin (Ig) V genes through translesion DNA synthesis–dependent point mutations (Ig hypermutation) and homologous recombination (HR)–dependent Ig gene conversion. The error-prone biochemical characteristic of the A family DNA polymerases Pol\( \nu \) and Pol\( \theta \) led us to explore the role of these polymerases in Ig gene diversification in DT40 cells. Disruption of both polymerases causes a significant decrease in Ig gene conversion events, although Pol\( \nu \)-/Pol\( \theta \)-/ cells exhibit no prominent defect in HR-mediated DNA repair, as indicated by no increase in sensitivity to camptothecin. Pol\( \nu \) has also been previously implicated in Ig gene conversion. We show that a PolH-/-/PolN-/-/PolQ-/- triple mutant displays no Ig gene conversion and reduced Ig hypermutation. Together, these data define a role for Pol\( \nu \) and Pol\( \theta \) in recombination and suggest that the DNA synthesis associated with Ig gene conversion is accounted for by three specialized DNA polymerases.

Introduction

The chicken DT40 B lymphocyte cell line provides a unique opportunity to analyze the role of individual DNA polymerases in homologous recombination (HR) and translesion DNA synthesis (TLS) because DT40 cells diversify Ig V genes through HR (Ig gene conversion) and nontemplated single-nucleotide substitutions (Ig hypermutation) during in vitro culture (Buerstedde et al., 1990; Sale et al., 2001). Ig gene conversion introduces tracts of templated mutations derived from an array of pseudo-V\( \lambda \) (pseudo-V\( \lambda \)) regions, located upstream of rearranged V\( \lambda \), to the V\( \lambda \) segment of the rearranged VJ\( \lambda \) (Reynaud et al., 1987). Because donor and recipient segments have an \( \sim 10\% \) sequence divergence, sequential Ig gene conversion events are able to substantially diversify the Ig V gene. However, Ig hypermutation is performed by TLS past abasic sites in DT40 cells (Simpson and Sale, 2003; Arakawa et al., 2006; Saberi et al., 2008).

Activation-induced deaminase (AID) is responsible for triggering Ig hypermutation and Ig gene conversion (Fig. 1; Arakawa et al., 2002; Harris et al., 2002). AID catalyses deoxycytidine to generate uracil followed by elimination of uracil by uracil glycosylase to induce abasic sites (Di Noia and Neuberger, 2002; Petersen-Mahrt et al., 2002). Replication blockages at abasic sites generated at the V(D)J segment and subsequent release from the blockage by HR and TLS may cause Ig gene conversion and Ig hypermutation, respectively, in DT40 cells (Fig. 1; Simpson and Sale, 2003; Saberi et al., 2008; Nakahara et al., 2009). In Ig gene conversion, replication blockage may induce template switch from the V(D)J segment to pseudo-V segments. Subsequent DNA synthesis using pseudo-V segments as a template may lead to gene conversion from the pseudo-V segments to the V(D)J segment (Buerstedde and Arakawa, 2006). Collectively, determination
although other DNA polymerases may also contribute to HR. Another unresolved question concerns the nature of the DNA polymerases that are involved in HR-dependent release of replication blockage. Computational analysis of the human genome revealed genes encoding two A-type DNA polymerases, POLN (Marini et al., 2003) and POLQ (Seki et al., 2003, 2004), in addition to the POLG gene, a unique DNA polymerase found in mitochondria. POLQ, but not POLN, contains a helicase domain near its N terminus, as does MUS308, the prototype orthologue of POLN/POLQ in Drosophila melanogaster (Boyd et al., 1990). Subsequent biochemical studies have shown that Polϕ and Polξ, which lack intrinsic exonucleolytic proofreading activity, can indeed perform TLS past abasic sites, undergo DNA synthesis with very low fidelity, and extend from mismatches (Seki et al., 2004; Takata et al., 2006; Arana et al., 2007, 2008; Seki and Wood, 2008). Genetic studies have addressed the function of POLN and POLQ using D. melanogaster, mice, and chicken DT40 cells (Boyd et al., 1990; Shima et al., 2004; Yoshimura et al., 2006). D. melanogaster deficient in MUS308 are hypersensitive to chemical cross-linkers, indicating the critical role played by the A-type polymerases in DNA synthesis of Ig V nucleotide sequences in DT40 cells provides a unique opportunity to identify both the gene conversion tracts and the spectrum of TLS-dependent mutations. This allows identification of the DNA polymerases involved in these Ig V diversification reactions.

HR is a multistep process that repairs double-strand breaks (DSBs) and releases replication blockage using intact homologous sequences as a template (Pâques and Haber, 1999; Wyman and Kanaar, 2006; Takeda et al., 2007). DSBs are processed during the early steps of HR, leading to the formation of 3′ single-strand overhangs, which associate with polymerized Rad51. The resulting complex, including the 3′ overhangs and Rad51, invades intact homologous duplex DNA to form a D loop structure. DNA synthesis from the invading 3′ overhang, followed by the recapture of the newly synthesized DNA strand by the other end of the DSB, completes DSB repair. This type of HR is called synthesis-dependent strand annealing and does not cause the generation of crossover DNA. Because the D loop is unstable, efficient DNA synthesis may significantly increase the rate of gene conversion (Pâques et al., 1998). DNA synthesis can be performed by DNA polymerases η and ζ (Polη and Polζ; Sonoda et al., 2003; Kawamoto et al., 2005; McIlwraith et al., 2005), although other DNA polymerases may also contribute to HR. Another unresolved question concerns the nature of the DNA polymerases that are involved in HR-dependent release of replication blockage.

