
***In vitro* reconstitution of RNA primer removal in Archaea reveals the existence of two pathways**

Ghislaine Henneke^{a, b, c, *}

^a Ifremer, UMR 6197, Laboratoire de Microbiologie des Environnements Extrêmes, BP 70, 29280 Plouzané, France

^b Université de Bretagne Occidentale, UMR 6197, Laboratoire de Microbiologie des Environnements Extrêmes, 29280 Plouzané, France

^c Centre National de la Recherche Scientifique, UMR 6197, Laboratoire de Microbiologie des Environnements Extrêmes, 29280 Plouzané, France

*: Corresponding author : Ghislaine Henneke, email address : gheenneke@ifremer.fr

Abstract:

Using model DNA substrates and purified recombinant proteins from *Pyrococcus abyssi*, I have reconstituted the enzymatic reactions involved in RNA primer elimination *in vitro*. In my dual-labelled system, polymerase D performed efficient strand displacement DNA synthesis, generating 5'-RNA flaps which were subsequently released by Fen1, before ligation by Lig1. In this pathway, the initial cleavage event by RNase HII facilitated RNA primer removal of Okazaki fragments. In addition, I have shown that polymerase B was able to displace downstream DNA strands with a single ribonucleotide at the 5'-end, a product resulting from a single cut in the RNA initiator by RNase HII. After RNA elimination, the combined activities of strand displacement DNA synthesis by polymerase B and flap cleavage by Fen1 provided a nicked substrate for ligation by Lig1. The unique specificities of Okazaki fragment maturation enzymes and replicative DNA polymerases strongly support the existence of two pathways in the resolution of RNA fragments.

Keywords: Archaea ; DNA ligase ; DNA polymerase ; nuclease ; Okazaki fragment

Abbreviations used: Cy5, indodicarbocyanine ; FAM, 6-carboxyfluorescein ; Fen1, flap endonuclease 1 ; Lig1, DNA ligase 1 ; *Pab*, *P. abyssi* ; PCNA, proliferating-cell nuclear antigen ; PIP, PCNA-interacting protein ; pol α , polymerase α ; pol B, family B DNA polymerase ; pol D, family D DNA polymerase ; pol δ , polymerase δ .

INTRODUCTION

Accurate elimination of RNA initiators at the replication fork is a crucial process to ensure genome integrity. In eukaryotic cells, RNA initiators, 7-12 nucleotides (nt) in length, are generated by the primase activity of DNA polymerase α /primase (pol α /primase) and prime DNA synthesis of both leading and lagging strands. These initiating RNA primers are then further extended to around 20 deoxynucleotides by the polymerase activity of pol α /primase before handing over to the replicative DNA polymerase [1]. Whereas the leading strand is continuously replicated by DNA polymerase ϵ (pol ϵ) [2], the lagging strand of opposite polarity is discontinuously synthesized by DNA polymerase δ (pol δ) [3] and consists of multiple segments called Okazaki fragments, each of roughly 130-150 nt [4]. There is considerable evidence that pol δ displaces the RNA initiator of the downstream Okazaki fragment and creates 5'-tails [5]. Short tail intermediates are cleaved by the flap endonuclease (Fen1) to generate a nick for ligation by DNA ligase 1 (Lig 1), a prevailing pathway in wild-type replicating cells supported by genetic and biochemical evidence [6-8]. Failure of this process occasionally leads to long flap formation [6], a situation that requires the coordinated activities of two nucleases, Dna2 and Fen1, and the single-stranded binding protein, replication protein A (RPA), to fully ensure RNA primer removal in yeast [9-12]. Human Dna2 has recently been described as a nuclease for processing mitochondrial Okazaki fragments [13]. Ultimately, the dysfunctional maturation mechanism can be counteracted by an alternative pathway involving two nucleases, RNase H and Fen1 [14]. In the second pathway preliminary removal of RNA initiators is conducted by RNase H, leaving a single ribonucleotide at the 5'-end of the downstream primer, before displacement DNA synthesis by pol δ holoenzyme. The resulting flap is then cleaved by Fen1, generating a nick for DNA ligation [15]. While Okazaki fragment processing has been intensively examined, RNA primer removal at the leading strand remains unknown in eukaryotic cells.

In archaeal cells, RNA-primed replication intermediates, similar to those formed during eukaryotic DNA replication, have been observed in euryarchaea and crenarchaea [16], arguing for either RNA or RNA-DNA primers at the archaeal replication fork. RNA priming seems to involve a eukaryotic-like primase complex [17-21]. However, there is currently no direct evidence that archaeal primases are responsible for RNA primer elongation. Biochemical data accumulated so far indicated that the family D DNA polymerase (pol D) would extend RNA primers both at the leading and lagging strands in euryarchaea, followed by DNA synthesis at the lagging strand [22]. Furthermore, switching from pol D to the replicative family B DNA polymerase (pol B) on the homotrimeric DNA sliding clamp (PCNA) has been shown to confer processive DNA synthesis at the leading strand [23]. As in bacteria and eukaryotes, the DNA sliding clamp is an essential protein [24-26], acting as a stimulatory factor to pol B and pol D in archaeal DNA transactions [27-29]. In crenarchaea, multiple family B DNA polymerases (designated B1, B2 and B3) [30, 31] have been identified and observed mediating interactions with their cognate PCNA [32, 33]. Crenarchaeal PCNA organizes as a heterotrimer and contacts simultaneously DNA polymerase B1, Lig1, or Fen1, imposing a defined architecture at the lagging strand [33, 34]. More recently functional assessment of this structural organization argues for the coordination of multiple enzyme activities by a single PCNA in Okazaki fragment maturation [35]. On the other hand, euryarchaeal PCNA interacts with maturation enzymes [25, 36, 37], raising the possibility that a eukaryotic-type Okazaki fragment maturation process is functional in these cells. However, the molecular mechanism required for RNA primer elimination at the leading strand remains to be determined in archaea.

Pyrococcus abyssi (*P. abyssi*), a hyperthermophilic and anaerobic euryarchaeon that grows optimally at 95°C and pH 6.8 [38], has proved to be an excellent model to analyze DNA

replication because it contains a simplified eukaryotic-like replication machinery [39]. Consistent with this statement, the structure and size of RNA-primed replication intermediates (~150 nt in length with RNA primer of ~12 nt) are similar to eukaryotic Okazaki fragments [16]. *P. abyssi* possesses a core version of the eukaryotic maturation enzymes: two DNA polymerases (pol B and pol D), Fen1, Lig1, PCNA and a type 2 RNase H (RNase HII). In order to examine the specific reactions involved in RNA primer elimination, these recombinant proteins have been purified and used to reconstitute Okazaki fragment processing *in vitro*. We found that two functional pathways may be responsible for RNA primer removal in *P. abyssi*.

