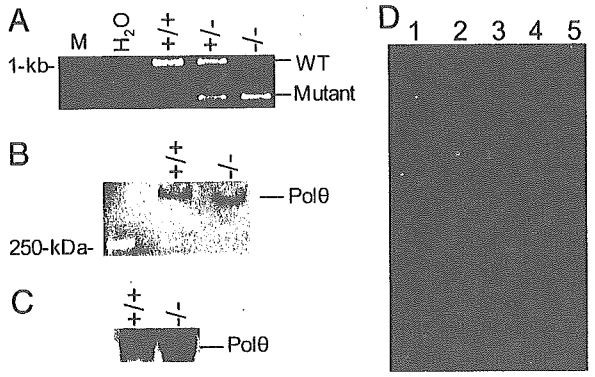

Fig. 2. Polq-knockout mice express a mutant Polθ devoid of polymerase activity. (*A*) RT-PCR analysis using primers s6623 and as7597 (Fig. 1*B*) to detect the truncated mRNA. (*B*) Western blot analysis of Polθ expression in the splenocytes. (*C*) WT and mutant Polθ were immunoprecipitated from mouse spleen with rabbit polyclonal antibodies against Polθ and subjected to Western blot with the same antibodies. (*D*) The immunoprecipitated WT and mutant Polθ were analyzed for DNA polymerase activity as described in *Materials and Methods*. Lane 1, primer template alone; lane 2, exonuclease-free Klenow fragment; lane 3, empty well; lane 4, WT Polθ; lane 5, mutant Polθ.

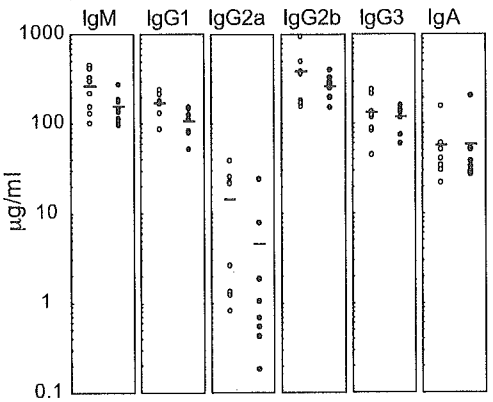

Fig. 4. Serum Ig levels are slightly reduced in Polq-inactive mice. Eight pairs of age-matched WT and Polq-inactive mice (9–10 weeks old) were bled, and the serum Ig levels were measured by ELISA. Open circles, WT; solid circles, Polq-inactive mice. Bars, average titer.

expression of the mutant mRNA, Western blot analysis revealed an immunoreactive protein in the mutant cells with a molecular weight similar to the WT Polθ (Fig. 2*B*). Because the antibodies used in this assay recognize an epitope near the C-terminal region of Polθ, we conclude that mutant cells express a truncated Polθ protein lacking the polymerase core domain at a slightly reduced level compared to WT cells.

To confirm that the mutant enzyme was devoid of DNA synthesis activity, we immunoprecipitated Polθ from WT and mutant splenocytes. Western blot analysis revealed that a similar amount of WT and mutant Polθ was immunoprecipitated from both samples (Fig. 2*C*). As expected, WT (Fig. 2*D*, lane 4), but not mutant (Fig. 2*D*, lane 5), Polθ exhibited a template-dependent polymerase activity. These results demonstrate that the mutant mice express a truncated Polθ that has greatly reduced DNA polymerase activity but is likely intact for other potentially important functions.

Normal B Cell Development and Maturation in Polq-Inactive Mice. FACS analysis of bone marrow cells revealed no significant differences in the percentages of $B220^+CD43^+$ progenitor and $B220^+CD43^-IgM^-$ precursor cells (Fig. 3*A*). B cell maturation in the spleen was also normal, because there was a similar ratio of $IgM^{high}IgD^{dull}$ (immature), $IgM^{high}IgD^{high}$ (transitional), and $IgM^{dull}IgD^{high}$ (mature) populations in WT and Polq-inactive mice (Fig. 3*B*). In addition, no apparent differences between WT and Polq-inactive mice were observed in the follicular ($CD23^{high}CD21^{dull}$) and marginal zone ($CD23^{dull}CD21^{high}$) B cells (Fig. 3*B*). In our analysis of six pairs of WT and Polq-inactive mice, we have found no significant differences in B and T lymphocyte development and function (data not shown). In addition to their normal frequency, Polq-inactive B cells were functionally normal, as assessed by proliferation in response to anti-IgM antibodies, LPS, and CD40 ligand (Fig. 3*C*) and to various combinations of these stimuli (not shown), although the response to low doses of anti-IgM stimulation appeared to be slightly reduced in Polq-inactive B cells. These results demonstrate that Polq-inactive B cells undergo normal differentiation and maturation and respond normally to an array of *in vitro* stimuli that signal through a variety of receptors, including the B cell antigen receptor, Toll-like receptor 4, and the surrogate for T cell help, CD40.

