ACS OMEGA

http://pubs.acs.org/journal/acsodf

Article

Helix Bundle Domain of Primase RepB′ Is Required for Dinucleotide Formation and Extension

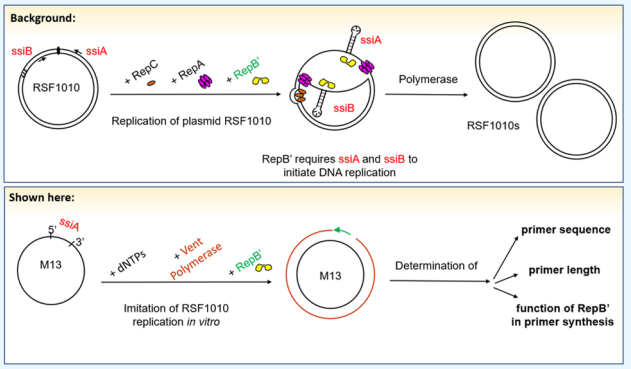
Sofia Banchenko, Christoph Weise, Erich Lanka, Wolfram Saenger, and Sebastian Geibel*

Cite This: *ACS Omega* 2021, 6, 28903−28911

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: During DNA replication, primases synthesize oligonucleotide primers on single-stranded template DNA, which are then extended by DNA polymerases to synthesize a complementary DNA strand. Primase RepB′ of plasmid RSF1010 initiates DNA replication on two 40 nucleotide-long inverted repeats, termed ssiA and ssiB, within the oriV of RSF1010. RepB′ consists of a catalytic domain and a helix bundle domain, which are connected by long α-helix 6 and an unstructured linker. Previous work has demonstrated that RepB′ requires both domains for the initiation of dsDNA synthesis in DNA replication assays. However, the precise functions of these two domains in primer synthesis have been unknown. Here, we report that both domains of RepB′ are required to synthesize a 10−12 nucleotide-long DNA primer, whereas the isolated domains are inactive. Mutational analysis of the catalytic domain indicates that the solvent-exposed W50 plays a critical role in resolving hairpin structures formed by ssiA and ssiB. Three structurally conserved aspartates (D77, D78, and D134) of RepB′ catalyze the nucleotidyl transfer reaction. Mutations on the helix bundle domain are identified that either reduce the primer length to a dinucleotide (R285A) or abolish the primer synthesis (D238A), indicating that the helix bundle domain is required to form and extend the initial dinucleotide synthesized by the catalytic domain.



INTRODUCTION

Transfer of genetic information from viral, prokaryotic, eukaryotic, and plasmid genomes to the daughter generation is ensured by the duplication (replication) of genomic DNA. During DNA replication, helicases separate the parental DNA, and primases synthesize *de novo* short oligonucleotide primers complementary to the single-stranded parental template DNA.[1] DNA polymerases require the 3′-end of the primer for nucleotide addition, in order to synthesize a complementary daughter DNA strand. Primers are elongated continuously on the leading strand and discontinuously on the oppositely oriented lagging strand resulting in the synthesis of Okazaki fragments, which require periodically primase activity.

The DNA replication of the broad host range RSF1010 has been extensively studied in *Escherichia coli*. Unlike its host genomes (i.e., *E. coli*), RSF1010 is replicated exclusively in the leading strand mode[2,3] (Figure S1A). RSF1010 encodes helicase RepA, primase RepB′, and initiator protein RepC, which are required for its own replication alongside the host replication machinery.[4,5] Binding of RepC to the origin of vegetative replication (oriV) initiates the unwinding of the double-stranded plasmid DNA (dsDNA) by RepA in opposite directions[6,7] and exposes two 40 nucleotide single-strand initiator sequences A and B (ssiA and ssiB), which are encoded only once on complementary DNA strands (Figure S1B,C). RepB′ requires ssiA and ssiB to initiate DNA replication.[2,8,9]

The unusually complex ssiA and ssiB are unique to IncQ and IncQ-like plasmids and exhibit hairpin loops at nucleotides 7−27 of ssiA/ssiB[10,11] (Figure S1B,C). While RepB′ binds specifically the first six nucleotides of ssiA that lie upstream of the hairpin,[10] *in vivo* studies suggested that primer synthesis may be initiated downstream of the hairpin within a triplet "GTG" (nt 31−33).[2] *In vitro* DNA replication experiments showed that RepB′ requires 2′-deoxynucleoside 5′-triphosphate (dNTPs) for primase activity.