EXPERIMENTAL

Enzymes and Proteins. All the enzymes used were prepared and purified as previously described [25, 40, 41] with the exception of Lig1 which was purified with Ni-NTA spin columns (Qiagen). The purity of proteins was controlled by SDS-PAGE (data not shown).

DNA substrates. Oligonucleotides were chemically synthesized and HPLC-purified by Eurogentec (Belgium), with all sequences listed in Supplementary Table 1. The location of the 5'-FAM or 3'-Cy5 fluorescent-label on primer is differentiated by a full circle or an asterisk, respectively. Construction of the primer-template minicircle was previously described [42]. To generate the substrates, complementary primers were annealed to the respective template as previously described [22, 42] and the resulting substrates were gel-purified from excess primer. The dual-labeled substrate allows simultaneous detection of upstream and downstream primers in a single reaction, providing multiple correlated data from a single reaction.

DNA replication assays. *PabpolB* assays (10 μ l) contained 50 mM Tris-HCl (pH 8.8), 50 mM KCl, 1 mM dithiothreitol, 2 mM MgCl₂, 0.25 pmol (3'-OH ends) of fluorescent-labeled primed synthetic substrate and 200 μ M of each dNTPs (additional components are mentioned in Figure legends). Various amounts of enzymes and proteins were incubated with appropriate substrate as indicated in Figure legends. Reactions were performed at 60°C for 30 min, unless otherwise stated. Reactions were terminated by addition of 10 μ l of formamide containing 10 mM EDTA, followed by heating at 95°C for 5 min. Labeled products were separated by denaturing PAGE as already described [42]. Products resulting from coupled-activity of *PabpolB* and *PabRNase HII* were resolved on 8 % native PAGE, followed by staining with SYBR Gold (Invitrogen). Product analyses were performed with an Imager Typhoon 9400 (GE Healthcare) and quantification was carried out using ImageQuant 5.2 software. In all cases, the percentage of substrate hydrolysis, displaced strands or ligation was determined by the products / (products + substrate) ratio. *PabpolD* assays (10 μ l) contained 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 5 mM MgCl₂, 0.25 pmol (3'-OH ends) of fluorescent-labeled primed synthetic oligonucleotide and 200 μ M of each dNTPs (unless otherwise stated). Reactions were performed and analyzed as described above.

RESULTS

***PabRNase HII* is required to initiate strand displacement DNA synthesis by *PabpolB*, followed by *PabFen1* cleavage**

We initially determined whether active ribonuclease function could be required to provide an appropriate substrate for *PabpolB* strand displacement as recently suggested [43]. To address this question, we used a minicircle DNA template annealed to an unlabeled RNA-DNA primer, since *PabRNase HII* cleavage would have hampered the detection of *PabpolB* displacement products. Our detection assay was based on fluorescent gel staining which allowed detection of the products from the combined enzymatic activities. As expected, *PabRNase HII* alone displayed efficient cleavage activity (Figure 1A, lane 3). In this case, the bands representing cleavage products shifted to lower positions (Figure 1A, compare lane 1 and lane 3). When *PabpolB* was titrated into the reaction, a distribution of longer products appeared (Figure 1A, lanes 4-7). Strand displacement mostly occurred on substrates in which RNA initiators, annealed to the minicircle DNA template, have been hydrolyzed, as shown by the decrease of short products. *PabRNase HII* specifically cuts one nucleotide upstream of the RNA-DNA junction, leaving a single ribonucleotide at the 5'-end of the primer on this labeled substrate (Supplementary Figure S1A). Control reactions were carried out with *PabpolB* alone and the results were consistent with its inability to displace RNA-DNA segments (Figure 1A, lane 2) (see also Supplementary Figure S2C). Moreover, addition of increasing amounts of *PabPCNA* did not initiate strand displacement activity by *PabpolB* (Supplementary Figure S2D). These results suggested that initial nuclease activity on RNA initiators by *PabRNase HII* is required to promote displacement of the resulting 5'-phosphorylated junction ribonucleotide attached to DNA by *PabpolB*. It is of interest to note that strand displacement activity by *PabpolB* was not dependent of the nature of the monoribonucleotide (data not shown). In a plausible model for RNA primer elimination, one could envisage that after strand invasion by the polymerase, the resulting flap could be cut by the 5'-flap endonuclease, *PabFen1*. This prediction was assessed on the minicircle DNA template annealed to a fluorescent 5'-end labeled monoribonucleotide DNA primer (Figure 1B). This substrate allows a direct measurement of strand displacement DNA synthesis coupled to flap cleavage. In the conditions tested, *PabpolB* alone carried out strand displacement DNA synthesis with accumulated products of about 97 nt in length (~16 % displ after 10 min), resulting from ~10 nt of strand displacement (Figure 1B, lane 2). When *PabFen1* was titrated into the reaction, the major distribution of cleavage products was in the 3-6 nt size range with a greater accumulation of shorter fragments at the highest enzyme concentration (Figure 1B, lane 7) (~15 % cleavage). Control assays containing *PabFen1* without DNA polymerase showed no cleavage products (Figure 1B, lane 8). Interestingly, a direct relationship between the loss of 5'-flaps and the formation of *Fen1* cleavage products was noticeable. These results indicate that the coordination between *PabpolB* and *PabFen1* is functional and preferentially leads to cleavage of short flaps. This was not the consequence of a limited size flap cleavage capability by *PabFen1* since the enzyme could catalyze the hydrolysis of 16 nt flaps (Supplementary Figure S1B). When *PabFen1* was supplemented at different time intervals, the distribution of cleavage products varied (Figure 1C). At time 0, 60 % of cleavage products were in the 1-12 nt size range. At time 5 min, a steady state situation between shorter and longer products was detected. At time 15 min, 62 % of cleavage products were 20-25 nt in size. Similar to what was observed when *PabFen1* was present from the beginning of the reaction, shorter products (1-12 nt) were preferentially generated. However, when *PabpolB* was allowed to displace longer DNA tracts (at least 25 nt in 15 min) prior to the addition of *PabFen1* longer cleavage products were formed. Thus, these results show that the flap continuously generated by *PabpolB* strand displacement can be cut by *PabFen1*.