Reduction in Serum Ig Levels in Polq-Inactive Mice. To analyze B cell function *in vivo*, we first measured the levels of serum Igs in nonimmunized mice. Except for IgA, we found a general reduction in serum Ig levels in Polq-inactive mice (Fig. 4). The average Ig levels ± SD (μg/ml) in WT and Polq-inactive mice were 262 ±

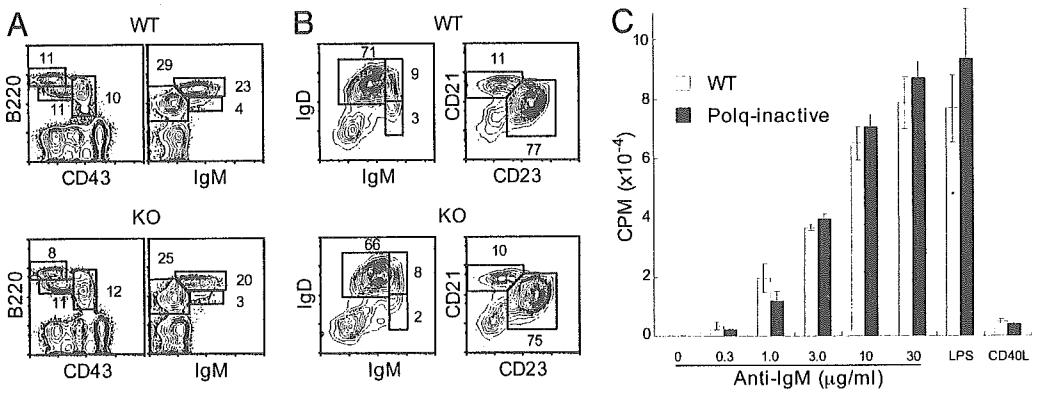

Fig. 3. Normal B cell development and *in vitro* responses in Polq-inactive mice. (*A*) FACS profiles of bone marrow cells in the lymphoid gate. (*B*) FACS profiles of $B220^+$ cells in spleen. (*C*) Proliferative responses of purified spleen B cells. Cells (5×10^5/ml, 100 μl per well in 96 flat-bottom plates) were cultured for 48 h in medium alone or in the presence of different doses of anti-IgM antibodies, 10 μg/ml LPS, or 1/3 dilution of CD40L and pulsed with ^{3}H-thymidine for the last 6 h.