Previous studies provided insights into the RepB′ primase mechanism. The X-ray structure of RepB′ exhibits a catalytic domain with a mixed α/β fold that is connected via an unstructured linker to a helix bundle domain. The crystal structure of the catalytic domain of RepB′ bound to the first 27 nucleotides of ssiA revealed that a specific binding site for the first six nucleotides and the active site are in proximity,

Received: July 21, 2021
Published: October 20, 2021

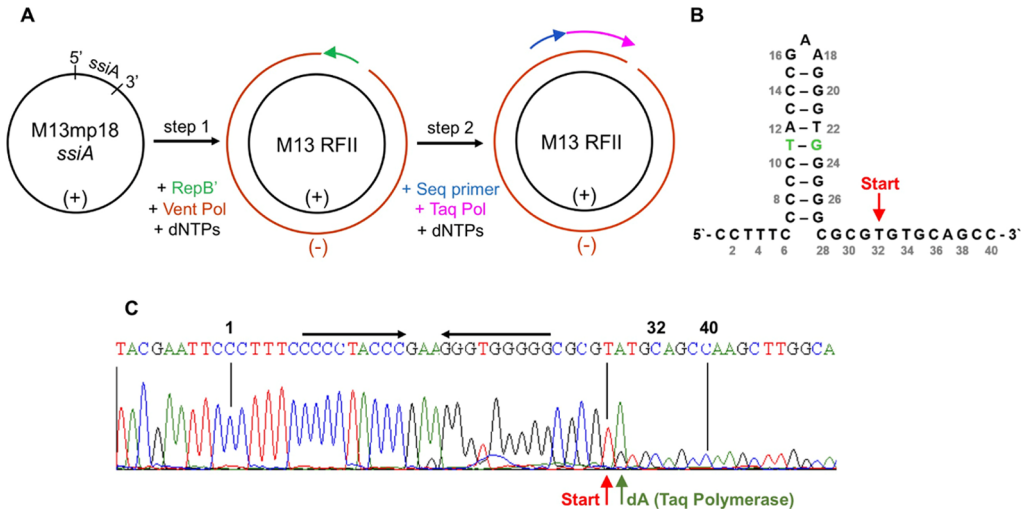


Figure 1. Primase RepB′ starts primer synthesis on thymine 32 of ssiA. (A) Principle of the DNA replication assay. Primase activity is indicated by the conversion of single- to double-stranded DNA (step 1). RepB′ synthesizes a complementary DNA primer (green arrow) on ssiA of the (+) strand of M13mp18ssiA ssDNA (black). The Vent DNA polymerase elongates the primer by addition of dNTPs complementary to the single-stranded M13mp18ssiA template (brown). The double-stranded DNA contains a single-strand nick and is labeled RFII, in order to distinguish it from the closed, circular M13dsDNA, which is labeled replicative form I (RFI). A run off DNA sequencing reaction was performed in order to find the first nucleotide of the RepB′ primer (step 2). (B) Schematic of the ssiA hairpin. The starting point for the primer synthesis on thymine 32 is indicated with a red arrow. (C) The sequencing signal drops at thymine 32. A mismatching adenine signal in position 33 of ssiA can be attributed to the Taq DNA polymerase, which adds 2′-deoxyadenosine 5′-monophosphate (dAMP) to the 3′ end of DNA.