Coordinated activities of *PabpolB* and *PabFen1* on monoribonucleotide-primed DNA replication intermediates produce ligatable nicks

To reconstitute the final step of RNA primer removal, a dual-labeled system was used to allow simultaneous detection of the resulting upstream and downstream products in a single reaction. The substrate mimics an RNA-primed replication intermediate which can be generated during the maturation process. On this substrate, complete strand displacement DNA synthesis by *PabpolB* (~66 % displ) was observed, as shown by the synthesis of full-length products (87 nt) (Figure 2, lane 5; see FAM panel). Strand displacement DNA synthesis of the monoribonucleotide DNA hybrid was attributed to pausing of the polymerase in the vicinity of the 57 nt position. Two pause sites at 57 nt and 62 nt, corresponding to the “nick” position (57 nt) and + 5 position (62 nt), were also observed (Figure 2, lane 5). Furthermore, a faint band appeared at a lower size (80 nt) than the full-length products (87 nt), indicating aborted DNA synthesis. This is indicative of the presence of persistent secondary structures formed by the DNA, as deduced by the primary sequence, presumably causing the dissociation of the polymerase from the DNA template. Addition of *PabFen1* did not alter the efficiency of *PabpolB* strand displacement DNA synthesis (~69 % displ), (Figure 2, lane 6; see FAM panel) nor the presence of pause sites. *PabFen1* cleavage activity mainly released products 3-12 nt in length (Figure 2, lane 6; see Cy5 panel). These results are in agreement with those obtained in Figure 1B showing that *PabFen1* cleaves the flap continuously generated by *PabpolB* strand displacement, releasing products of a preferential size range.

PabPCNA has been described as a molecular platform since it specifically interacts not only with DNA polymerases but also with maturation enzymes [25, 27]. When added to *PabpolB* in the maturation assay, strand displacement DNA synthesis was not affected (~71 % displ) (Figure 2, lane 7; see FAM panel), although stimulation of primer extension was observed with the control (Supplementary Figure S2D). On the other hand, *PabPCNA* helped to bypass secondary structures (62 and 80 nt), resulting in a higher amount of full-length products (87 nt) (Figure 2, compare lanes 5 and 7; see FAM panel). In the presence of *PabFen1* and *PabPCNA*, cleavage efficiency remains constant (~42 %) but shorter cleavage products were released by *PabFen1* (3-15 nt) (Figure 2, compare lanes 6 and 8 in Cy5 panel).

When *PabLig1* was included in the strand displacement reaction, a dual-labeled band migrating at a higher position than the full-length synthesis product (87 nt) was observed (Figure 2, lane 15; see FAM and Cy5 panels). This DNA fragment, running slower than the full-length extension product because of the attached Cy5, represented ligation products. In these conditions, the total percentage of ligated products was 61 %. Ligation obviously resulted from a combination of *PabpolB* strand displacement and *PabFen1* cleavage, as deduced by the loss of *PabFen1* cleavage products (Figure 2, lane 6 compared with lane 15 in Cy5 panel). Moreover, *PabpolB* strand displacement DNA synthesis was strongly stimulated by *PabLig1* (~91 % displ), as observed by the accumulation of full-length products and the concomitant disappearance of pause sites (Figure 2, compare lanes 5 and 14; see FAM panel). This unanticipated result suggests that the polymerase and DNA ligase likely cooperate to enhance strand displacement. Addition of *PabPCNA* did not impact on ligation efficiency (Figure 2, compare lanes 15 and 17 in Cy5 panel). This observation is interesting, since functional interaction between *PabPCNA* and *PabLig1* was detectable (Supplementary Figure S4B). Altogether, these data demonstrate the coordination of strand displacement and flap cleavage activities by *PabpolB* and *PabFen1* to achieve efficient DNA ligation by *PabLig1* on DNA replication intermediates.

Coordinated activities of *PabpolD* and *PabFen1* on RNA-primed DNA replication intermediates produce ligatable nicks

Previous reports indicated that *PabpolD* is the lagging strand DNA polymerase, performing RNA primer extension and strand displacement in the presence of *PabPCNA* [22, 23]. Others demonstrated that Okazaki fragments are initiated with short RNA primers (~12 nt) followed by extension with DNA [16]. To verify whether *PabpolD* was capable of displacing the RNA initiator at the 5'-end of the downstream primer, our reconstituted dual-labeled system was used. Time course experiments indicated that *PabpolD* displaced RNA-DNA primers (Supplementary Figure S2A), this activity was further stimulated by *PabPCNA* (Supplementary Figure S2B). However, when these reactions were performed with *PabpolB* alone (Supplementary Figure S2C) or in the presence of *PabPCNA* (Supplementary Figure S2D), strand displacement DNA synthesis never occurred. The incubation of *PabFen1* and *PabpolD* in the maturation assay yielded extension products up to 87 nt (Figure 3, lane 6; see FAM panel) and cleaved flaps 2-12 nt in length (see Cy5 panel). Cleavage products accumulated (12 %) to the same extent than strand displacement products (17 % displ) generated by *PabpolD*. Primer extension and DNA synthesis through pause sites in the vicinity of the 57 nt (nick) position were moderately enhanced in the presence of *PabFen1* (Figure 3, compare lanes 5 and 6; see FAM panel). When *PabPCNA* was added to the same reactions, strand displacement DNA synthesis (70 % displ) and *PabFen1* cleavage activity (42 %) were markedly increased, but the pattern of cleavage products was not affected (Figure 3, lane 6 compared with lane 8 in FAM and Cy5 panels). Thus, our results show that *PabFen1* cuts the flaps (not exceeding 12 nt in length) that are continuously generated by *PabpolD*. In order to reconstitute the final step of Okazaki fragment maturation, DNA ligase 1 was added. As expected, ligation only occurred in the presence of *PabFen1* (Figure 3, lane 15; see Cy5 panel). All ligated products derived from the combination of flap creation and total elimination of the RNA initiator. Consistently, control experiments indicated that *PabLig1* can not catalyze the formation of the phosphodiester bond between DNA and RNA (Supplementary Figure 4C). On the other hand, DNA ligation proceeded with 29 % efficiency when the monoribonucleotide downstream primer was used (Supplementary Figure 3, lane 15; see Cy5 panel). This suggests that elimination of the RNA portion of the downstream primer, prior to strand displacement DNA synthesis, likely contributes to enhance the maturation reaction. Although unexpected, addition of DNA ligase 1 clearly stimulated strand displacement DNA synthesis by *PabpolD* (6-fold) (Figure 3, lane 14 compared with lane 5 in FAM panel). Moreover, addition of *PabPCNA* resulted in an increase of strand displacement products (70 % displ) and *PabFen1* cleavage efficiency (40 %), while not affecting DNA ligation (Figure 3, lane 17 in both panels). Altogether, our data demonstrate that sequential enzymatic reactions by *PabpolD*, *PabFen1* and *PabLig1* lead to the generation of displaced strands which can be processed into ligatable products to create the continuous double-stranded DNA.

