**DNA Polymerases η and θ Function in the Same Genetic Pathway to Generate Mutations at A/T during Somatic Hypermutation of Ig Genes**

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Somatic hypermutation of the Ig genes requires the activity of multiple DNA polymerases to ultimately introduce mutations at both A/T and C/G base pairs. Mice deficient for DNA polymerase η (POLH) exhibited an ~80% reduction of the mutations at A/T, whereas absence of polymerase θ (POLQ) resulted in ~20% reduction of both A/T and C/G mutations. To investigate whether the residual A/T mutations observed in the absence of POLH are generated by POLQ and how these two polymerases might cooperate or compete with each other to generate A/T mutations, here we have established mice deficient for both POLH and POLQ. Polq−/−/Polh−/− mice, however, did not show a further decrease of A/T mutations as compared with Polh−/− mice, suggesting that POLH and POLQ function in the same genetic pathway in the generation of these mutations. Frequent misincorporation of nucleotides, in particular opposite template T, is a known feature of POLH, but the efficiency of extension beyond the misincorporation differs significantly depending on the nature of the mispairing. Remarkably, we found that POLQ catalyzed extension more efficiently than POLH from all types of mispaired termini opposite A or T. Moreover, POLQ was able to extend mispaired termini generated by POLH albeit at a relatively low efficiency. These results reveal genetic and biochemical interactions between POLH and POLQ and suggest that POLQ might cooperate with POLH to generate some of the A/T mutations during the somatic hypermutation of Ig genes.

The immunoglobulin genes are assembled in developing B cells by recombination-activating gene-mediated rearrangement of the germline V, D, and J gene segments (1–3). This process generates a primary repertoire of B cells expressing diversified cell surface immunoglobulins. Upon antigen stimulation and in the presence of T cell help, B cells are activated and form germinal centers (GC) in the secondary lymphoid organs such as spleen, lymph node, and Peyer's patches (4). Here they undergo further diversification of their Ig genes, namely somatic hypermutation (SHM) and class switch recombination (CSR). SHM introduces mainly point mutations in the variable (V) region genes and can alter the affinity of the antibodies produced by B cells, whereas CSR replaces the Ig gene constant regions to acquire different antibody effector functions. Both SHM and CSR are initiated by activation-induced cytidine deaminase (AID), which is thought to catalyze the deamination of cytosine (C) to uracil (U) and generate a U:G DNA lesion (5–7) in the switch regions for CSR and in the V genes for SHM. The precise mechanism of SHM remains elusive, but mutations are ultimately introduced by multiple DNA polymerases during the replication and repair of the U:G mismatch (8, 9).

Approximately 10 low fidelity DNA polymerases have thus far been identified in mammalian cells (10, 11). Studies with gene-targeted mice have revealed that DNA polymerases η (POLH), θ (POLQ), and REV1 have unique roles in Ig gene SHM. A deficiency in POLH caused an ~80% reduction of mutations at A/T, suggesting that POLH is the major enzyme generating A/T mutations and that its activity cannot be compensated for by other polymerases (12–14). The absence of POLQ resulted in decreases of both A/T and C/G mutations, although the magnitude of the reduction differed in two independent studies (15–17). REV1 deficiency resulted in a specific reduction of C to G and G to C transversions (18, 19), consistent with its being a deoxycytidyl transferase but had no apparent effect on A/T mutations. The catalytic subunit of DNA polymerase ζ, REV3, also appears to have a role in SHM (20, 21), although a definitive genetic model has not been available because deficiency in REV3 led to early embryonic lethality (22–25). These results collectively suggest that multiple DNA polymerases participate in distinct...
but overlapping mutagenic pathways to generate different types of base substitutions.