suggesting that the missing nucleotides 28−40 of ssiA may bind along the catalytic center and place the "GTG" triplet (nt 31−33) close to the three catalytic aspartates 77, 78, and 134.[10] Comparisons of RepB′ with known structures of primases and polymerases revealed that these three aspartates are conserved and may coordinate two metal ions for the catalysis of the nucleotidyl transfer reaction.[12−14] DNA replication assays confirmed that RepB′ requires these three aspartates for the initiation of dsDNA synthesis.[10] However, the catalytic domain and the helix bundle domain are inactive in isolation, and DNA replication is only initiated in the presence of both domains.[10] The helix bundle domain of RepB′ binds ssiA with significantly lower affinity (∼27 μM) compared to the catalytic domain (∼2 μM), indicating a more transient interaction with ssiA compared to the catalytic domain.[10] Therefore, it was proposed that the catalytic domain remains bound to the 5′-end of ssiA/ssiB during primer synthesis, while the helix bundle domain stabilizes the growing primer on the DNA template.[10]

Both the catalytic and helix bundle domains of RepB′ have structural orthologues in the archaeo/eukaryotic primase class despite the lack of sequence conservation.[15] X-ray crystallography and biochemical studies of heterotrimeric primase PriSXL from *Sulfolobus solfataricus* revealed that the helix bundle subunit PriX provides the nucleotide binding site for primer initiation, while the catalytic subunit PriS harbors the nucleotide binding site for nucleotide addition.[16] As observed for RepB′, both subunits are far apart from each other in PriSXL, indicating that the complex needs to change into a closed conformation that juxtaposes the NTPs bound to the initiation and catalytic sites for dinucleotide synthesis and subsequent elongation to synthesize a primer.

A nuclear magnetic resonance study on the primase part of primase-polymerase ORF904 from the archaeal plasmid pRN1 showed the structure of the helix bundle domain of ORF904 bound to template DNA and two ATP molecules. Further experimental works indicated that this structure represents an early step of the catalytic cycle before the template DNA, and the two nucleotides are transferred to the catalytic domain for dinucleotide formation.[17]

Several studies have shown that primases act as a caliper to limit the primer length.[18−20] Primases are classified into prokaryotic DnaG type and archeal/eukaryotic Pri-type primases. Although structurally distinct, both classes have in common that they require a catalytic and an accessory domain for primer synthesis. The latter one is a helix bundle domain in Pri-type primases and a zinc-binding domain in DnaG-type primases, where it has been shown to initiate and regulate the primer synthesis and length. Recent studies have mainly discussed two models for primer termination by Pri-type primases. In the first model, the helix bundle domain interacts with the 5′ end of the nascent primer, and the catalytic domain adds nucleotides to the 3′ end of the primer, while the flexible element (i.e., the linker) between the two domains is suggested to restrict the primer synthesis to a defined primer length.[18] Accumulating evidence favors a second model where the formation of the primer-template duplex imposes a rotation of the catalytic and helix bundle domains around the helical duplex, while both domains are bound to the primer at different ends. This rotation eventually causes steric restraints[21] or clashes[22] determined by the primase structure causing the termination of primer synthesis.

While most primases have no sequence specificity or recognize only triplets on template DNA, ssiA and ssiB are unusually long and complex and may have additional structural requirements for primer synthesis. Here, we set out to dissect the structure of RepB′ by analyzing its primer synthesis in combination with *in vitro* DNA replication assays.

RESULTS

RepB′ Begins Primer Synthesis on Thymine 32 of ssiA. As the starting point and the length of the RepB′ primer were unknown, we sought to investigate the primer synthesis

