In vitro reconstitution of *Thermococcus* species 9°N Okazaki fragment maturation

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ABSTRACT

During replication, Okazaki fragment maturation is a fundamental process that joins discontinuously synthesized DNA fragments into a contiguous lagging strand. Efficient maturation prevents repeat sequence expansions, small duplications and generation of double-stranded DNA breaks.To address the components required for the process, Okazaki fragment maturation was reconstituted *in vitro* using purified proteins from *Thermococcus* species 9°N. The similarities to both bacterial and eukaryotic systems and evolutionary implications of archaeal Okazaki fragment maturation are discussed.

INTRODUCTION

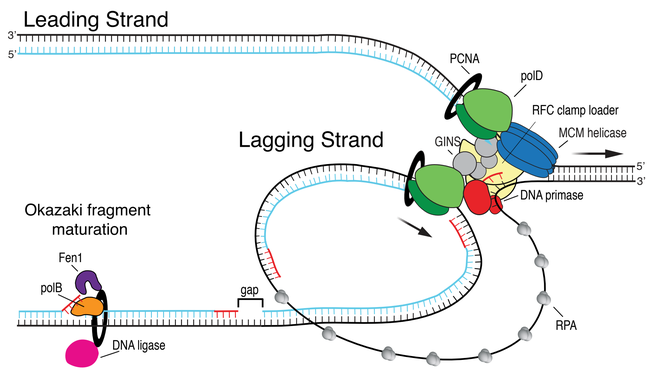

FIGURE 1. Model of leading and lagging strand replication in *Thermococcus*. A model of *Thermococcus* replication is based on genetic deletion (1) and protein interaction (2) data and this study. MCM helicase (blue) unwinds DNA in the 3'-5' direction. PolD (green) in complex with PCNA (black) synthesizes the leading strand continuously. DNA primase (red) primes lagging strand synthesis and polD/PCNA extends until it stops before the 5'-terminus of the downstream fragment. PolD then releases the replication loop leaving a gap that is filled in with polB (orange) with PCNA. PolB strand displacement creates a flap that is cleaved by Fen1 (purple) and the nick ligated by DNA ligase (pink).

DNA replication is a conserved process throughout all domains of life. Due to the antiparallel nature of double-stranded DNA and the uni-directionality of DNA polymerases, the leading strand replicates continuously while the lagging strand is synthesized discontinuously from a series of Okazaki fragments. The Okazaki fragment on the lagging strand starts as a short RNA primer synthesized by the primase. Then the processivity factor (the sliding clamp) assembles around the primer and binds DNA polymerase. The DNA polymerase-sliding clamp extends the RNA primer to synthesize thecomplementary strand. To form an uninterrupted lagging strand, RNA primers are removed, the gap in the DNA is filled and the Okazaki fragments are joined together. Each domain of life accomplishes this objective using different mechanisms and components. In bacteria, the lagging strand is synthesized by DNA polymerase III holoenzyme (pol III) while DNA polymerase I (pol I) is the major polymerase that carries out Okazaki fragment maturation. Pol I uses its polymerase activity to extend nicks or gaps left by pol III and its 5'-3' exonuclease activity to degrade the downstream RNA primer. The nick is sealed by DNA ligase. In eukarya, the same requirements are fulfilled using a different repertoire of enzymes. The lagging strand polymerase, pol δ, synthesizes the lagging strand and displaces the RNA primers into a flap structure. Flap endonuclease (Fen1) removes the flap and the nick is sealed by DNA ligase to generate a continuous double-stranded DNA.

Lagging strand synthesis and Okazaki fragment maturation are not as well understood in euryarchaea. The majority of characterized archaeal species (excluding the known Crenarchaea) encode both members of Family B DNA polymerase (polB) as well as the archaeal specific Family D DNA polymerase (polD). Several lines of evidence suggest that polD is the main replicative polymerase for both the leading and lagging strand synthesis. In some species, polD is the only essential DNA polymerase for cell viability while in others, both polB and polD are required. PolD forms complexes with several key replisome proteins *in vivo* while polB does not. In addition, the ability of polD to efficiently extend an RNA primer fulfills a requirement for both a leading and lagging strand DNA polymerase. Thus, polD was suggested to replicate at least the lagging strand and likely the leading strand as well.

In this study, the roles of polD and polB during Okazaki fragment maturation were evaluated using *in vitro* assays with proteins purified from *Thermococcus* species 9°N or cell extracts. The data suggest that Okazaki fragment maturation is a hybrid of the bacterial and eukaryal systems.