Figure 1. Molecular mechanisms for Ig gene diversification, yielding substitutions at C/G pairs and Ig gene conversion in DT40 cells. AID-mediated deamination of C generates a U/G mispair. Uracil DNA glycosylase (UNG) can excise the U residue to generate an abasic site (AP), which causes replication blockage. (bottom right) Release of replication blockage by TLS causes mutations at C/G pairs, depending on which nucleotide is inserted opposite the abasic site. (bottom left) Alternatively, HR-dependent template switch from V(D)J segment to pseudo-V genes induces Ig gene conversion. Colored boxes indicate pseudo-V genes. DNA polymerases with a previously identified role in Ig gene diversification are shown in black (Kawamoto et al., 2005; Okada et al., 2005; Ross and Sale, 2006; Saberi et al., 2008), and those identified in this study are shown in red. This figure is based on previously described results (Sale, 2004; Buerstedde and Arakawa, 2006).
Remarkably, no Ig gene conversion events were detectable in POLQ−/−/POLQ−/− DT40 cells and POLQ−/− mice shows that Polv and Polθ do not play the same critical role as Mus308 in cellular responses to chemical cross-linkers, and Polθ makes significant contributions to the DNA repair of base damage and probably to TLS (Shima et al., 2004; Yoshimura et al., 2006).

We examined Ig V diversification in POLN−/−, POLQ−/−, and POLN−/−/POLQ−/− DT40 clones. Compared with wild-type cells, POLN−/−/POLQ−/− DT40 cells exhibited a significant reduction in the rate of Ig gene conversion, which was associated with increased length of gene conversion but not Ig hypermutation rate. This observation is in marked contrast to the absence of prominent defects in POLN−/−/POLQ−/− cells in general HR, including gene targeting and camptothenicin sensitivity. Moreover, POLN−/−/POLQ−/− clones showed significant reduction in the number of C to G mutations, indicating that Polv and Polθ play a role in TLS past the abasic site. Because Polv is involved in Ig hypermutation and Ig gene conversion (Kawamoto et al., 2005), we disrupted the POLH gene in the POLN−/−/POLQ−/− background. Ig hypermutation rate was significantly decreased in the resulting POLH−/−/POLN−/−/POLQ−/− cells. Remarkably, no Ig gene conversion events were detectable in POLH−/−/POLN−/−/POLQ−/− cells, indicating that the three DNA polymerases were responsible for Ig gene diversification (Fig. 1). Only POLH−/−/POLN−/−/POLQ−/− cells showed increased sensitivity to camptothenicin, a topoisomerase I inhibitor. In conclusion, Polv and Polθ may have adapted to perform a specialized function for Ig V diversification and can contribute to HR-dependent repair only when other HR-related DNA polymerases are absent.

**Results**

**Cells deficient in POLN and POLQ exhibit a decrease in the rate of Ig gene conversion**

To investigate the role of Polv and Polθ in Ig gene diversification, we induced Ig gene conversion and Ig hypermutation by ectopically expressing AID in POLN−/−, POLQ−/−, POLN−/−/POLQ−/−, and wild-type DT40 cells through retrovirus infection (Shinkura et al., 2004; Saberi et al., 2008). We determined the VJ, nucleotide sequences of more than three cloned expanded populations from each genotype. The overexpression of the AID transgene increased the rate of Ig gene conversion in wild-type cells by 17-fold to 9.8 × 10−3 per nucleotide per division (Fig. 2, A and B; and Fig. 3 C). POLH−/− cells showed a decrease in Ig gene conversion rate as we have previously shown (Kawamoto et al., 2005). Wild-type, POLN−/−, and POLQ−/− clones exhibited indistinguishable rates of Ig gene conversion, whereas the rate of Ig gene conversion in POLN−/−/POLQ−/− cells is 4.9 times lower than in wild-type cells (P < 0.0002; Fig. 2 C and Fig. 3 C). This finding reveals an unexpected function of Polv and Polθ in HR (Fig. 1). Moreover, the synergistic effect of POLN and POLQ mutations on the Ig gene conversion rate indicates that Polv and Polθ may complement each other in HR-dependent Ig V diversification.