Initial cleavage event by *PabRNase HII* facilitates RNA primer removal of Okazaki fragments

Although our findings showed that *PabFen1* provides the nuclease activity for removal of RNA initiators on Okazaki fragments, a role for *PabRNase HII* could not be totally excluded. Our recent results have shown that *PabRNase HII* made structure-specific endonucleolytic cleavage in the RNA initiator, leaving a single ribonucleotide at the 5'-end of the RNA-DNA junction [43] (see also Supplementary Figure 1A), an activity which can occur once at the leading strand and much more frequently at the lagging strand. Therefore, we assessed the ability of the nuclease to degrade the RNA initiator using the reconstituted assay in the presence of all the maturation enzymes and either *PabpolD* or *PabpolB*. *PabRNase HII* could

hydrolyze the substrate generating a primary product, exactly the length (19 nt) of the DNA portion with a single ribonucleotide remaining at the 5'-end (Figure 4, lanes 2 and 11 in Cy5 panel). Neither specificity nor efficiency of cleavage by *PabRNase HII* was modified upon different combinations of maturation enzymes and replicative DNA polymerases (Figure 4, lanes 3-7 and lanes 12-16; see Cy5 panel). However, *PabpolD* strand displacement DNA synthesis was stimulated upon addition of *PabRNase HII* (Figure 4, compare lanes 8 and 9; FAM panel), as shown by the absence of pause products in the vicinity of the downstream primer (57 nt in length) and the increase of intensity of the full-length extension products (up to 81 % displ). Interestingly, the amount of ligation products, which resulted from total RNA elimination, increased by approximately three times in the presence of *PabRNase HII* (Figure 4, compare lanes 8 and 9; Cy5 panel).

Consistent with the results shown in Figure 1A, *PabRNase HII* was initially required to provide the monoribonucleotide substrate intermediate, thus promoting strand displacement DNA synthesis by *PabpolB* (Figure 4, lane 16; FAM panel). Indeed, all extension products accumulated at the position of the nick (57 nt) when *PabRNase HII* was omitted from the maturation assay (Figure 4, lane 17; FAM panel), and none of the maturation enzymes tested were able to initiate strand displacement activity. These results confirm the inability of *PabpolB* to displace RNA primers as shown in Supplementary Figure S2C-D. Accordingly, maturation assays performed initially with *PabRNase HII* followed by the addition of all maturation enzymes and *PabpolB* resulted in the generation of ligation products (Figure 4, lane 18; Cy5 panel). Altogether, these results show that the reconstitution of RNA primer excision with initial RNase HII cleavage event is functional with both replicative DNA polymerases, and that it implies timely orchestrated enzymatic reactions.

DISCUSSION

In this report, we have successfully reconstituted the reactions of RNA primer removal *in vitro* by using purified enzymes from the hyperthermophilic euryarchaeon *Pyrococcus abyssi*. We reported that pol B strand displacement DNA synthesis was restricted to a single ribonucleotide attached to a DNA segment. In this pathway, the initial cleavage event by RNase HII was shown to be required to provide a suitable substrate for pol B strand displacement. These data indicate that the combined activities of pol B and RNase HII are involved in the elimination of the RNA initiator. Moreover, they are in agreement with the recent findings that assigned pol B a role in leading strand DNA synthesis [22, 23] and described RNase HII as a potent maturation enzyme [43]. As a result of the action of the two enzymatic activities, flap structural intermediates were recognized and released by Fen1. Flap removal was entirely dependent on the tight coordination of strand displacement DNA synthesis and flap cleavage. In this mechanism, Fen1 cuts the flaps that are continuously generated by pol B, releasing products 2-12 nt in length. However, it is plausible that a delay in Fen1 action (shown in this paper) or uncontrolled strand displacement as observed in eukaryotes [44], results in the formation of lengthy flap structures. Although the occurrence of this event awaits further *in vivo* assessment in archaea, the resolution of these structural intermediates might involve a specific molecular mechanism yet undiscovered. Eventually, DNA ligase 1 was recruited to seal the resulting DNA nick. Unexpectedly, Lig1 was found to stimulate strand displacement DNA synthesis by *PabpolB*. Time course experiments indicated that Lig1 accelerates the rate of Fen1 cleavage activity, thus facilitating the displacement of the 5'-end monoribonucleotide primer by pol B (data not shown). Therefore, Lig1 seems to be an important component of maturation processing in archaea.

On model Okazaki fragment substrates [16], pol D exclusively carried out RNA strand displacement DNA synthesis in the presence of PCNA, acting as a stimulatory clamp for strand displacement and primer utilization. The dynamic process of strand displacement DNA synthesis by pol D coupled with Fen1 cleavage was clearly identified. These combined activities gave rise to a preferential size distribution of released flaps, with flaps not exceeding 12 nt in length. Consistent with the total elimination of the RNA portion of Okazaki fragments, ligation only occurred on substrates containing DNA ends with a 3'-OH termini and a 5'-phosphate. Moreover, we observed that RNase HII could participate early in this pathway, cutting specifically at the phosphodiester bond of the nucleotide 5' of the RNA-DNA junction. Removal of the last 5'-ribonucleotide was then performed by the action of pol D and Fen1 prior to DNA ligation. Although unanticipated, we found that Lig1 and RNase HII stimulated strand displacement activity by pol D, and that Fen1 slightly enhanced primer utilization. Such functional interactions have already been described in eukaryotes [45]. Further investigation is now required to analyze the specificity of the interplay between these enzymatic activities. This would contribute, for instance, to explain the weak ligation efficiency observed with all maturation enzymes on RNA-primed DNA replication intermediates.

Based on these findings, a model for RNA primer removal during DNA replication is represented in Figure 5. In the RNase HII-pathway, RNase HII initially degrades the RNA initiator. Following primer extension, either pol B or pol D encounters the double-stranded region, displacing the monoribonucleotide primer. The resulting flap is then recognized and cleaved by Fen1. As a final step, the resulting DNA nick is sealed by Lig1 to yield the continuous double-stranded DNA. In the absence of RNase HII, pol D encounters and then displaces the downstream RNA-DNA primer. Elimination of RNA flaps relies on the combined activities of pol D strand displacement DNA synthesis and Fen1 cleavage. As a final step, a DNA nick is created and subsequently sealed by Lig1.