RESULTS

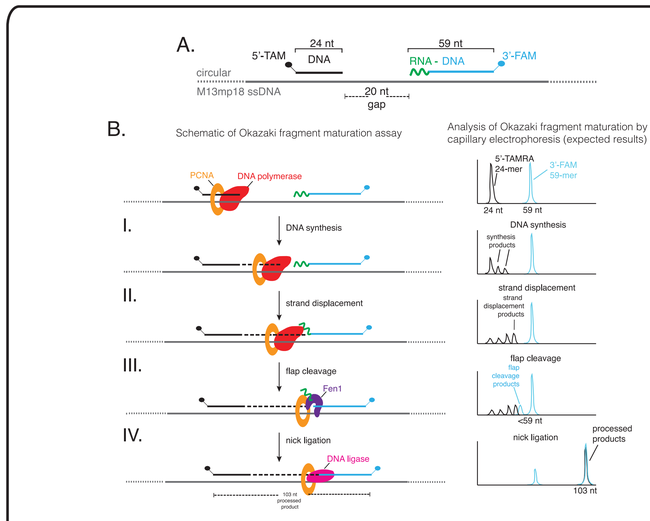

FIGURE 2. Dual-label fluorescence assay to monitor Okazaki fragment maturation. (A) An Okazaki fragment maturation substrate was prepared by annealing a 5'-TAM-labeled extension primer (24 nt, shaded black), a 3'-FAM-labeled blocking oligonucleotide containing 10 nt 5' RNA (green) followed by 49 nt DNA (blue) and circular single-stranded M13mp18 DNA. (B) A simplified schematic of Okazaki fragment maturation and expected results of capillary electrophoresis. (I.) Together with PCNA/RFC, a DNA polymerase initiates synthesis from the 5'-TAM-extension primer resulting in products longer than 24 nt. (II.) DNA polymerase strand displacement of the downstream Okazaki fragment creates flap structures. (III.) Fen1 cleavage products are observed as shorter 3'-FAM-labeled products. (IV.) The remaining DNA:DNA nick is then sealed by DNA ligase to generate a dual 5'-TAM- and 3'-FAM-labeled processed Okazaki fragment (103 nt).

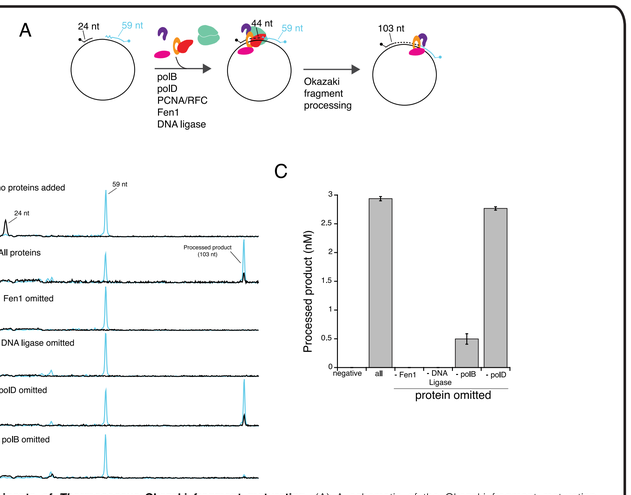

FIGURE 3. Determinants of *Thermococcus* Okazaki fragment maturation. (A) A schematic of the Okazaki fragment maturation experiment is shown. The processing substrate was prepared by annealing a 5'-TAM-labeled extension primer (shaded black) and 3'-FAM-labeled blocking oligonucleotide (shaded blue) to ssM13 as described in the text. 9°N polB, polD, PCNA/RFC, Fen1, and DNA ligase were incubated with the processing substrate at 60°C for 15 minutes. Processed products (103 nt) result from complete Okazaki fragment maturation. (B) Okazaki fragment maturation assays were performed without any proteins added, all proteins added (9°N polB, polD, PCNA/RFC, Fen1, and DNA ligase), or in reactions that omit one protein as noted in the figure. Reaction products were analyzed by capillary electrophoresis. 5'-TAM-labeled extension primer is shaded black and 3'-FAM-labeled blocking oligonucleotide is shaded blue. Processed products (103 nt) result from a complete Okazaki fragment maturation assay and are labeled with both 5'-TAM (black) and 3'-FAM (blue). (C) Processed products from reactions omitting various replication proteins in panel (B) were quantitated and plotted. The data shown are averages with standard deviations from three independent experiments.