We next evaluated the contribution of Polv and Polθ to Ig gene conversion without overexpressing AID. We generated POLN−/−, POLQ−/−, and POLN−/−/POLQ−/− cells from surface IgM (slgM)–negative wild-type cells carrying a +G frameshift mutation in the rearranged VJ3 segment at the same site as in the CL18 clone (Fig. 4 A and Fig. 5 B; Buerstedde et al., 1990). We monitored the gain of slgM expression resulting from the Ig gene...
conversion–mediated elimination of the frameshift mutation. We measured sIgM gain fluctuation using subclones derived from a single sIgM-negative cell after 3 wk of clonal expansion. Because sIgM gain was hardly detectable in the POLN−/− or POLQ−/− cells (Fig. 4 B), we augmented the rate of Ig gene conversion by treating cells with trichostatin A (TSA), a histone deacetylase inhibitor. TSA increases Ig gene conversion 50-fold without inducing Ig hypermutation, probably by relaxing the local chromatin structure specifically at the ψV segments and thereby promoting the interaction between the ψV donor segments and V(D)J segments (Seo et al., 2005; Nakahara et al., 2009). When compared with wild-type cells, the POLN−/− and POLQ−/− cells exhibited a significant decrease in the appearance of sIgM-positive revertant fractions (Fig. 4 C). It should be noted that such a decrease was not observed in AID-overexpressing cells (Fig. 3 C). This apparent discrepancy might be explained by the idea that the rate-limiting step of Ig gene conversion may be the interaction between the ψV segments and V(D)J segments in AID-overexpressing cells, whereas a subsequent DNA synthesis step may be rate limiting in TSA-treated cells. Thus, a compromised DNA synthesis step in POLN−/− and POLQ−/− cells may cause significant decreases in the rate of gene conversion only in TSA-treated cells but not in AID-overexpressing cells. In addition to significantly reduced Ig gene conversion in POLN−/− and POLQ−/− cells, remarkably, the reversion fraction was nearly undetectable in the POLN−/−/POLQ−/− cells (Fig. 4 C).

To confirm the role of Polv in Ig gene conversion, we reconstituted POLN−/− cells with the human POLN transgene (Takata et al., 2006). The POLN transgene rescued the rate of Ig gene conversion to a level comparable with that of wild-type cells (Fig. 4 D), confirming an important role for Polv in Ig gene conversion. When taken together, nucleotide sequence analysis of Ig Vδ in AID-overexpressing cells (Fig. 3) and measurement of sIgM gain (Fig. 4) indicate the involvement of both Polv and Polθ in Ig gene conversion (Fig. 1).

Increased length of gene conversion tracts in POLN−/−/POLQ−/− cells
To assess the nature of Ig gene conversion events, we examined the type of Ig gene conversion that causes sIgM gain. We sorted sIgM-positive cells from three clonally expanded populations from each genotype. To measure the length of the gene conversion tracts, we analyzed those tracts where the +G frameshift mutation of the rearranged VJδ segment (Buerstedde et al., 1990) is replaced by the ψVδ segment (Fig. 5 A). To achieve this, we subcloned each genotype, cultured individual subclones in the presence of TSA, sorted sIgM-positive revertants at 3 wk, and determined the nucleotide sequence in the VJδ segment. The sequences around the parental frameshift mutation were replaced by ψVδ in most of the VJδ segments analyzed, presumably because the VJδ segment around the frameshift mutation has the highest sequence similarity (92.4%) to the ψVδ segment (Buerstedde et al., 1990).

The length of the gene conversion tract involving ψVδ was estimated as reported previously for the analysis of POLH−/− cells (Kawamoto et al., 2005). Conducting a sequence comparison between (a) a pseudo-Vδ (donor), (b) the VJδ segment containing the frameshift mutation (recipient), and (c) their converted sequences (Fig. 5 B) enabled us to determine which sequences in the recipient were replaced by which donor in each product. For example, sequence analysis of tracts in wild-type cells indicated that the 5′-TACGGCT-3′ sequences in the recipient were replaced by ψVδ-derived 5′-TATGCT-3′ sequences in all analyzed products (Fig. 5 B; note that the bolded G causes the frameshift and corresponds to the black triangle in Fig. 5 C). Thus, the CG of the recipient is replaced by the T of the donor through Ig gene conversion, which restores the reading frame of the Ig Vδ gene. These converted sequences were flanked by identical sequences that were shared by all donor, recipient, and converted sequences (Fig. 5 B, blue). Outside this region of ~80 nucleotides, there are occasional mismatches,
of sIgM gain variants from the following number of sIgM-negative subclones: wild type, 35; POLQ\textsuperscript{−/−}, 22; POLN\textsuperscript{−/−}, 13; and POLN\textsuperscript{−/−}/POLQ\textsuperscript{−/−}, 34. Wild-type data were described previously (Kawamoto et al., 2005; Nakahara et al., 2009). The horizontal bars in the panel indicate median percents. (C) The frequency of sIgM gain revertants of the indicated genotype was determined at 3 wk after addition of 1.25 ng/ml TSA. We determined the percentage of sIgM gain variants from the following number of sIgM-negative subclones: wild type, 14; POLQ\textsuperscript{−/−}, 9; POLN\textsuperscript{−/−}, 10; and POLN\textsuperscript{−/−}/POLQ\textsuperscript{−/−}, 6.