Unlike the heterotrimeric crenarchaeal PCNA which has been recently shown to coordinate multiple enzymatic activities in the removal of RNA primers on a single ring [35], the homotrimeric euryarchaeal PCNA seems to have evolved a distinct mechanism. Through this study, we clearly demonstrated that PCNA could contribute to further enhance DNA replication through double-stranded DNA structures rather than stimulation of primer utilization by pol B, while stimulating both properties by pol D. Interestingly, a previous report identified one and two functional PCNA binding motifs in pol B and pol D, respectively [27]. PCNA binding was different for the two DNA polymerases, and it was suggested that the mechanism involved in polymerase recruitment and stimulation at the replication fork might be different. Our maturation assay gave the first evidence that PCNA can indeed confer differential properties to the DNA synthesis activities of the replicative DNA polymerases. On the other hand, the role of PCNA as a processivity factor helping the polymerase to disrupt hydrogen bonding of the downstream primer during strand displacement DNA synthesis was preserved [22]. When all enzymes were combined, PCNA exerted a stimulatory effect on Fen1 cleavage but did not influence DNA ligation by Lig1. It is probable that PCNA interaction with DNA polymerases and Fen1 might sterically prevent the simultaneous binding of additional partners. Similarly, it has been shown that the eukaryotic DNA ligase 1 do not function with the highest efficiency in the presence of the homotrimeric PCNA. It was proposed that Fen1 and/or the replicative DNA polymerase δ likely competed with DNA ligase 1 for the same binding site(s) on PCNA [6]. Therefore, it appears that the presence of three identical surfaces on PCNA may restrict and direct the access of target proteins. In this respect, Creze and coworkers recently described the functional and structural characterization of the complex formed between PCNA and NucS, a structure-specific DNA endonuclease. They demonstrated that only one molecule of the NucS homodimer binds to the outside surface of the PCNA homotrimer via the PIP/IDCL interface, leading to the conclusion that this particular stoichiometry could be due to long range conformational changes on PCNA that in turn could regulate the recruitment of additional partners [46]. Other factor(s) (e.g., local protein concentrations and/or affinities of the PIP motifs of the various proteins) might also be required for *in vivo* coordination of these functional interactions during the processing maturation in euryarchaea. It is interesting to note that post-translational modifications of proteins involved in DNA transactions have, as yet, not been identified in archaea.

Our *in vitro* reconstitution assay has provided mechanistic insights into how proteins known to be essential in archaea [24, 25] ensure the accurate and timely processing of RNA primers, an activity which can occur once at the leading strand or much more frequently at the lagging strand. In agreement with the recent study of Beattie and Bell [35], it appears that archaea evolved a minimal 5'-RNA containing tail as a safety control. In this mechanism, Fen1 cleaves the tails as soon as they are produced by the replicative DNA polymerases. However, it remains possible that other factors may be involved in this pathway. RNase HIII is one of the plausible candidates which could participate in RNA primer removal early in the maturation reaction, before displacement by replicative DNA polymerases. Intriguingly, these findings resemble predicted mechanistic pathways of RNA primer processing in mitochondria [47]. Thus, euryarchaea could serve as a good model to understand these highly ordered processes occurring not only in the nucleus of eukaryotes but also in mitochondria.

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FIGURE LEGENDS

Figure 1. *PabRNase HII* is required to initiate strand displacement DNA synthesis by *PabpolB*, followed by *PabFen1* cleavage. (A) The unlabeled minicircle primer-template (P2:T1) was preincubated at 60°C for 10 min in *PabpolB* reaction buffer before addition of dNTPs and *PabpolB*. Incubation was continued at 60°C for 30 min (lanes 4-7). Analysis was on an 8 % nondenaturing PAGE with SYBR Gold present. The structure of the RNA-DNA primer annealed to the minicircle DNA template is depicted at the top. Lane 1, control reaction without protein. Lanes 2 and 3 are the control reactions with enzymes alone, *PabpolB* and *PabRNase HII*, respectively. The *arrow* indicates nicked DNA minicircles (B) The structure of the 5'-FAM-labeled monoribonucleotide DNA primer annealed to the minicircle DNA template (P3:T1) is illustrated at the top. Reactions were run at 60°C for 10 min and products analyzed by denaturing PAGE as described under Experimental. *PabpolB* was incubated with varying amounts of *PabFen1* (lanes 3-7). Lanes 2 and 8 are the control reactions with individual enzymes, *PabpolB* and *PabFen1*, respectively. Lane 1, control reaction without enzyme. Lane *M* contains the 5'-FAM labeled 57 nt (C) Delayed addition of *PabFen1* to *PabpolB* strand displacement assays. Reactions used 5'-FAM-labeled monoribonucleotide DNA primer annealed to the minicircle DNA template (P3:T1) and 1 pmol of *PabFen1*, which was added at the time interval indicated after the start of reaction with *PabpolB*. Total incubation time is 30 min. Products were electrophoresed and quantified as described in Experimental. Relative distribution of flap cleavage products is expressed as the ratio of different product population sizes (1-12 nt, 12-19 nt and 20-25 nt) / total number of flap cleavage products.

Figure 2. Coordinated activities of *PabpolB* and *PabFen1* on monoribonucleotide-primed DNA replication intermediates produce ligatable nicks. The dual-labeled substrate (P1:P4:T1) is shown at the top. The monoribonucleotide portion is indicated by a thick line, and DNA is indicated by a solid black line. Reactions were performed in the presence of various combinations of *PabpolB* (0.5 pmol), *PabFen1* (0.5 pmol), *PabLig1* (2 pmol) and *PabPCNA* (0.5 pmol) in *PabpolB* buffer containing ATP (0.1 mM). Upper and lower gels refer to upstream (5'-FAM label) and downstream (3'-Cy5 label) resulting products, respectively. Lane 1 contains oligonucleotide ladders (32-, 57- and 87-nt) 5'-FAM labeled. *Plus* (+) denotes presence and *minus* (-) denotes absence of enzyme.

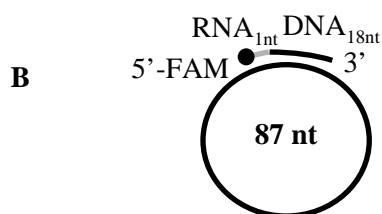
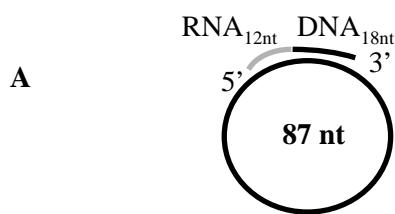
Figure 3. Coordinated activities of *PabpolD* and *PabFen1* on RNA-primed DNA replication intermediates produce ligatable nicks. The dual-labeled substrate (P1:P2:T1) is shown at the top. The RNA portion is indicated by a thick line, and DNA is indicated by a solid black line. Reactions were performed in the presence of various combinations of *PabpolD* (0.25 pmol), *PabFen1* (0.5 pmol) and *PabPCNA* (0.5 pmol) in *PabpolD* buffer containing ATP (0.1 mM) under the conditions described in Experimental. Upper and lower gels refer to upstream (5'-FAM label) and downstream (3'-Cy5 label) resulting products, respectively. Lane *M* contains oligonucleotide markers (32-, 57- and 87-nt) 5'-FAM labeled. *Plus* (+) denotes presence and *minus* (-) denotes absence of enzyme.