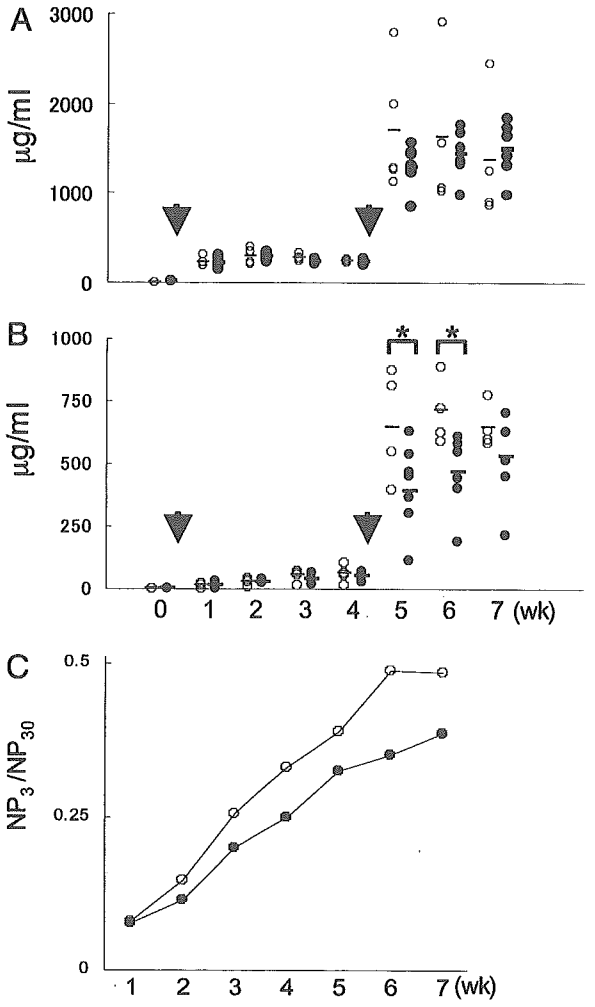

Fig. 5. Immune responses and affinity maturation in Polq-inactive mice. Mice (five WT and eight Polq-inactive) were immunized with 100 μg of NP-CGG precipitated with alum and boosted 4 wks later. Arrows indicate immunization times. Mice were bled weekly, and NP-specific serum IgG1 antibodies were measured by ELISA. Open and solid circles represent WT and Polq-inactive mice, respectively. (*A*) Titers of total (high- and low-affinity) NP-specific antibodies. (*B*) Titers of high-affinity NP-specific antibodies. *, $P < 0.1$ (unpaired Student's *t* test). (*C*) The average ratio of high-/low-affinity antibodies.

132 and 154 ± 57 for IgM, 169 ± 47 and 107 ± 34 for IgG1, 14.1 ± 14 and 4.52 ± 8.1 for IgG2a, 381 ± 257 and 261 ± 81 for IgG2b, 130 ± 66 and 118 ± 34 for IgG3, and 56.0 ± 42 and 57.2 ± 59 for IgA, respectively. The reduction in the levels of IgM and IgG1 was statistically significant ($P = 0.05$ and 0.009, respectively), whereas the reduction in the levels of IgG2a, IgG2b, and IgG3 was not.

A Partial Impairment in the Production of High-Affinity Antibodies in Polq-Inactive Mice. We then examined the primary and secondary immune responses to the T-dependent antigen NP-CGG. Polq-inactive mice produced slightly reduced amounts of total (low- and high-affinity) anti-NP antibodies in both primary and secondary immune responses, as measured in an ELISA assay with NP_{30}-BSA (Fig. 5*A*). However, the production of high-affinity antibodies, as measured with NP_3-BSA, was more clearly reduced in the Polq-inactive mice (Fig. 5*B*). In fact, the observed reduction in the titers of total anti-NP antibodies could be explained by the reduction in the titers of high-affinity anti-NP antibodies. The reduced affinity maturation in Polq-inactive mice was further illustrated by comparing the ratio of NP_3- and NP_{30}-binding antibody titers (Fig. 5*C*). We also examined the appearance of the $B220^+PNA^+$ GC B cells after NP-CGG immunization. Polq-inactive and WT mice contained a similar frequency of GC B cells in spleen on days 10 and 14 after antigen injection (data not shown). These results may suggest a defect in selection of high-affinity B cells in the GC or that the Polq-inactive B cells have intrinsic defects in the SHM of Ig V genes.

A Selective Reduction of Mutations at C/G in Ig Genes of Polq-Inactive Mice. To gain insight into the mechanisms underlying the reduced levels of high-affinity antibodies, we immunized mice with NP-CGG, isolated the GC B cells, and examined SHM. We restricted our analysis to the intronic sequence downstream of the J_H4 region rather than the coding sequence of the $V_H186.2$ gene, which is the dominant V gene segment used in the NP response, because the $V_H186.2$ sequence is under strong antigen-driven selection, hence the unselected mutation pattern would likely be obscured (22, 25, 26). We analyzed 98 and 97 unique clones from WT and Polq-inactive mice, respectively (Fig. 6*A*; data for sequences are available upon request). Polq-inactive mice exhibited a slight reduction in the total mutation frequency in the J_H4 intron (Table 1). However, when the mutation frequency of C/G vs. A/T mutations was calculated, we found that, whereas mutation frequency at A/T was similar between WT and Polq-inactive mice (0.522% and 0.514%, respectively), C/G mutation frequency was reduced by 41% (0.279% vs. 0.476% in WT mice, Table 1; Fig. 6*B*). In fact, the reduction in the overall mutation frequency in Polq-inactive mice was solely attributable to the reduction in mutations at C/G. The specific reduction of mutations at C/G was further illustrated by calculating the ratio of AT:CG mutations, which was 1.097 in WT and 1.843 in Polq-inactive mice (Table 1). Similarly, the relative representation of mutations at C/G and A/T was skewed (Fig. 6*C*; $P < 0.001$, χ^2 test). The reduction of mutations at C/G was more focused on the intrinsic SHM hotspots (Fig. 6*B*). In particular, the C/G mutations at the four hotspots located at nucleotide positions 39, 47, 56, and 62 were all significantly reduced in Polq-inactive mice (Fig. 6*B*, $P < 0.05$). Both transition and transversion mutations were similarly affected in the Polq-inactive mice (Fig. 6*C*). No differences were observed in the ratio of transitions to transversions within C/G and A/T. These results indicate that the polymerase activity of Polθ is important for the generation of mutations at C/G.