We were able to define a minimum tract length (distance between the furthest mismatched nucleotides in the Ig gene conversion product and recipient) and a maximum tract length (distance between the closest mismatched nucleotides in the Ig gene conversion product and donor; Fig. 5 C). We confirmed that the starting sequences of the frameshift in wild-type and each mutant were identical (Fig. S1). We next calculated the maximum length of the gene conversion tract for each genotype. 25% of the analyzed gene conversion tracts contained the donor sequences at the two mismatches, the 48th and 182nd nucleotides in the wild-type clones. This result defines the maximum length of the conversion tract in wild-type cells (134 nucleotides). Likewise, 3% of the gene conversion tracts exhibited a maximum length of 125 nucleotides in the POLN\textsuperscript{−/−} cells. Remarkably, the POLN\textsuperscript{−/−}/POLQ\textsuperscript{−/−} clones exhibited a dramatic increase in the length of the gene conversion tract compared with cells carrying the other genotypes, with 88% of the gene conversion tracts exhibiting a maximum length of 256 nucleotides. Tract length in POLN\textsuperscript{−/−}/POLQ\textsuperscript{−/−} clones is statistically longer than that of POLQ\textsuperscript{−/−} clones, and POLN\textsuperscript{−/−}/POLQ\textsuperscript{−/−} clones have the longest tract (242 vs. 116; P < 0.01). Moreover, all of the gene conversion sites in the donor and recipient DNA. The sequence shared by both the donor \(\Psi V\) and the recipient gene is indicated by the shaded boxes. Crossover sites must be within these boxes. The dotted vertical lines indicate the position of mismatches between donor \(\Psi V\) and recipient \(\Psi V\). [These positions are 48, 97, 108, 143, 172, 182, 222, 237, and 304 bases from the 76th base of the intron between the leader and the V\_J segment. Black triangles indicate the position of the frameshift (corresponding to the bolded G in B) of the recipient V\_J segment. The number of analyzed \(\Psi V\) segments rearranged with the \(\Psi V\) donor in wild-type, POLQ\textsuperscript{−/−}, POLN\textsuperscript{−/−}, and POLN\textsuperscript{−/−}/POLQ\textsuperscript{−/−} cells was 20, 39, 30, and 17, respectively.]
tracts exhibited a longer minimum length in three subclones derived from the POLN<sup>−/−</sup>/POLQ<sup>−/−</sup> cells than in the wild-type cells. These results suggest that other unidentified polymerases may carry out DNA synthesis with higher processivity in the absence of Polλ and Polθ and thereby significantly increase the length of the gene conversion tracts in POLN<sup>−/−</sup>/POLQ<sup>−/−</sup> cells (Fig. 5 C).

**No detectable Ig gene conversion events in POLH<sup>−/−</sup>/POLN<sup>−/−</sup>/POLQ<sup>−/−</sup> cells**

Because both POLN<sup>−/−</sup>/POLQ<sup>−/−</sup> and POLH<sup>−/−</sup> DT40 clones exhibited a significant decrease in the rate of Ig gene conversion, we wanted to explore the functional redundancy between the two A-type DNA polymerases and Polη. To this end, we disrupted the POLH gene in two independently isolated POLN<sup>−/−</sup>/POLQ<sup>−/−</sup> clones. The resulting three POLH<sup>−/−</sup>/POLN<sup>−/−</sup>/POLQ<sup>−/−</sup> clones consistently showed a marked retardation of cellular proliferation (Fig. 6 A) accompanied by increased levels of spontaneous chromosomal aberrations (Fig. 6 B). Thus, the three DNA polymerases functionally overlap in terms of the maintenance of chromosomal DNA. We next measured Ig gene conversion in POLH<sup>−/−</sup>/POLN<sup>−/−</sup>/POLQ<sup>−/−</sup> clones. Remarkably, we were not able to detect any Ig V gene conversion events in POLH<sup>−/−</sup>/POLN<sup>−/−</sup>/POLQ<sup>−/−</sup> clones (Fig. 2 C and Fig. 3 C). Moreover, ambiguous A/T mutations, which are products of HR (Saber et al., 2008), were abolished in the POLH<sup>−/−</sup>/POLN<sup>−/−</sup>/POLQ<sup>−/−</sup> cells (Fig. 3 B). These observations demonstrate that these three error-prone DNA polymerases are sufficient for all Ig gene conversion events in DT40 cells.

**HR-dependent repair of POLN<sup>−/−</sup>/POLQ<sup>−/−</sup> and POLH<sup>−/−</sup>/POLN<sup>−/−</sup>/POLQ<sup>−/−</sup> cells**

Although HR plays a key role in cellular tolerance to cisplatin (Nojima et al., 2005), POLN<sup>−/−</sup>/POLQ<sup>−/−</sup> cells exhibit a normal tolerance to chemical cross-linking agents (Yoshimura et al., 2006). The apparent discrepancy between this observation and the important role played by Polν and Polθ in Ig gene conversion led us to reevaluate the efficiency of HR reactions in cells deficient in POLN and POLQ. To this end, we measured the frequency of gene targeting (Table I) and cellular tolerance to cisplatin and camptothecin, a topoisomerase I poison (Fig. 6, C and D), and the HR-dependent repair of ISceI restriction enzyme–induced DSBs (Fig. 7).