Similar to mice and humans lacking POLH, mice deficient for either MSH2 or MSH6 have a large reduction of A/T mutations (26–28). The MSH2-MSH6 heterodimer binds to mispaired bases, including the U:G mismatch that is potentially generated by AID-mediated deamination of C, and initiates mismatch repair (MMR). Moreover, MSH2-MSH6 was found to interact with and stimulate the polymerase activity of POLH (29), suggesting that POLH introduces mutations at A/T during MMR of the AID-induced U:G lesions. Although mutations at A/T were greatly reduced in mice lacking POLH, MSH2, or MSH6, ~20% of the normal level of A/T mutations were still generated in these mice, and it remains unclear which polymerase mediates these residual A/T mutations. Because Polq−/− mice, which completely lack POLQ expression, exhibited ~20% reduction of mutations at A/T (17), one possibility is that POLQ might be involved in the generation of the residual A/T mutations observed in the absence of POLH. To investigate this possibility, we established mice deficient for both POLH and POLQ and analyzed the frequency and patterns of Ig gene mutations. Our results suggest that POLH and POLQ function in the same genetic pathway to generate mutations at A/T.

**EXPERIMENTAL PROCEDURES**

**Establishment of Polq−/−Polh−/− Mice—** Polq−/− and Polh−/− mice were generated in a 129/C57BL/6 mixed background and have been backcrossed with C57BL/6 mice for five and nine generations, respectively. Polq−/− mice were bred with Polh−/− mice to generate Polq+/- Polh+/- mice, which were then crossed to obtain WT, Polq−/-, Polh−/-, and Polq−/− Polh−/- mice. The genotypes of these mice were verified by genomic PCR of tail DNA as described previously (17, 30). For CSR, proliferation assays, and induction of class switch recombination assays, mice were bred with WT, Polq−/-, Polh−/-, and Polq−/− Polh−/- mice to generate B cells responsive to various activation signals. As shown in Fig. 1A, the ratio of the immature (B220+ Igs+ IgM+) and recirculating (B220+ Igs+ IgM+) B cells was similar among these mice (Fig. 1B). B cell differentiation in the spleen was also normal as judged by the similar ratio of CD23+ CD21+ follicular and CD23−CD21+ marginal zone B cells in all types of mice (Fig. 1C). T cell differentiation also appeared normal because there was a similar representation of CD4+ and CD8+ T cells in the spleen (Fig. 1D).

**Normal B and T Cell Development and Maturation in Polq−/− Polh−/− Mice—** FACS analysis of the bone marrow cells of WT, Polq−/-, Polh−/-, and Polq−/− Polh−/- mice revealed no obvious differences in the percentages of CD43+ IgM− progenitor, CD43+ IgM− precursor, and CD43+ IgM+ B cells after gating on the B220+ cells (Fig. 1A). The ratio of the immature (B220+ Igs+ IgM+) and recirculating (B220+ Igs+ IgM+) B cells was similar among these mice (Fig. 1B). B cell differentiation in the spleen was also normal as judged by the similar ratio of CD23+ CD21+ follicular and CD23− CD21+ marginal zone B cells in all types of mice (Fig. 1C). T cell differentiation also appeared normal because there was a similar representation of CD4+ and CD8+ T cells in the spleen (Fig. 1D).

**Normal B Cell Responses and Class Switch Recombination in Polq−/− Polh−/− Mice—** Having confirmed that B and T cell development were phenotypically indistinguishable among the different mice, we analyzed B cell function as assessed by proliferative responses to various activation signals. As shown in Fig. 2A, Polq−/− Polh−/− B cells responded normally to anti-IgM antibodies, CD40L, LPS, and their combination. In addition, these B cells switched normally from IgM to IgG1 upon in vitro stimulation with LPS plus IL-4 or CD40L plus IL-4 (Fig. 2B). These results demonstrate that deficiency in both POLH and POLQ did not have any obvious effect on B cell development, maturation, activation, or class switch recombination of the Ig genes.
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Mutation Frequency in Polq<sup>−/−</sup> Mice—To examine the somatic hypermutation of Ig genes, mice were immunized with 4-hydroxy-3-nitrophenyl-acetyl coupled to chicken γ-globulin precipitated with alum, and 2 weeks later their splenocytes were stained with B220 and PNA to identify GC B cells. A similar frequency of the B220<sup>+</sup> PNA<sup>+</sup> cells was observed in WT, Polq<sup>−/−</sup>, Polh<sup>−/−</sup>, and Polq<sup>−/−</sup>Polh<sup>−/−</sup> mice (supplemental Fig. S1). The B220<sup>+</sup> PNA<sup>+</sup> GC B cells were sorted, and the purity was generally more than 90% (typical profiles shown in supplemental Fig. S1).