https://doi.org/10.1021/acsomega.1c03881
ACS Omega 2021, 6, 28903−28911

of RepB′ first. An *in vivo* study suggested that RepB′ begins the primer synthesis within nucleotides 31−33 of ssiA and ssiB.[2] To verify the exact starting point of the primer synthesis, we established a DNA replication assay using purified RepB′, Vent DNA polymerase, dNTPs, and single-stranded DNA of the phage M13mp18 (M13, Max-Planck strain 18) encoding ssiA in the (+) strand (M13mp18ssiA) (Figure 1A,B). This reaction produced a dsDNA (M13 RFII, replicative form II) containing a nick in the newly synthesized (−) DNA strand where the primer synthesis started (Figure 1A, step1). Next, we carried out a run off sequencing reaction toward the ssDNA nick using a sequencing primer complementary to the nicked (−) strand of the M13 RFII DNA (Figure 1A, step2). The fluorescent signal of the sequencing reaction fell off significantly after thymine in position 32 of ssiA, showing that the first nucleotide in the RepB′ primer is a 2′-deoxyadenosine 5′-triphosphate (dATP) (Figure 1C). An additional mismatching adenine signal in position 33 that overlaps with the lower fluorescence signal of guanine 33 can be attributed to the Taq polymerase used in the sequencing reaction, which adds adenosine monophosphate to free 3′-OH termini independent of the DNA template.

RepB′ Synthesizes DNA Primers of up to 12 Nucleotides. To investigate the primer synthesis, RepB′ was incubated with a mix of dNTPs supplemented with [$^{32}\alpha$P] 2′-deoxycytidine 5′-triphosphate (dCTP) and M13mp18ssiA (Figure 2A−C). Reactions were analyzed on a denaturing polyacrylamide gel. At equimolar concentrations of all four dNTPs, two primers with lengths of 11 and 12 nucleotides were observed (Figure 2B). As dATP is the first nucleotide to be incorporated, we increased the dATP concentration 10−100 times in relation to dCTP, 2′-deoxyguanosine 5′-triphosphate (dGTP) and 2′-deoxythymidine 5′-triphosphate (dTTP) (100 μM each).

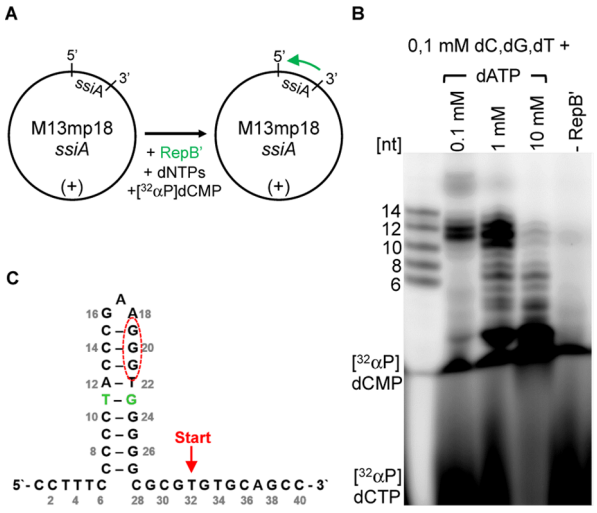


Figure 2. Primase RepB′ synthesizes 11 to 12 nucleotide-long primers. (A) Schematic of the primer synthesis reaction. (B) DNA primer synthesis of RepB′ on circular single-stranded M13mp18ssiA. Oligonucleotide length standard: 6, 8, 10, 12, and 14 nucleotides (lane 1). Reactions contained M13mp18ssiA, RepB′ and 10 μM dCTP (1 μM [$^{32}\alpha$P] dCTP + 9μM dCTP), dGTP, and dTTP. The dATP concentration was varied from 10 μM (lane 2) to 1 mM (lane 3) and to 10 mM (lane 4). The negative control contained all four dNTPs (10 μM) and M13mp18ssiA DNA but no RepB′ (lane 5). The primer length was either determined by counting premature primer abortions or by comparison to the oligonucleotide marker. (C) Schematic of the ssiA sequence. Starting (red arrow) and end (red oval) points of the RepB′ primer are indicated. The wobble base pair is shown in green.