Camptothecin stabilizes a complex of topoisomerase I covalently linked to nicked DNA. This complex interrupts replication and causes DSBs in one of the sister chromatids. These DSBs are repaired exclusively by HR using the intact sister chromatid (Hochegeger et al., 2006; Pommier, 2006; Saberi et al., 2007). We found that neither POLN<sup>−/−</sup>, POLQ<sup>−/−</sup>, nor POLN<sup>−/−</sup>/POLQ<sup>−/−</sup> clones exhibited an increased sensitivity to camptothecin (Fig. 6 C).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>OVALBMN locus</th>
<th>POLK locus</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>18/19 (95%)</td>
<td>2/23 (8.7%)</td>
</tr>
<tr>
<td>POLQ&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>20/21 (95%)</td>
<td>ND</td>
</tr>
<tr>
<td>POLN&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>22/23 (96%)</td>
<td>8/51 (16%)</td>
</tr>
<tr>
<td>POLN&lt;sup&gt;−/−&lt;/sup&gt;/POLQ&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>29/31 (94%)</td>
<td>1/10 (10%)</td>
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Wild-type and knockout cells were transfected with targeting constructs of the indicated genotype. The number of targeted clones/number of drug-resistant clones analyzed is shown.
Likewise, the frequency of targeted integration into the OVALBUMIN and the POLK locus was not diminished in any of the mutant clones (Table I). Collectively, although Polκ and Polθ play a critical role in Ig gene conversion, these DNA polymerases have little, if any, contribution to HR-mediated repair. Alternatively, the functional overlap between the two A-type DNA polymerases and other DNA polymerases may mask the contribution of Polκ and Polθ to HR-dependent repair.

We next explored the functional relationship of Polν and Polθ with Polη in HR-mediated repair. To this end, we measured cellular sensitivity to camptothecin and cisplatin. Intriguingly, the POLH-/POLN-/POLQ- clones exhibited an increase in sensitivity to camptothecin, a phenotype that was not shared by the POLH+/ POLN+/POLQ+ clones (Fig. 6 C). These observations reveal that the two A-type DNA polymerases can play a role in general HR only when Polη is deleted. In contrast, POLH-/POLN-/POLQ- clones showed a normal sensitivity to cisplatin (Fig. 6 D). Presumably, other polymerases may have substituted for Mus308, the prototype polymerase of D. melanogaster, which plays a critical role in providing a cellular tolerance to cross-linking reagents.

To analyze HR-dependent DSB repair in an artificial substrate, we inserted an HR substrate, SCneo, which carries the rare cutting endonuclease site, ISceI, into the OVALBUMIN locus of wild-type and all mutant clones (Johnson and Jasin, 2000; Fukushima et al., 2001). This construct carries two mutant neomycin-resistance genes (S2neo and S2neo-3), which are localized in tandem and are complementary to each other (Fig. 7 A). After transient expression of ISceI, the induced DSB in S2neo is repaired by HR, with the S2neo-3 gene serving as the donor for gene conversion, leading to the restoration of a functional neomycin-resistance gene. Thus, we can measure the frequency of HR-dependent DSB repair by counting the number of neomycin-resistant (neoR) colonies. The frequency of HR-dependent repair was decreased three to six times in POLQ-/-, POLN-/-, and POLN+/ POLQ-/- cells in comparison with wild-type cells (Fig. 7 B). Collectively, Polν and Polθ contribute to some of the HR reactions.

Polν, Polθ, and Polη are involved in TLS-dependent hypermutation at IgV

Biochemical studies and our previous study on POLN+/ POLQ-/- DT40 cells suggest a role for Polν and Polθ in TLS (Seki et al., 2005; Takata et al., 2006; Yoshimura et al., 2006; Arana et al., 2007). To verify this conclusion, we examined nontemplated single-base substitutions (Ig hypermutation) in cells deficient in POLH, POLN, and POLQ. AID overexpression for 2 wk increased the rate of Ig hypermutation from 1.3 × 10^-6 to 6.3 × 10^-5 per nucleotide per division (Fig. 2, A and B; and Fig. 3 C). The rate of Ig hypermutation exhibited only a modest change in the single- and double-mutant clones when compared with wild-type cells; 1.7 times lower for POLH+/ POLN-/- cells, 1.4 times higher for POLQ-/- cells, 1.1 times higher for POLN+/ POLQ+/- cells, and 1.6 times lower for POLN+/ POLQ-/- cells (Fig. 2 C and Fig. 3 C). On the contrary, the rate of Ig hypermutation in wild-type cells was 3.5 times higher than in POLH-/ POLN+/ POLQ-/- cells (Fig. 2 C and Fig. 3 C).

The POLN+/ POLQ-/- cells showed a marked reduction in the number of C to G mutations when compared with wild-type cells, indicating that Polν and Polθ play a role in TLS past the abasic site (P < 0.0095; Fig. 3 A). The C to G, C to T, and G to C mutations tended to also be decreased in POLH+/ POLN+/ POLQ-/- clones when compared with wild-type cells. These data support the notion that A family DNA polymerases Polν and Polθ, together with Y family DNA polymerase Polη, contribute to TLS-dependent Ig hypermutation in DT40 cells (Fig. 1).