We analyzed I<sub>1,4</sub> intronic sequences from three WT, two Polq<sup>−/−</sup>, four Polh<sup>−/−</sup>, and two Polq<sup>−/−</sup>Polh<sup>−/−</sup> mice that were derived from breeding of Polq<sup>+/−</sup> by Polh<sup>+/−</sup> mice. The detailed results from individual mice in each group (except for Polq<sup>−/−</sup> mice where the data were obtained from GC B cells pooled from two mice) are shown in supplemental Tables S1–S4. For WT and Polq<sup>−/−</sup> mice, we also included data from our previous study (17), allowing the analysis of a larger number of mutations of each type of base substitution. The results from each group were combined and are shown in Table 1. In agreement with earlier studies, the overall mutation frequency in the I<sub>1,4</sub> intronic region was ~1% in WT mice (0.996%; Table 1). Mutation frequency at C/G (0.493%) was similar to that at A/T (0.503%), so the ratio of C/G:A/T mutations was 49.5:50.5. Consistent with our recent observations, the overall mutation frequency in Polq<sup>−/−</sup> mice dropped to 0.766%, and mutations at C/G and A/T were decreased to 0.377 and 0.389% (24 and 23% reduction compared with WT mice, respectively). As expected, mutations at A/T were reduced by 83% in Polh<sup>−/−</sup> mice (0.084% versus 0.503% in WT mice) but interestingly mutations at C/G were unaffected (0.504% versus 0.493% in WT mice), suggesting that POLH was not involved in the generation of C/G mutations and that the decrease of mutations at A/T was not compensated for by an increase in C/G mutations. The overall mutation frequency dropped to 0.588% (41% reduction compared with WT mice) in Polq<sup>−/−</sup> mice exclusively because of the decreased A/T mutations. Because Polq<sup>−/−</sup> and Polh<sup>−/−</sup> mice each exhibited a decrease of mutations at A/T, we expected a greater reduction of A/T mutations in Polq<sup>−/−</sup>Polh<sup>−/−</sup> mice. Unexpectedly, Polq<sup>−/−</sup>Polh<sup>−/−</sup> mice did not exhibit any further decrease of A/T mutations as compared with Polh<sup>−/−</sup> mice (Table 1). These observations suggest that POLH and POLQ likely function in the same genetic pathway, so the double deficiency does not give an additive effect.

Taking advantage of the large number of mutations we have analyzed, we next compared the actual frequency of each type of nucleotide substitution (Fig. 3A). Compared with WT mice (open bars), Polq<sup>−/−</sup> mice showed a moderate reduction of mutations of virtually all types of base substitutions (gray bars), indicating that POLQ is involved in the generation of mutations at both A/T and C/G and that its role is not biased toward a particular type of base substitution. Although Polh<sup>−/−</sup> mice showed a preferential reduction of mutations that occurred at A/T, the magnitude of reduction differed depending on the type of base substitutions (striped bars). Thus the A to G and T to C substitutions, which together accounted for nearly half of the A/T mutations in WT mice, were almost totally abolished in Polh<sup>−/−</sup> mice (94 and 93% reductions, respectively). A slightly less dramatic reduction was observed for A to T and T to A changes (85 and 90% reductions, respectively), whereas A to C and T to G substitutions were decreased by only ~50%. Therefore, the A to G and T to C transitions are almost exclusively dependent on the activity of POLH, whereas half of the A to C and T to G are likely generated by another polymerase(s). These results are in good agreement with earlier observations in Polh<sup>−/−</sup> mice (12, 14). As is the case for the overall mutation frequency at A/T (Table 1), the frequency of each type of base substitution at A/T in Polq<sup>−/−</sup>Polh<sup>−/−</sup> mice was very similar to that observed in Polh<sup>−/−</sup> mice, further suggesting that POLH and POLQ function on the same genetic pathway in the generation of A/T mutations.

Mutation Patterns in Polq<sup>−/−</sup>Polh<sup>−/−</sup> Mice—We further analyzed mutation patterns (Fig. 3B), which do not take into consideration the absolute mutation frequency but instead reflect the relative representation of each type of nucleotide substitution among all mutations. The patterns of base substitutions were quite similar between WT and Polq<sup>−/−</sup> mice except there was a slight increase of G to C transversions and a moderate decrease of G to T and T to G transversions, as we recently described (17). By contrast, Polh<sup>−/−</sup> mice exhibited dramatically decreased mutations at A/T and an increased representation of C/G mutations as reported previously (12–14). Again, the mutation patterns in Polq<sup>−/−</sup>Polh<sup>−/−</sup> mice were
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quite similar to that in Polh−/− mice except for a decrease of C to A in the former mice.