Figure 4. Initial cleavage event by *PabRNase HII* facilitates RNA primer removal of Okazaki fragments. The dual-labeled substrate (P1:P2:T1) is shown at the top. Initial cleavage events were performed by incubating *PabRNase HII* (1 pmol) at 60°C for 10 min. Subsequent reactions were carried out at 60°C for 30 min in the presence of various combinations of either *PabpolD* (0.25 pmol) (lanes 2-9) or *PabpolB* (0.5 pmol) (lanes 11-18), *PabFen1* (0.5 pmol), *PabPCNA* (0.5 pmol) and *PabLig1* (2 pmol) in the respective

polymerase buffer containing ATP (0.1 mM) under the conditions described in Experimental. Upper and lower gels refer to the upstream (5'-FAM label) and downstream (3'-Cy5 label) resulting products, respectively. *M* corresponds to oligonucleotide ladders (32-, 57- and 87-nt) 5'-FAM labeled. *Plus* (+) denotes presence and *minus* (-) denotes absence of enzyme.

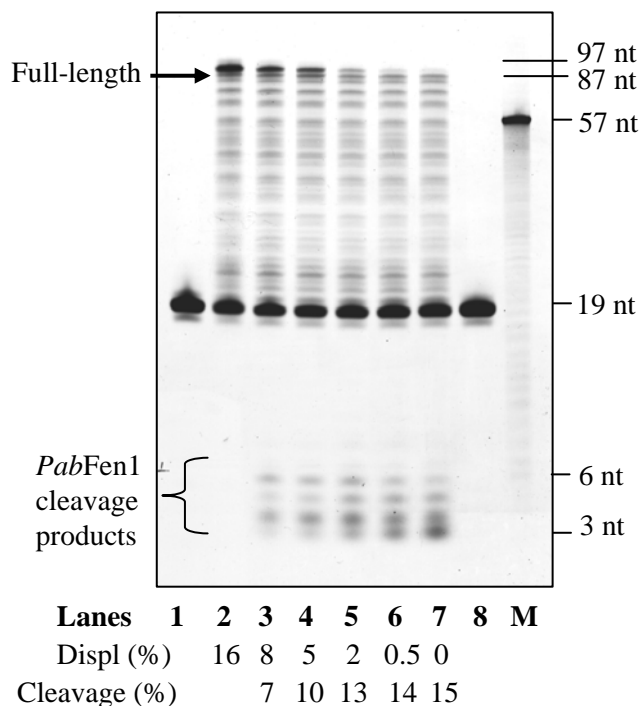
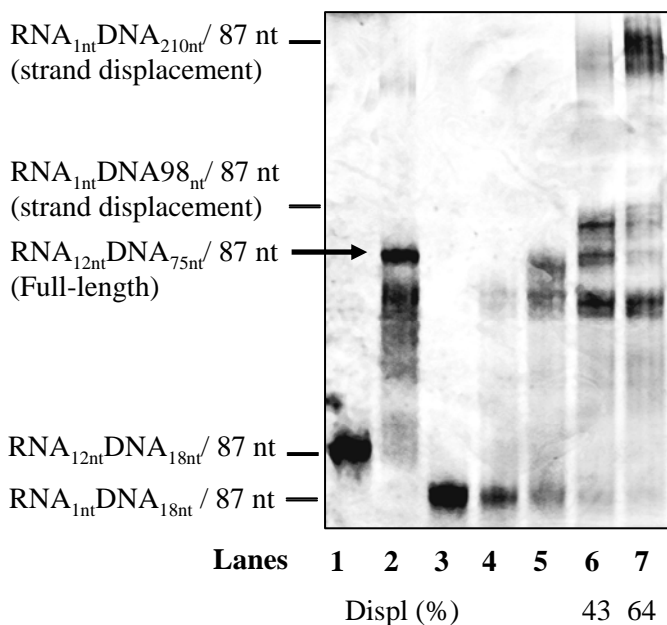
Figure 5. Model for RNA primer removal in archaea. Complete RNA primer elimination is ensured by continuous pol D strand displacement DNA synthesis and 5'-RNA flap cleavage by Fen1 (steps 1 and 2). These coupled activities are iterated until all RNA is degraded. Then, DNA ligase 1 is recruited to seal the resulting nick, generating the continuous double-stranded DNA (step 3). Alternatively, the processing maturation requires a site-specific cleavage by RNase HII of the RNA initiator (step 1'). Removal of the last 5' ribonucleotide is accomplished by Fen1 cleavage and strand displacement DNA synthesis by pol B or pol D (steps 2' and 3'). A nick is then generated between the two DNA termini (step 3') which are joined by DNA ligase 1 (step 4').

FIGURE 1



<i>PabpolB</i> (pmol)	0	2	0	0.25	0.5	1	2
1 pmol <i>PabRNaseHIII</i>	-	-	+	+	+	+	+

<i>PabFen1</i> (pmol)	0	0	0.06	0.125	0.25	0.5	1	1
0.5 pmol <i>PabpolB</i>	-	+	+	+	+	+	+	-



C

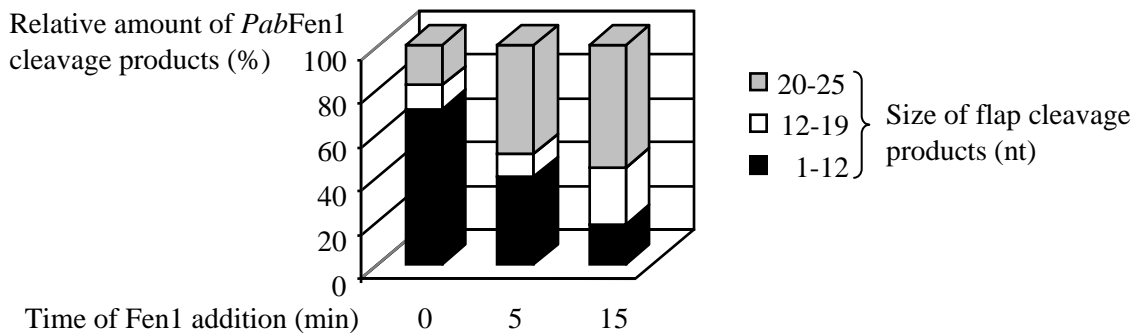
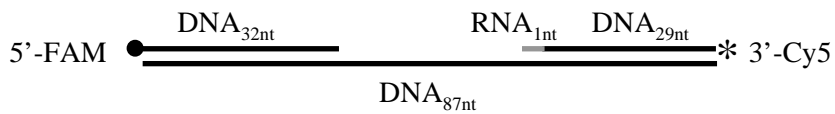
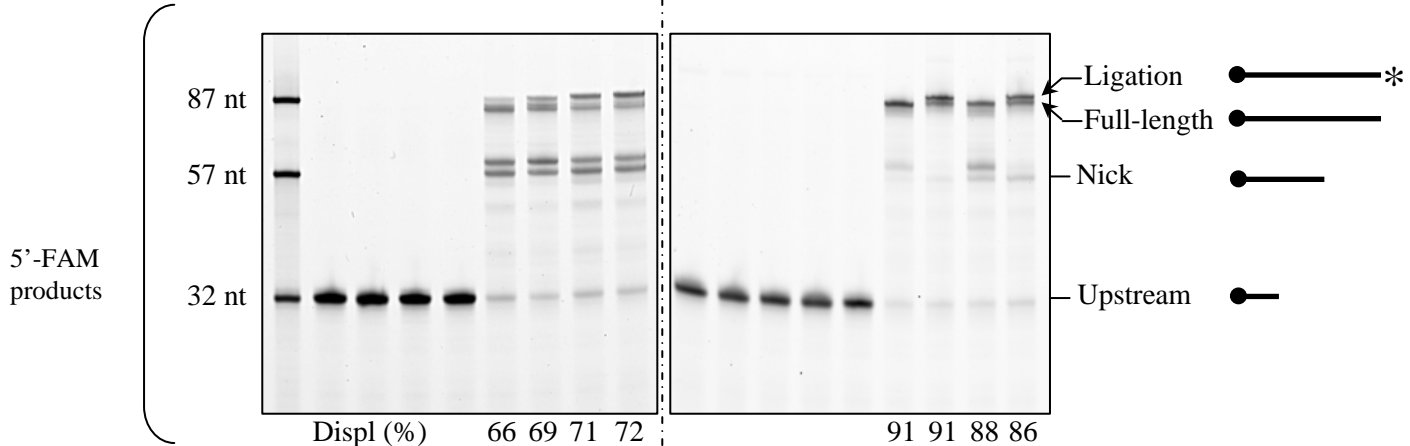