Discussion

We previously demonstrated that Polθ can play a role in base excision repair and probably in TLS (Yoshimura et al., 2006). In this study, we demonstrate a dominant role played by Polθ and Polν in HR-dependent Ig V gene conversion and in TLS-dependent Ig hypermutation. These DNA polymerases make only a small contribution to HR, as indicated by the normal range of cellular tolerance to the anticancer agents cisplatin and camptothecin and by normal efficiency of gene targeting (Fig. 6, C and D; and Table I). Nonetheless, the hypersensitivity of POLH-/ POLN+/ POLQ-/- cells but not of POLH-/- or POLN+/ POLQ-/- cells to camptothecin reveals that the two A-type DNA polymerases can contribute to HR-dependent DNA repair if other HR-related DNA polymerases are not available (Fig. 6 C). In addition, reduced HR-dependent DSB repair in POLN+/ POLQ-/- cells as well as in Polv-depleted human cells (Zietlow et al., 2009; Moldovan et al., 2010) indicates a contribution of Polv and Polθ preferentially to HR in DSB repair (Fig. 7 B). Conceivably, the choice of DNA polymerases in the DNA synthesis
step of HR may be dependent on the type of DNA damage, which may explain why \textit{POLN}^{-/-} and \textit{POLQ}^{-/-} cells showed defective HR-dependent DSB repair without displaying sensitivity to cisplatin or camptothecin.

Accumulating evidence indicates that individual DNA polymerases can perform multiple roles, e.g., Polκ for nucleotide excision repair and TLS (Ogi and Lehmann, 2006; Ogi et al., 2010), Polη for HR and TLS (Kawamoto et al., 2005), and Polζ for HR and TLS (Sonoda et al., 2003). Thus, we can also add the role played by Polγ and Polθ in HR and TLS to the list of multiple functions performed by individual DNA polymerases.

\textbf{Polγ and Polθ may promote DNA synthesis in Ig gene conversion}

Our results support the premise that Polγ and Polθ play a role in the DNA synthesis step of HR. First, Ig gene conversion was significantly decreased in \textit{POLN}^{-/-}/\textit{POLQ}^{-/-} cells (Fig. 2 C, Fig. 3 C, and Fig. 4 C). Second, this decrease may be associated with a marked increase in the length of the gene conversion tract (Fig. 5 C). Third, no Ig gene conversion events were detectable in \textit{POLH}^{-/-}/\textit{POLN}^{-/-}/\textit{POLQ}^{-/-} clones (Fig. 2 C and Fig. 3 C). Therefore, Polη may be responsible for gene conversion events having the longer gene conversion tract. Purified Polη can undergo DNA synthesis using a D loop structure as a template (McI1wraith et al., 2005). Thus, like Polη, the two A-type DNA polymerases may contribute to the DNA synthesis step of Ig gene conversion.

During metazoan evolution, Polγ and Polθ appear to have obtained a specialized HR function (HR-dependent Ig V diversification in B lymphocyte precursors), whereas other DNA polymerases may have substituted for Mus308, the prototype DNA polymerase, which plays a critical role in cellular tolerance to DNA-damaging agents. It should be noted that Ig gene conversion is distinctly different from other HR reactions because it allows for recombination between two diverged sequences. In fact, although a 0.1% sequence divergence strongly suppresses HR reactions (te Riele et al., 1992), Ig gene conversion is efficiently performed between homologous sequences with 10% divergence. This divergence may strongly suppress DNA synthesis in the D loop structure such that a mismatch between primer and template strands interferes with DNA synthesis from the primer and also destabilizes the D loop (Johnson and Jasin, 2000; Biet et al., 2003). Accordingly, during evolution, Polγ and Polθ may have acquired a specialized character suitable for this extremely difficult task, HR-dependent Ig V diversification. Ig gene conversion plays an important role in diversifying Ig V gene not only in chickens but also in the rabbit, whereas primates and rodents rely on V(D)J recombination to diversify Ig V (Lundqvist et al., 2006; Mage et al., 2006). Evidence that Ig gene conversion contributes to Ig V diversification in other mammals such as pig, cattle, sheep, and horses is less convincing (Butler et al., 2006; Zhao et al., 2006). Because performing effective DNA synthesis in Ig gene conversion is a difficult challenge for DNA polymerases, this study suggests the critical role played by Polη, Polγ, and Polθ in Ig V diversification in some domesticated animals as well as in DT40 cells. Conceivably, vertebrates are faced with more complex tasks, such as acquired immune reactions, in addition to the maintenance of genomic DNA (Fig. 6 B). In addition to new DNA polymerases evolving to perform DNA damage responses, the function of existing polymerases appears to have shifted during metazoan evolution from involvement in the DNA damage response to the diversification of Ig V genes.