Higher dATP concentrations stimulated the primer synthesis considerably and led to the synthesis of shorter primers. At a concentration of 1 mM dATP, RepB′ predominantly produced primers of 2, 10, 11, and 12 nucleotides, while at a concentration of 10 mM dATP, all [$^{32}\alpha$P] dCTP was incorporated into di-, tri-, and hexa-oligonucleotides. The continuous synthesis of short oligonucleotides requires dissociation of these primers from M13mp18ssiA, which then becomes available as the ssDNA template again. This result further indicates that RepB′ contains a nucleotide binding site that preferentially binds dATP. Once dATP is bound, synthesis of a new primer is initiated. For the subsequent experiments, we used an excess of 1 mM dATP in the reaction mix as this produced the highest amounts of full-length primer.

To confirm dATP as a starting nucleotide of the primer, we carried out primer synthesis experiments using different combinations of dNTP (Figure S2A,B). Full primase activity was only retained in the presence of dATP, whereas no primers were detected in the reaction without dATP. In the absence of dGTP, RepB′ synthesized mainly dinucleotides and to a lesser extent trinucleotides as well as DNA primers with a length of up to 12 nucleotides. The accumulation of trinucleotides in the reaction without dGTP was expected because the position C30 of ssiA requires the incorporation of complementary 2′-deoxyguanosine 5′-monophosphate (third position in the DNA primer). However, the synthesis of trinucleotides and longer DNA primers in the absence of dGTP shows that RepB′ also incorporates non-complementary dNTPs into the DNA primer, albeit inefficiently. Therefore, it is likely that in positions C28 and C30 of ssiA, dAMP has been incorporated into the RepB′ DNA primer to form a C-A wobble base pair. Omission of dTTP had no effect on the primer length because adenine does not occur in the sequence of ssiA, which serves as a template for primer synthesis (Figure S2B).

Potential Role of W50 in Unwinding the ssiA Hairpin. The primer length and the starting point of the primer synthesis indicate that RepB′ must unwind the ssiA hairpin structure, in order to synthesize primers in full length because the first base pair (C7-G27) of the hairpin is formed six nucleotides upstream of the primer start site (Figure 2B). The crystal structure of the catalytic domain of RepB′ bound to the first 27 nucleotides of the ssiA hairpin[10] shows that the solvent-exposed tryptophan 50 interacts with the first base pair and may play a role in unwinding the hairpin structure (Figure 3A). RepB′ W50 is conserved among one of two subgroups of IncQ plasmids (Figure S3), which were previously identified by sequence alignments,[10] whereas in the second subgroup it is replaced by a glutamate (Figure S3A). Interestingly, structural comparisons show structurally conserved hydrophobic tyrosines in the catalytic domains of archeal and phage Pri-type primases, supporting the idea that W50 may interact with template DNA (Figure S3B).

To investigate whether W50 affects primer synthesis as well as the initiation of DNA replication, we used site-directed mutagenesis to generate mutant RepB′ W50A (Figure S3C). We found that RepB′ W50A synthesized mostly short primers of 2, 5, and 6 nucleotides, whereas the synthesis of full-length primers was diminished significantly (Figure 3A). The