FIGURE 2



<i>PabpolB</i>	-	-	-	-	+	+	+	+	-	-	-	-	-	+	+	+	+
<i>PabFen1</i>	-	+	-	+	-	+	-	+	-	-	+	-	+	-	+	-	+
<i>PabPCNA</i>	-	-	+	+	-	-	+	+	-	-	-	+	+	-	-	+	+
<i>PabLig1</i>	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+



Lanes M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

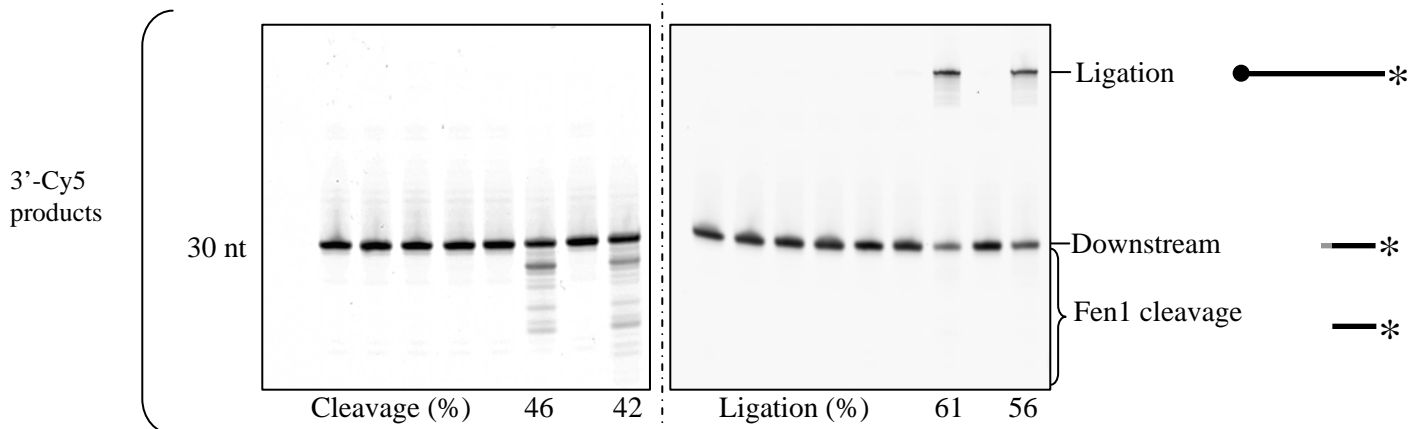


FIGURE 3

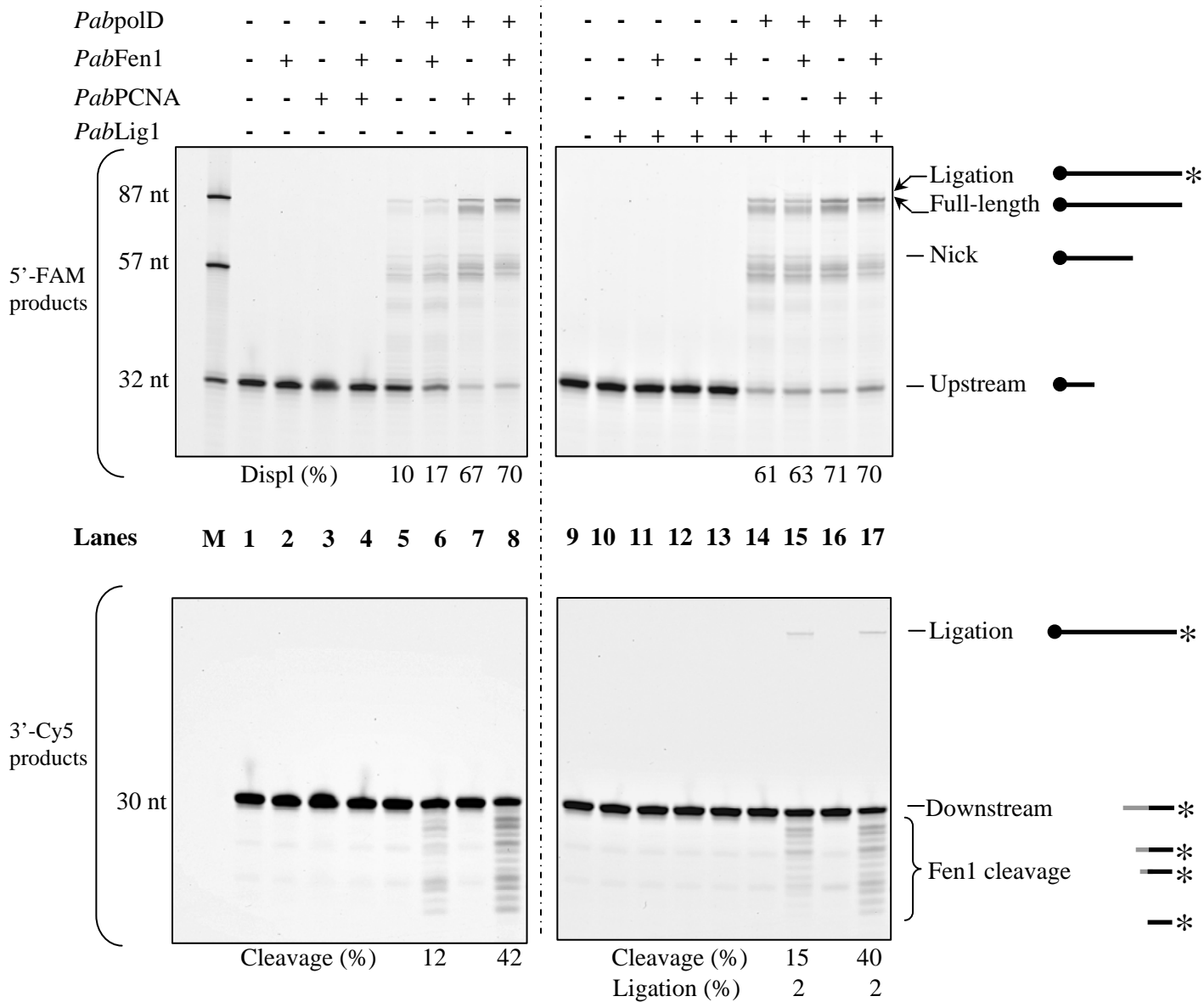
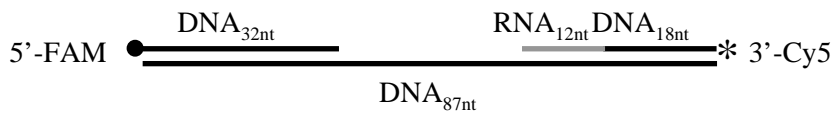
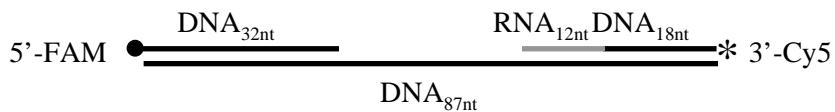