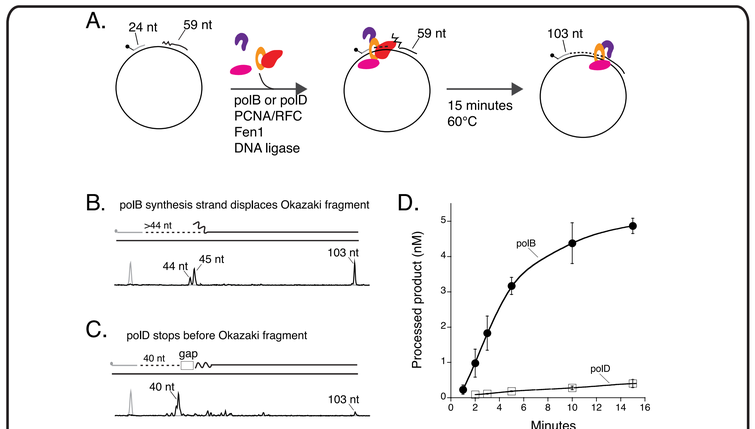

FIGURE 4. PolB strand displacement is required for efficient Okazaki fragment maturation. (A) A schematic of the Okazaki fragment maturation experiment is shown. A DNA polymerase with PCNA/RFC extends a primer. Processed Okazaki fragments result from DNA polymerase strand displacement of the downstream blocking oligonucleotide followed by processing by Fen1 and DNA ligase. Okazaki fragment maturation assays were carried out with either (B) polB or (C) polD as the DNA polymerase. (B) PolB strand displacement synthesis (>44 nt) efficiently creates flap structures that are subsequently processed by Fen1 and DNA ligase (103 nt). (C) A majority of polD synthesis stops upstream of the blocking oligonucleotide (40 nt) leaving a 4 nt gap. A residual amount of polD synthesis continues to generate flaps for Okazaki fragment maturation (103 nt). (D) Quantification of Okazaki fragment maturation. The data shown are averages with standard deviations from three independent experiments with either polB (filled circles) or polD (open squares).

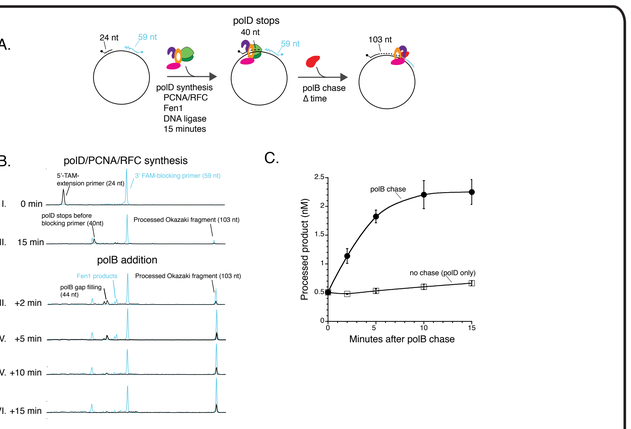

FIGURE 5. PolB completes Okazaki fragment maturation after polD synthesis stops. (A) Schematic of Okazaki fragment maturation experiment. PolD, PCNA/RFC, Fen1 and DNA Ligase are incubated with substrate for 15 minutes at 60°C as described in Materials and Methods. After 15 minutes of incubation, polB was added and reaction aliquots were sampled after 2, 5, 10, and 15 minutes and halted with 0.25 M EDTA. (B) Reaction products were resolved and analyzed by capillary electrophoresis. DNA polymerase synthesis products are 5'-TAM-labeled (black), Fen1 products are 3'-FAM-labeled (blue) and Okazaki fragment maturation products are labeled with both 5'-TAM and 3'-FAM (black and blue). (C) The data shown are averages with standard deviations from three independent experiments with either polB chase (filled circles) or no chase (polD alone, open squares).