https://doi.org/10.1021/acsomega.1c03881
ACS Omega 2021, 6, 28903−28911

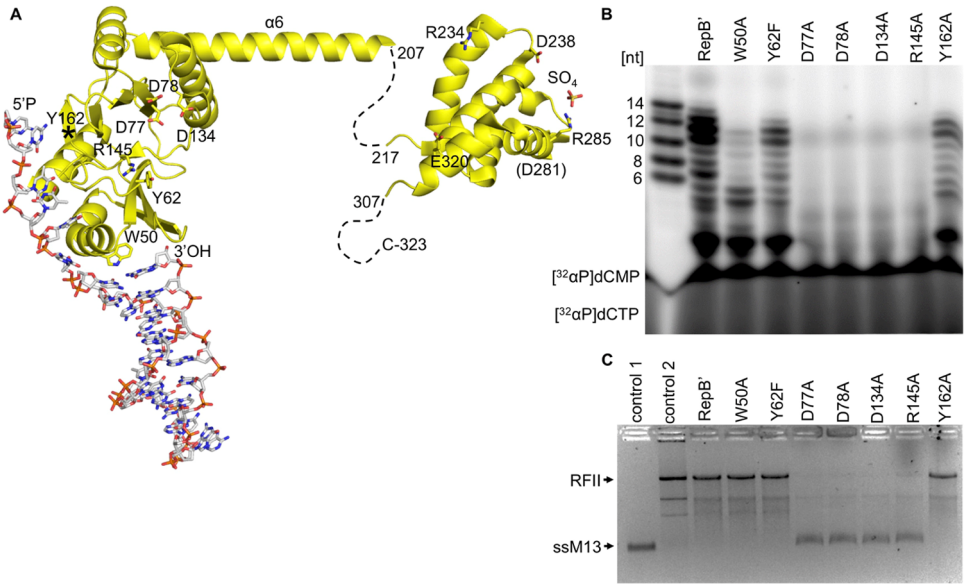


Figure 3. Primase activity of RepB′ variants W50A, Y62F, D77A, D78A, D134A, R145A, and Y162A and the possible role of W50 in hairpin unwinding. (A) Model of RepB′ bound to the first 27 nucleotides of ssiA based on crystal structures of full-length RepB′ and its catalytic domain bound to the first 27 nucleotides of ssiA.[10] Mutated amino acids are shown as sticks. (B) Primer synthesis assay. Reactions contained M13mp18ssiA, dNTPs, [$^{32}\alpha$P] dCTP, and one of the following RepB′ variants: W50A, Y62F, D77A, D78A, D134A, R145A, and Y162A. Oligonucleotide standard: 6, 8, 10, 12, and 14 nucleotides. (C) *In vitro* DNA replication assay. The assay tests the ability of the primase to initiate dsDNA synthesis on M13mp18ssiA in the presence of Vent DNA polymerase (see Figure 1A, step 1). Reactions contained Vent DNA polymerase, dNTPs, M13mp18ssiA, and one of the following primase variants: W50A, Y62F, D77A, D78A, D134A, R145A, and Y162A. Controls: reaction without RepB′ (lane 1) and M13mp18ssiA dsDNA (RFII) (lane 2).