POLQ Efficiently Catalyzed the Extension from Mispaired Termini Opposite A or T—The observation that Polq−/− Polh−/− mice did not show any further decrease in A/T mutations as compared with Polh−/− mice suggested that POLQ functions in the same genetic pathway as POLH. Because the reduction of A/T mutations was more dramatic in Polh−/− than in Polq−/− mice, one potential pathway would place POLQ downstream of POLH, where it could cooperate with POLH to generate a subset of the A/T mutations. POLQ has already been shown to efficiently catalyze not only the insertion but also the extension step for bypass of a noninstructive abasic site and a thymine glycol (32). We therefore hypothesized that POLQ might also have the ability to extend DNA synthesis from mispaired termini opposite an A or T, which could be generated by POLH because it frequently incorporates incorrect nucleotides during copying of undamaged DNA (33, 34). To test this possibility, we compared the ability of POLH and POLQ to extend DNA synthesis from different types of mispaired termini opposite A or T. Under conditions where both polymerases exhibited similar activities from normal, matched termini (Fig. 4, A, lanes 11 and 12, and B, lanes 2 and 3), we found that POLQ exhibited greater activity than POLH in extending from mispaired termini opposite either A (Fig. 4A) or T (Fig. 4B). POLH was relatively efficient at extension from a G opposite T (Fig. 4B, lane 8) or a T opposite T (Fig. 4B, lane 11) but was inefficient in extending other types of mispaired termini opposite A or T (Fig. 4). In contrast, POLQ was proficient in extending all types of mispaired termini opposite A or T, suggesting that POLQ is an efficient mismatch extender. In this assay, the processivity of POLQ was greater than that of POLH, and POLQ was able to extend one nucleotide further than the template length (Fig. 4, A and B) by nontemplated addition at a blunt end (32). To examine whether POLQ could directly extend the mispaired termini generated by

**FIGURE 2. Normal proliferative responses and class switch recombination by B cells in Polq−/− Polh−/− mice.** A, purified spleen B cells were cultured for 2 days in the presence of the indicated stimuli and pulsed with [3H]thymidine for the last 6 h. White bars, WT; gray bars, Polq−/−; black bars, Polh−/− Polq−/− mice. B, class switch from IgM to IgG1 after in vitro stimulation with LPS + IL-4 (upper panels) or CD40L + IL-4 (lower panels). The experiments were repeated three times with similar results.

**TABLE 1**

| Mutation frequency in WT and Polq−/−, Polh−/−, and double mutant mice |
|---------------------------------------------------------------|
| **Ij4 intron (509 bp)** | WT | Polq−/− | Polh−/− | Polq−/− Polh−/− |
|--------------------------|----|--------|--------|-----------------|
| Number of clones | 639 | 531    | 477    | 250             |
| Mutated clones (%) | 487 (76.2%) | 405 (76.3%) | 349 (73.2%) | 176 (70.4%) |
| Total length of mutated sequences | 247,883 | 206,145 | 177,641 | 89,584 |
| Total number of mutations | 2470 | 1579 | 1045 | 530 |
| Overall mutation frequency (%) | 0.996 | 0.766 | 0.588 | 0.392 |
| Mutation frequency at C/G (%) | 0.493 | 0.377 | 0.504 | 0.495 |
| Mutation frequency at A/T (%) | 0.503 | 0.389 | 0.084 | 0.097 |
| Ratio of mutations at C/G:A/T | 49.5:50.5 | 42.5:50.8 | 85.7:14.3 | 83.6:16.4 |