Discussion

In the present study, we generated and analyzed mice expressing a mutant Polθ specifically devoid of polymerase activity. To our knowledge, this represents a unique mutant mouse line to have reduced C/G but relatively normal A/T mutations. The active site, rather than a gene ablation mutagenesis strategy, was adopted, because we reasoned that in the complete absence of Polθ, its function might be compensated for by other DNA polymerases. At least 10 lesion bypass DNA polymerases have been identified in higher eukaryotes. The presence of the mutant Polθ could potentially prevent irrelevant DNA polymerases from taking part in the process of SHM and allow us to explore the requirement for the DNA synthesis activity of Polθ in SHM. In this regard, it is possible that Polθ-null mice might show a quite different phenotype as compared with our Polq-inactive mice.

B cell development and maturation were normal in Polq-inactive mice. In addition, the Polq-inactive and WT B cells responded similarly to several different *in vitro* stimuli. Interestingly, we observed a reduction in serum Ig levels in nonimmunized mice, suggesting that Polq-inactive B cells might be partially impaired in CSR. In this regard, it is notable that mice deficient in msh2 or msh6, components of the mismatch repair pathway, exhibit a specific reduction in mutations at A/T and

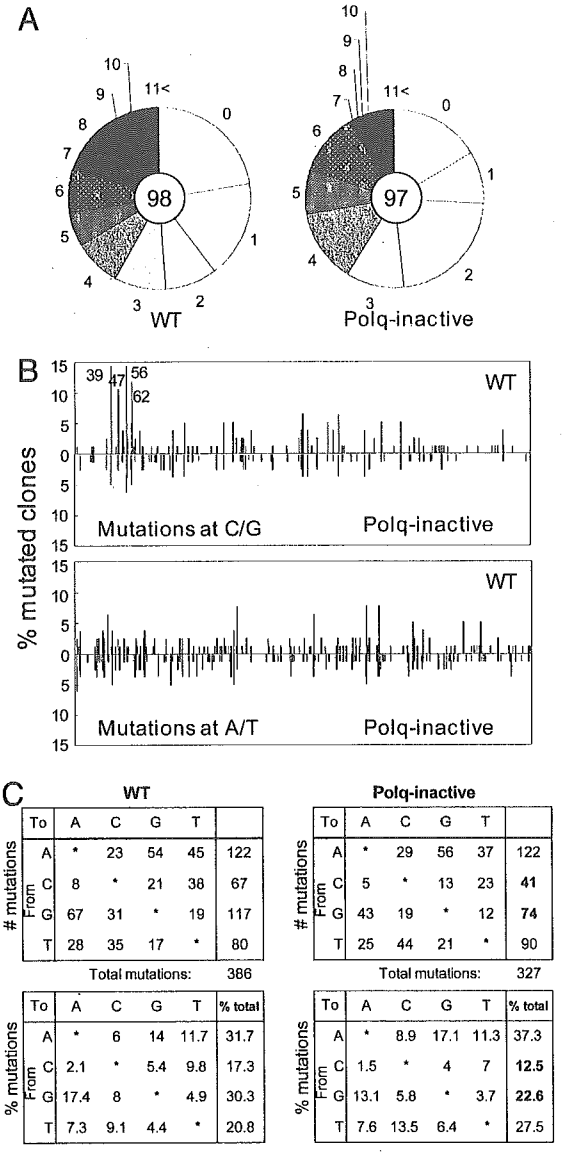

Fig. 6. Specific reduction of C/G mutations in the J_H4 intronic sequences in Polq-inactive mice. (*A*) Pie charts depict the accumulation of mutations in unique sequences. The total number of unique clones analyzed is shown in the center of each circle. (*B*) Distribution of C/G (*Upper*) and A/T (*Lower*) mutations over the J_H4 intronic region. The nucleotide number shown in *Upper* indicates the four hotspots (39, TGTT; 47, AGTT; 56, AGCA and 62, TGCA) (25, 27, 29, 38, 39).

also have defects in CSR (27, 28). Although Polθ contains a helicase-like domain, helicase activity, which is known to play a critical role in DNA replication and recombination, has not been experimentally verified. Because Polq-inactive mice express a mutant protein with intact helicase and other potentially important functional domains, it is unclear how a mutant Polθ devoid of DNA synthesis activity might affect CSR. The reduction in the titer of serum IgM, which is mainly derived from B1 B cells, further suggests that Polq-inactive mice may have defects in the differentiation of specific B cell subpopulations.