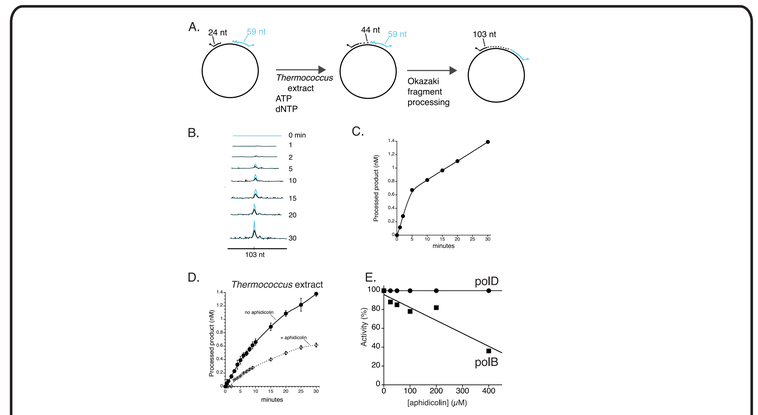

FIGURE 6. Okazaki fragment maturation in *Thermococcus* extracts. Thermococcus extract was prepared and incubated with the Okazaki fragment maturation substrate at 60°C (schematically illustrated in panel A). Reaction aliquots were sampled at 1, 5, 10, 15, 20, 25, and 30 minutes and halted with EDTA. Processed Okazaki fragment products (103 nt) were detected by capillary electrophoresis (B) and quantified (C). (D) Tko extract was incubated with the Okazaki fragment maturation substrate at 60°C in the presence (200 μM; open diamonds) or absence of aphidicolin (filled circles) and analyzed as above. (E) DNA synthesis by purified polB (10 nM) (filled squares) and polD (10 nM) (filled circles) was measured in the presence of increasing concentrations of aphidicolin (0 – 400 μM) and plotted as a percentage of activity in a reaction without aphidicolin.

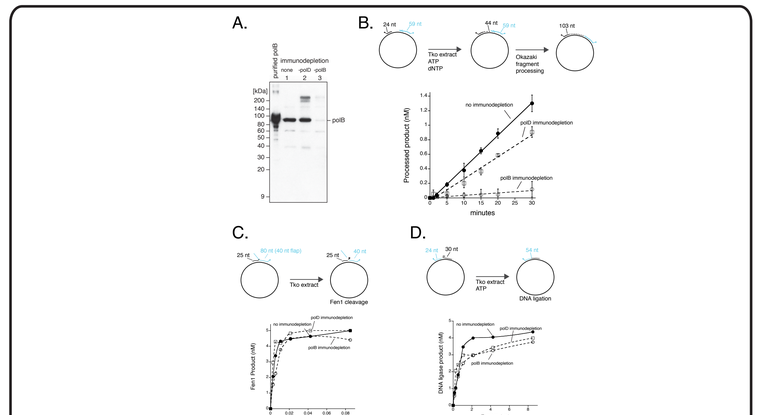

FIGURE 7. Okazaki fragment maturation in polB or polD immunodepleted Tko extracts. Either anti-polB or anti-polD antibodies were used to immunodeplete polB or polD from Tko extract. (A) Purified polB or immunodepleted Tko extracts were separated by electrophoresis, transferred onto membranes, and probed by incubation with rabbit polyclonal antisera raised against pol B. Tko extracts without immunodepletion (filled circles) or immunodepleted for polB (open circles) or polD (open squares) were assayed for (B) Okazaki fragment processing activity, (C) Fen1 activity and (D) DNA ligase activity as described in the text.

CONCLUSIONS

- PolD is essential for viability in *Thermococcus* and may synthesize both the leading and lagging strands.
- PolD synthesis stops before downstream Okazaki fragments and requires polB for strand displacement and subsequent processing.
- In *Thermococcus*, efficient Okazaki fragment processing requires polB, flap endonuclease and DNA ligase.
- Okazaki fragment maturation in *Thermocccus* shares similarities to both bacterial and eukaryal systems.

SIMPLIFIED MODEL OF OKAZAKI FRAGMENT MATURATION

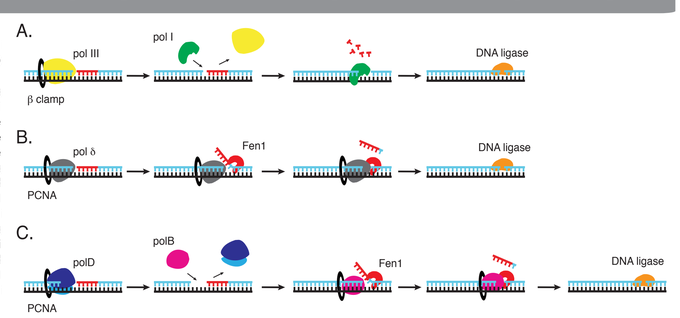

FIGURE 8. Simplified models of Okazaki fragment maturation in (A) bacteria, (B) eukarya and (C) *Thermococcus* species 9°N (A) In bacteria, pol III synthesizes the lagging strand. Pol I replaces pol III to complete Okazaki fragment maturation. Pol I 5'-3' exonuclease removes the RNA primer as its DNA polymerase activity fills the gap. DNA ligase seals the Okazaki fragments. (B) The eukaryal lagging strand DNA polymerase, pol δ, strand displacement activity generates a flap for Fen1 cleavage. DNA ligase seals the Okazaki fragments. (C) PolD synthesizes the lagging strand and stops at a downstream Okazaki fragment. PolB replaces polD and its strand displacement activity generates a flap for Fen1 cleavage. DNA ligase seals the Okazaki fragments.