* Pooled data of this study (three mice, 430 clones) and a previous study (209 clones) (17).
* Pooled data of this study (two mice, 160 clones) and a previous study (371 clones) (17).
* This study (four mice).
* This study (two mice).
* The data are corrected for base composition.
* The values in bold type indicate significant differences from WT mice (p < 0.001, χ2 test).
POLH, we further analyzed the mismatch insertion and extension in the same reaction. POLH alone was able to incorporate an incorrect nucleotide opposite an A but was inefficient in extending after the misincorporation site (Fig. 4C, lane 10). POLQ alone was able to insert the wrong nucleotide opposite A and further carried out the extension to the end of the template albeit at relatively low efficiency (Fig. 4C, lane 16). When POLH and POLQ were both present in the same reaction, a more efficient extension was observed (Fig. 4C, lane 11). At a higher concentration, POLH alone catalyzed greater mismatch insertion and apparent extension (Fig. 4C, lane 12), and the presence of POLQ resulted in further extension (Fig. 4C, lane 13). A mutant POLH, in which the conserved aspartic acid (D) and glutamic acid (E) residues at amino acid positions 115 and 116 were both converted to alanine (AA), had no detectable polymerase activity (lanes 6 and 14) and did not increase but rather slightly inhibited the activity of POLQ (compare lane 7 versus lane 8 and lane 15 versus lane 16). These observations indicate that the increased mismatch extension observed in the presence of both POLH and POLQ was not due to POLH-mediated stabilization of POLQ.

**DISCUSSION**

It is now well appreciated that multiple DNA polymerases participate in the SHM of Ig genes. It has been unclear, however, whether these polymerases collaborate or compete with each other to generate the various types of mutations. The present study provides genetic and biochemical evidence that POLH and POLQ function in the same pathway in the generation of A/T mutations. POLH and POLQ are unable to functionally compensate for each other because the absence of each enzyme alone caused a decrease of A/T mutations. Therefore, they likely play distinct roles within the same genetic pathway. Previously, it has been shown that Ig gene somatic mutations at A/T correlated well with the spectrum of substitutions at A/T base pairs introduced by POLH (35, 36). Moreover, POLH was shown to physically and functionally interact with the MSH2-MSH6 complex, which has the ability to bind to a U:G mismatch (29). These observations collectively suggest that POLH is the primary enzyme that is recruited to the AID-induced U:G mismatches and introduces mutations at downstream A/T base pairs. Although POLH was able to efficiently incorporate incorrect nucleotides, in particular opposite a template T, the extension efficiency after the misincorporation differed significantly depending on the type of mismatch and the sequence context (31, 33, 34). Thus the extension from a G opposite template T was relatively efficient, whereas the extension from other mismatched termini, including those opposite a template A, was inefficient. The results of the present study demonstrate that POLQ can efficiently catalyze extension from all types of mispaired termini opposite either A or T. The biochemical properties of POLH and POLQ, along with the frequency and patterns of the Ig gene SHM in mice singly or doubly deficient for these enzymes, raise the possibility that POLH and POLQ might function sequentially, with POLH first incorporating the wrong nucleotides opposite A or T. Some of these mismatches would then be extended by POLQ. The finding that POLH and POLQ together gave rise to
a slightly better mismatch extension as compared with POLH or POLQ alone is consistent with such a possibility. However, we were unable to observe a strong synergistic effect by POLH and POLQ on mismatch extension, even though we tested many different concentrations of these two enzymes. One possible explanation for the relatively small effect is that our assay contained only polymerases but not other accessory factors such as proliferating cell nuclear antigen, which plays a crucial role in polymerase switch (37, 38) and has been implicated in Ig gene SHM (39). Another possibility is that the collaboration
between POLH and POLQ only occurs under certain specific intracellular conditions during SHM of Ig genes and is thus difficult to demonstrate by simply including both enzymes in the same reaction in vitro. Further experiments are necessary to clarify whether POLH and POLQ indeed cooperate to generate mutations at A/T in vivo.

A two-polymerase bypass model has been proposed for the bypass of DNA lesions, including abasic sites, UV-induced (6-4) photoproducts, a thymine glycol and N-2-acetylamino-fluorene (40). In these studies, the extension step was primarily carried out by *Saccharomyces cerevisiae* Rev3L, the catalytic subunit of POLZ. Our results identify a proficient mismatch extender in mammalian cells and potentially imply such a sequential action by two different polymerases during the SHM process. Because POLQ only contributes to ~20% of the A/T mutations, additional polymerases are likely to catalyze the extension step from POLH-generated mispaired termini. Interestingly, *S. cerevisiae* Rev3L is also an efficient mismatch extender (41), raising the possibility that its mammalian counterpart may be another enzyme involved in this process. To explore this possibility, it may be possible to generate mice that express a catalytically inactive REV3 and examine whether these mice also have decreased A/T mutations, and such experiments are underway.