FIGURE 4



<i>PabpolB</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
<i>PabpolD</i>	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-
<i>PabFen1</i>	-	-	-	+	-	+	-	+	+	-	-	-	+	-	+	-	+	+
<i>PabPCNA</i>	-	-	-	-	+	+	-	+	+	-	-	-	-	+	+	-	+	+
<i>PabLig1</i>	-	-	+	-	-	+	-	+	+	-	-	+	-	-	+	-	+	+
<i>PabRNase HII</i>	-	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	-	+

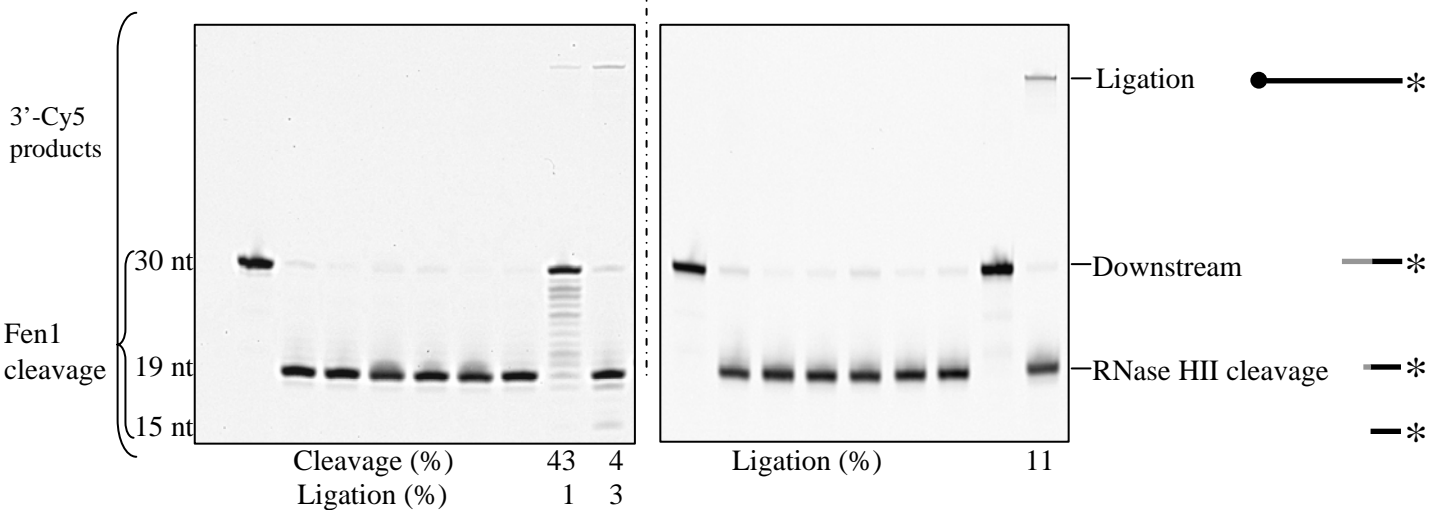
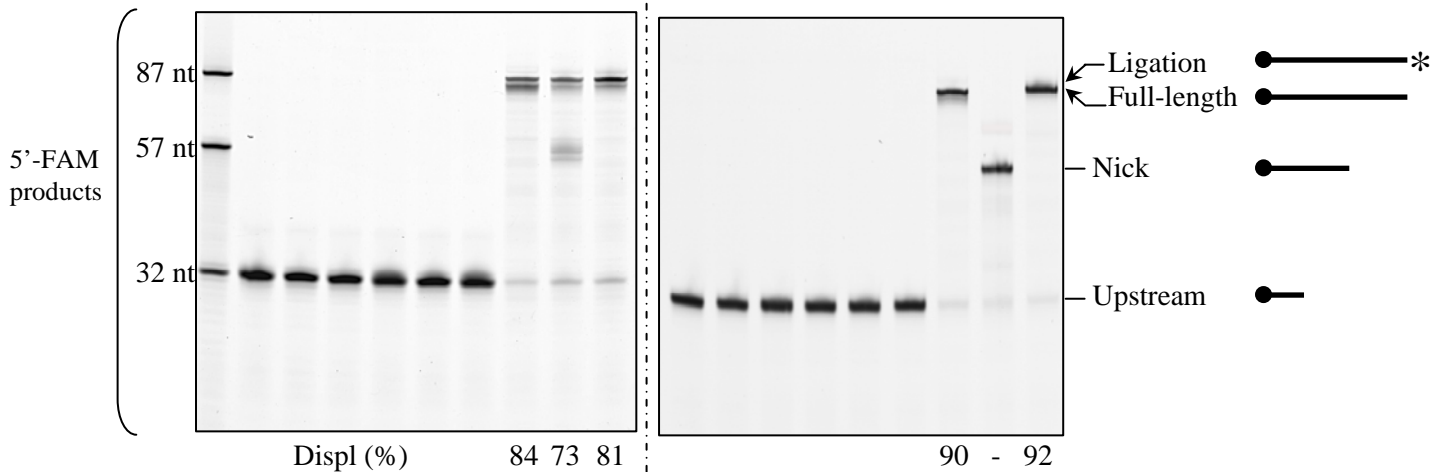


FIGURE 5