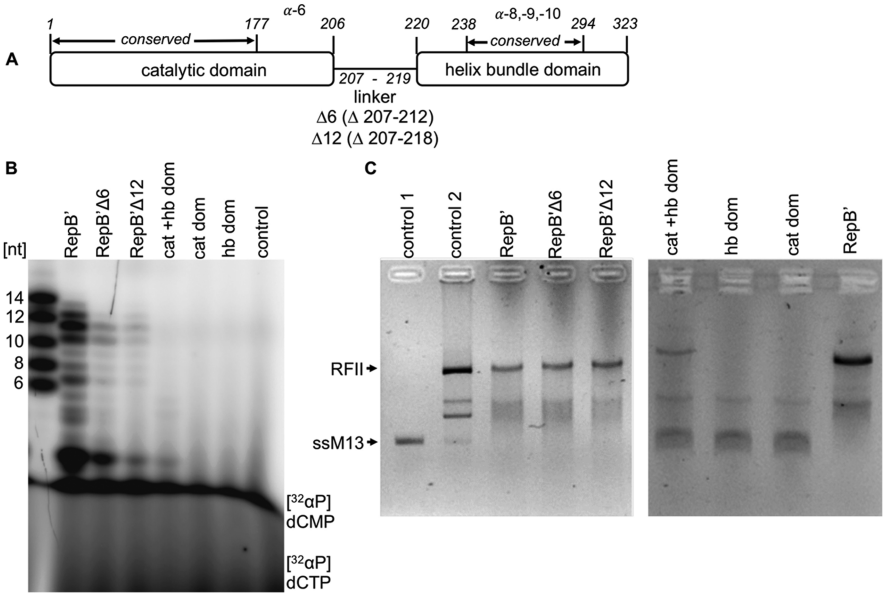


Figure 4. RepB′ requires catalytic and helix bundle domains for primer synthesis. (A) Schematic of RepB′. Boundaries of domains and the linker are indicated. Domains shared with other Pri-type primases are labeled as conserved. (B) Primer synthesis of RepB′Δ6, RepB′Δ12, and (cat) catalytic and (hb) helix bundle domains of RepB′. The reactions contained M13mp18ssiA, dNTPs, [$^{32}\alpha$P] dCTP, and one of the following RepB′ variants: RepB′Δ6, RepB′Δ12, catalytic domain (cat), helix bundle domain (hb), or cat + hb domains. Oligonucleotide marker (lane 1), control reaction without RepB′ (C, lane 8). (C) Left: *in vitro* DNA replication assay, representing the dsDNA synthesis by RepB′Δ6 and RepB′Δ12. Same controls are used as in Figure 3 (C1, C2, and RepB′). Right: *in vitro* DNA replication assay showing the catalytic domain (cat), helix bundle domain (hb), or cat + hb domains, as well as RepB′.

predominant species of penta- and hexaoligonucleotides indicate that the primer synthesis is aborted before the hairpin or after breaking the first base pair of the hairpin, pointing to a role of W50 in unwinding the ssiA hairpin. RepB′ W50A was still able to initiate DNA replication (Figure 3B).

Three Conserved Aspartates and One Arginine in the Catalytic Center of RepB′ Are Required for Nucleotidyltransferase Activity. A comparison of RepB′ with known primase and DNA polymerase structures revealed the spatially conserved amino acids D77, D78, D134, and R145,[10] which form a nucleotide binding pocket on the catalytic domain

https://doi.org/10.1021/acsomega.1c03881
ACS Omega 2021, 6, 28903−28911

(Figure 3A). In order to investigate whether these amino acids are required for nucleotidyltransferase activity, primer synthesis of RepB′ mutants D77A or D78A, D134A, and R145A was analyzed on M13mp18ssiA (Figure 3B). The RepB′ mutants D77A or D78A or D134A did not synthesize detectable amounts of primers and—as reported previously[10] —could not initiate DNA replication (Figure 3B). We conclude that the amino acids D77, D78, and D134 catalyze the nucleotidyltransferase activity of RepB′. Although primer synthesis of RepB′ R145A was below the detection level, the mutant was still able to initiate DNA replication at a low level as previously shown,[10] indicating basal nucleotidyltransferase activity (Figure 3B). The strongly reduced primase activity of RepB′ R145A suggests impaired binding or positioning of the incoming dNTP in the catalytic center.

Next, we investigated whether the two conserved amino acids Y62 and Y161. Both amino acids are located in proximity of the three catalytic aspartates and could aid in positioning of the template DNA along the catalytic center. As Y62 is strictly conserved in IncQ primases as well as in most known Pri-type primase structures, we hypothesized a possible conserved function of Y62 (Figure S3A,C). Y162 is located between the specific ssiA binding site and the catalytic site of RepB′ and therefore could interact with incoming nucleotides or with the 5′-end of ssiA, which is bound by the catalytic domain of RepB′. Y162 is strictly conserved among IncQ primases (Figure S3A) but not in Pri-type primases, suggesting a specific role for IncQ primases (Figure S3B). However, both RepB′ mutants, Y62F and Y162A, retained full activity in our primase and replication assays and therefore are unlikely to play critical roles in template or nucleotide binding in RepB′ as well as IncQ primases (Figures 3B,C; S3C).

Helix Bundle Domain Is Essential for Primer Synthesis. Both the catalytic domain and the helix bundle domain are required to initiate DNA replication.[10] To investigate whether the helix bundle domain is involved in primer synthesis or required for handing over the synthesized primer to the DNA polymerase, we analyzed primer synthesis of the catalytic (residues 1−212) and helix bundle domains (residues 213−323). When either of the two domains was tested in isolation, neither DNA replication[10] nor primer synthesis was detected (Figure 4A−C), whereas both domains combined produced low amounts of di-, tri-, and tetranucleotides (Figure 4B). We conclude that the helix bundle domain is required for initiating primer synthesis. The lower primer synthesis of the separated domains compared to wild type RepB′ may be attributed to the missing linker, which imposes proximity on both domains.