The A to G and T to C transitions were almost completely abolished in Polh−/− and Polq−/−Polh−/− mice. Because POLH most frequently misincorporates a G opposite template T and extends efficiently after the misincorporation, the A to G and T to C substitutions may largely be generated by POLH alone, and the contribution of other polymerases may be small. A to T and T to A changes were also greatly decreased, whereas A to C and T to G substitutions were decreased by only ~50%. Therefore, the remaining A/T mutations observed in Polh−/− and Polq−/−Polh−/− mice, mostly A to C and T to G substitutions, are likely generated by another polymerase.

The great reduction of A/T mutations in Polh−/− mice has been observed previously by several groups (12–14), but it remained unclear whether the overall mutation frequency was reduced or not. Our data clearly demonstrate that mutations at C/G were not affected by the absence of POLH. We have used GC B cells derived from spleen 2 weeks after immunization with a foreign antigen so that the mutations we observe are induced in a relatively short period by a defined antigen. With this method, the overall mutation frequency in WT mice was consistently ~1% and was highly reproducible among different studies (15, 17, 42, 43), allowing us to compare not only the overall mutation frequencies but also the frequency of each type of base substitutions. The current study revealed ~40% reduction of the overall mutation frequency in Polh−/− mice exclusively because of the greatly reduced A/T mutations. It will be interesting to investigate whether such a substantial reduction in the mutation frequency in Ig genes would affect B cell terminal differentiation, including memory B cell formation and plasma cell differentiation, as well as the affinity maturation of antibodies against various antigens.

Although deficiency in the MSH2 or MSH6 components of the MMR resulted in an ~80% reduction of A/T mutations, ~20% of A/T mutations were still observed. Interestingly, these residual A/T mutations were completely abolished in mice lacking both MSH2 and the uracil DNA glycosylase (UNG) (44). These observations suggest that POLH and POLQ are the major and minor pathways for the generation of A/T mutations, respectively (Fig. 5). After the submission of our manuscript, Delbos *et al.* (45) reported that mice deficient for both MSH6 and POLQ completely lacked A/T mutations. These results suggest that the residual A/T mutations generated in the UNG-dependent pathway also require POLH. Because UNG removes U to generate abasic sites, the residual A/T mutations are thus likely generated during the replication and/or error-prone processing of these abasic sites. Abasic sites are normally repaired by the base excision repair pathway. One possibility is that POLH may be recruited to the long patch base excision repair to generate A/T mutations (Fig. 5). Another possibility is that POLH may replicate over the abasic site to generate mutations at C/G and continue the polymerization to generate A/T mutations before being replaced by a high fidelity DNA polymerase (Fig. 5, *dashed arrow*). This possibility, however, seems unlikely because we did not observe any decrease of C/G mutations in the absence of POLH.

Based on genetic data, Neuberger *et al.* (6, 8) proposed a model for Ig gene SHM. Accordingly, AID is targeted to the Ig gene locus by a yet unknown transcription-dependent mechanism and deaminates C to generate a U:G lesion. Mutations are induced during the replication and repair processes of this U:G mismatch. First, direct replication of the U:G mismatch results in C to T and G to A transitions. U can also be excised by UNG, and the replication of the resulting abasic site generates transitions and transversions at C/G. The U:G mismatch can also be resolved by either MMR or UNG-dependent pathways to generate mutations at A/T. In both pathways, POLH is the primary enzyme that introduces mismatches opposite A/T. Our data further suggest that POLH, POLQ, and perhaps REV3 function to catalyze the extension from a part of the mismatches generated by POLH (Fig. 5). Mice deficient for both POLH and POLQ have provided us with intriguing insights into how these two polymerases interact genetically and possibly collaborate to generate A/T mutations. It will therefore be interesting and informative to analyze mice deficient for other combination pathways.
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of polymerases that are known to have a role in SHM of Ig genes and to investigate how the switching between these polymerases is achieved.

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