\textbf{Contribution of Polγ, Polθ, and Polθ to TLS past abasic sites}

In addition to the DNA synthesis step of Ig gene conversion, the following data suggest that Polθ and Polθ may carry out TLS past abasic sites in Ig V hypermutation in DT40 cells. \textit{POLN}^{-/-}/\textit{POLQ}^{-/-} cells showed a significant reduction in the number of C to G mutations in comparison with wild-type cells. In addition, \textit{POLH}^{-/-}/\textit{POLN}^{-/-}/\textit{POLQ}^{-/-} cells showed 3.5 times lower total number of mutations in comparison with wild-type cells. Therefore, we conclude the involvement of these three DNA polymerases in nontemplated Ig V hypermutation in DT40 cells (Fig. 1). It should be noted that the role of TLS in Ig V hypermutation in human and mouse B lymphocytes is unclear. In other words, although several studies demonstrate the role of TLS polymerases in murine Ig V hypermutation (Masuda et al., 2006; Martomo et al., 2008; Faili et al., 2009; Schenten et al., 2009), it is unclear whether TLS polymerases carry out DNA synthesis on intact template strands or bypass unknown DNA lesions to accumulate mutations.

DT40 cells deficient in Rev1, another TLS polymerase, exhibit a 75% drop in the number of Ig V hypermutation events in comparison with wild-type cells, indicating a higher contribution of Rev1 to Ig V hypermutation compared with Polγ, Polθ, and Polθ (Ross and Sale, 2006). Intriguingly, Polθ contains the Rev1-interacting motif x-x-x-F-F-y-y-y-y (x, no specific residue; y, no specific residue but not proline), which is conserved among species (three motifs in human and chicken and four motifs in mouse; Ohashi et al., 2009). The biological significance of possible interactions between Rev1 and Polθ should be elucidated in the future.

The data of nontemplated mutations do not completely agree with biochemical studies of Polγ and Polθ. Although only Polθ, but not Polγ, can efficiently bypass abasic sites in vitro (Seki and Wood, 2008), our study indicates the contribution of both DNA polymerases to this bypass reaction in vivo (Fig. 3 A). Furthermore, although in vitro studies predict that a defect in Polθ causes reduction in C to T and G to A transition mutations (Seki et al., 2004), this prediction is not verified by the spectrum of nontemplated mutations in our study (Fig. 3 A). This is also the case with \textit{POLQ}-deficient mice, which show ambiguous results on Ig hypermutation, making it difficult to conclude whether Polθ is involved in Ig hypermutation (Zan et al., 2005; Masuda et al., 2006; Martomo et al., 2008). Purified Polγ is an extremely low fidelity enzyme incorporating T opposite template G with a frequency of 0.45 (Takata et al., 2006; Arana et al., 2007), whereas C to T or G to A transition mutations are not significantly decreased in the absence of Polθ in this study (Fig. 3 A). However, it should be noted that some C to T and G to A transitions might be derived from non-processed uracil (simply generated by AID), which instructs template T and may be bypassed by many polymerases and not just Polγ and Polθ. In contrast with these discrepancies, Polθ-mediated
preferential incorporation of A opposite abasic sites in vitro (Kokoska et al., 2003; Zhao et al., 2004) is in agreement with the decreased number of C to T or G to A transition mutations caused by the loss of Poln in POLN<sup>−/−</sup> POLQ<sup>−/−</sup> cells (Fig. 3 A).

Further studies are required to discuss the relevance of in vitro studies to TLS in vivo because of complex functional interactions among multiple polymerases.

Materials and methods

Cell culture and DNA transfection

DT40 cells were cultured in RPMI 1640 medium supplemented with 10 µM β-mercaptoethanol, 10% fetal calf serum (Sigma-Aldrich), and 1% chicken serum (Sigma-Aldrich) at 39.5°C (Sonoda et al., 1998). 10<sup>5</sup> cells were suspended in 0.5 ml PBS containing 10–30 µg linearized plasmid for each transfection and electroporated with a gene pulser apparatus (BioRad Laboratories) at 550 V and 25 μF. After electroporation, cells were transferred into 20 ml fresh medium and incubated for 24 h. Cells were resuspended in 80 ml medium containing the appropriate drugs and divided into four 96-well plates. After 7–10 d, drug-resistant colonies were transferred to 24-well plates (Buerstedde and Takeda, 1991).

Measurement of targeted integration frequencies

To analyze integration events at the OVALBUMIN locus and POLK locus, targeted construct DNAs were transfected into cells, and Southern blot analysis was performed after selection of clones resistant to the appropriate antibiotics (Buerstedde and Takeda, 1991).