Available evidence supports the DNA deamination model in which activation-induced cytidine deaminase deaminates C to U and generates a U/G mismatch (27, 29–33). This U/G lesion is thought to be resolved by three distinct but related pathways (8, 34). The phase 1a pathway generates G to A and C to T transitions by directly replicating over the U/G mismatch where

Table 1. Somatic mutations in J_H4 intronic sequences (509 base pairs)

	WT	Polq inactive
Number of clones	98	97
Mutated clones, %	76 (77.6)	81 (83.5%)
Total length of mutated sequences	38,684	41,229
Total number of mutations (at AT:CG)	386 (202:184)	327 (212:115)
Total mutation frequency, %	0.998	0.793
Mutation frequency at A/T, %	0.522	0.514
Mutation frequency at C/G, %	0.476	0.279
Ratio of AT: CG mutations	1.097	1.843

U is recognized as T. The phase 1b pathway generates both transitions and transversions at C/G by replicating over the noninstructive abasic site formed after excision of U via uracil DNA glycosylase. U/G mispair can also be recognized by components of the mismatch repair pathways and trigger a short-patch mutagenic DNA repair, leading to mutations at A/T pairs (phase 2 pathway).

In all these scenarios, mutations are finally introduced by DNA polymerases. Indeed, a number of low-fidelity DNA polymerases have been implicated in SHM of Ig genes. Inhibition of DNA polymerase ζ (Polζ) expression by antisense RNA was shown to reduce overall mutation frequency, but not mutation pattern, in the CL-01 B cell line and in transgenic mice (35, 36). DNA polymerase ι (Polι) has also been suggested to play a role in SHM in a GC-type Burkitt's lymphoma line (37). However, 129 mice, which lack a functional Polι due to a nonsense mutation in the *Poli* gene, exhibit a normal frequency and pattern in Ig gene mutations (32, 38, 39). Deficiency in DNA polymerase η (Polη) has been shown to correlate with dramatically reduced mutations at A/T pairs in both human and mice (32, 33, 40–42). Polη does not seem to be involved in the mutations at C/G pairs, because neither the frequency nor the spectrum at C/G mutations was altered in the absence of Polη (32, 33). These observations suggest that different DNA polymerases might be involved in different mutagenic pathways. Until now, however, a DNA polymerase(s) specifically involved in C/G mutations has not been identified.

In the present study, we have shown that Polθ, or more precisely its DNA synthesis activity, is important for the generation of mutations at C/G pairs. These findings, together with the observations that human POLQ is the only enzyme that efficiently catalyzes both the insertion and the extension steps for bypass of abasic sites (16), implicate Polθ in the phase 1b mutagenic pathway. Biochemical analysis has demonstrated that POLQ preferentially inserts A opposite an abasic site (6). If POLQ has the same catalytic specificity *in vivo*, then it would generate primarily G to A and C to T transitions. If so, inactivation of Polθ polymerase activity should have resulted in fewer transitional mutations at C/G. However, both transition and transversion mutations at C/G were similarly reduced in the Polq-inactive mice. This observation suggested that C/G transversions were also affected in Polq-inactive mice. It is conceivable that Polθ may be able to insert nucleotides other than A opposite abasic sites under *in vivo* conditions where other necessary accessory factors are present. Indeed, recombinant human Polη and DNA polymerase κ (Polκ) exhibited dramatically different catalytic properties, including DNA synthetic activity and ability to bypass an abasic site, when assayed in the presence of accessory factors PCNA, replication factor C and replication protein A (43, 44). It is also possible that Polθ may catalyze the extension step from mismatch termini formed by other low-fidelity polymerases, which may insert C and T, as well

as A opposite an abasic site and generate both transition and transversion mutations.

Disruption of Polθ polymerase activity resulted in approximately one-third reduction in both transition and transversion mutations at C/G pairs. Assuming that the transition mutations generated by phase 1a pathway are unaffected in Polq-inactive mice, Polθ polymerase activity should generate most of the transition mutations and a part of the transversion mutations at C/G in the phase 1b pathway. Therefore, a substantial portion of the transversion mutations at C/G must be catalyzed by as-yet-unidentified DNA polymerases. Candidate enzymes include Rev1, which predominantly generates transversions at C/G (45) and interestingly is up-regulated in GC B cells (unpublished results), and Polζ, which is a mismatch extender (46) and has been implicated in both A/T and C/G mutations (35, 36).

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