Generation of gene-disrupted cells

We transiently expressed CreER and exposed POLN<sup>−/−</sup> and POLN<sup>−/−</sup>/POLQ<sup>−/−</sup> cells to 4-hydroxytamoxifen (OH-TAM) to delete the POLN or POLQ genes (Buerstedde and Takeda, 1991). We obtained and counted colonies on October 29, 2017 • jcb.rupress.org Downloaded from

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Analysis of AID-induced Ig hypermutation and Ig gene conversion at V<sub>J</sub>, segments

DNA was extracted from more than three clones each of wild-type, POLQ<sup>−/−</sup>, POLN<sup>−/−</sup>, and POLN<sup>−/−</sup>/POLQ<sup>−/−</sup> cells at 2 wk after subcloning (Fig. S2). PCR-amplified fragments of V<sub>J</sub> segments were cloned into plasmid and subjected to base sequence analysis. The rearranged V<sub>J</sub> was amplified using the CVL6S (5′-CAAGAATCTCAGGGGCCGTCACTG-3′) and CVL5 (5′-GGGCAAGAATCTCCAGGCCTCCGCGCC-G-3′) primers as previously described (Sale et al., 2001). After purification with a gel extraction kit (Qiagen, QIAGEN), PCR products were sequenced in the TOPO PCR2.1 cloning vector (Invitrogen) and sequenced with the M13 forward or reverse primer and a sequencer (ABI PRISM 3100; Applied Biosystems). Sequence alignment with GENETYX-MAC (Software Development) allowed the identification of changes from the consensus sequences in each clone. Frequencies of Ig hypermutation, ambiguous mutations, and Ig gene conversion were determined as previously described (Sale et al., 2001). In brief, all sequence changes were assigned to one of three categories: Ig gene conversion, nontemplated point mutation, or an ambiguous category. This discrimination rests on the published sequences of the V<sub>j</sub> genes that could act as donors for gene conversion. For each mutation, the database of the V<sub>j</sub> pseudogene was searched for a potential donor. If no pseudogene donor containing a string of >9 bp could be found, the mutation was categorized as a nontemplated point mutation. If such a string was identified and there were further mutations that could be explained by the same donor, all of these mutations were assigned to a single long tract gene conversion event. If there were no further mutations, it was possible that the isolated mutation could have arisen through a conversion mechanism or could have been nontemplated and was therefore categorized as ambiguous.

The rate of hypermutation and Amb mutation was calculated using the following equation: rate of mutation = mutation frequency/length of sequenced Ig light chain locus (430 bp)/sequenced sample numbers/number of cell divisions (42 cycles for wild-type, 37 cycles for POLN<sup>−/−</sup>/POLQ<sup>−/−</sup>, single-gene-disrupted clones, 34 cycles for POLN<sup>−/−</sup>/POLQ<sup>−/−</sup> double-mutant cells, and 26 cycles for POLN<sup>−/−</sup>/POLQ<sup>−/−</sup>/POLQ<sup>−/−</sup> triple-mutant cells for 14 d). The rate of Ig gene conversion was calculated using the following equation: rate of gene conversion = gene conversion frequency/sequenced sample numbers/number of cell divisions.

Analysis of the rate of slmG gain

The generation frequency of slmG gain revertants was monitored by flow cytometric analysis of cells that had been expanded for 3 wk after subcloning and stained with FITC-conjugated goat anti-chicken IgM (Bethyl Laboratories, Inc.). To enhance Ig gene conversion, 1.25 ng/ml TSA was added to the slmG-negative cell populations, and the fraction of slmG-positive revertants was monitored over time as described previously (Seo et al., 2005).

Nucleotide sequence analysis of rearranged V<sub>J</sub>, segments derived from slmG gain fractions

Fractions of three independent slmG gain revertant populations were obtained from each genotype using MACS separation columns (Miltenyi Biotec; Fig. S2).
Genomic DNA was amplified by PCR with Pyrobest DNA polymerase (30 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min). The rearranged V\(\gamma\)3 was amplified using the CVILF6 (5'-CAGGGACCTCGGCGGGGCT- CACTGATTCGGCC-3') and CVIR3 (5'-GGCCAACCTCCCCAGCTC- GCCGGCAGTCCAAG-3') primers as previously described [Sale et al., 2001]. After purification with a gel extraction kit (Qiagen), PCR products were cloned into the TOPO pcR2.1 cloning vector. Sequence alignment with GENETYX-MAC allowed the identification of changes from the consensus sequences in each clone. Frequencies of Ig hypermutation and Ig gene conversion were determined as previously described [see Analysis of AID-induced ig...]

Online supplemental material
Fig. S1 shows the \(V\gamma\) sequence alignment of wild-type, POLQ\(^{-/-}\), POLN\(^{-/-}\), and POLQ\(^{-/-}\)/POLN\(^{-/-}\) clones before TSA treatment. Fig. S2 shows the number of subclones and sequences analyzed in individual experiments. Online supplemental material is available at http://www.jcb. org/cgi/content/full/jcb.200912012/DC1.

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References

Buerstedde, J.M. and S. Takeda. 1991. Increased ratio of targeted to random hypermutation and Ig gene conversion were determined as previously described [see Analysis of AID-induced ig...]

Marini, F., N. Kim, A. Schuffert, and R.D. Wood. 2003. POLN, a nuclear PolA family DNA polymerase homologous to the DNA cross-link sensitiv-

Hypermethylation greatly reduces the Ig repertoire. PLoS Genet. 5:1384–1391. doi:10.1371/journal.pgen.1000642


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