Linker Connecting Catalytic and Helix Bundle Domains of RepB′ Is Required for Full Primase Activity. To test how the linker length (residues 207−218) affects the primase activity, we generated RepB′Δ6 and RepB′Δ12 with linker truncations of 6 (residues 207−212) and 12 amino acids (residues 207−218). Both RepB′Δ6 and RepB′Δ12 synthesized a lower amount of primers than wild-type RepB′ (Figure 4B) and initiated DNA replication (Figure 4C). RepB′Δ6 synthesized higher amounts of primers than RepB′Δ12, indicating that the efficiency of primer synthesis also depends on the flexibility mediated by the linker between the two domains.

Helix Bundle Domain Is Required for Dinucleotide Synthesis and Primer Elongation. To gain insights into the function of the helix bundle domain during primer synthesis, we sought to map functional amino acids on its surface. Structural comparisons with primases of the archeal/eukaryotic primase family revealed three conserved pockets with differential amino acid composition on the surface of the helix bundle domain of RepB′ (Figure S4A,B). In primase-polymerase ORF904, two of these pockets serve as binding sites for two ATPs, whereas the other pocket binds template DNA. The comparison revealed only one invariant aspartate in the nucleotide binding pocket 1 of ORF904 (D308 ORF904 and D238 RepB′), whereas no invariant amino acids were found in the nucleotide binding pocket 2 and the DNA template binding pocket. In ORF904, aspartate D308 is required for primer initiation. In PriX, an arginine has replaced the aspartate in pocket 1 and the bound AMPNPP adopts a different orientation compared to the ATP in ORF904. As we could not identify strictly conserved amino acids by structural comparisons, we carried out protein sequence comparisons of RepB′ with primases encoded by the RSF1010-related IncQ and IncQ-like plasmids (Figure S3A). These comparisons revealed several conserved amino acids on the surface of the helix bundle domain. To validate their role in primer synthesis, an alanine scan of amino acids R234, D238, D281, R285, and E302 was performed. The RepB′ variants R234A, D281A, and E302A synthesized reduced the amounts of DNA primers compared to wild-type RepB′ (Figure 5A) and retained full primase activity in the replication assay (Figure 5B).

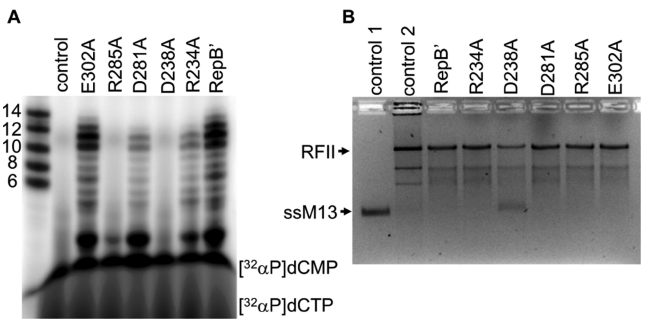


Figure 5. Helix bundle domain of RepB′ is required for dinucleotide formation and primer extension. Selected conserved amino acids on the helix bundle domain of RepB′ were mutated to alanine, and their impact on the primer synthesis (A) and DNA replication (B) was tested. Primer synthesis of mutant D238A could not be detected, while the mutant R285A synthesizes a small amount of dinucleotides. However, both variants, D238A and R385A, were able to initiate DNA replication.

In contrast, primer synthesis for RepB′ D238A was not detectable (Figure 5A). This is in line with the conservation of D238 in nucleotide binding pocket 1 of ORF904. Thus, this result suggests that the helix bundle domain of RepB′ provides the second nucleotide binding site required for dinucleotide formation.

RepB′ R285A produced only low amounts of dinucleotides (Figure 5A). Longer primers were not detected, suggesting that R285 is involved in the elongation step after dinucleotide formation. Interestingly, although not structurally conserved, R285 is placed into the DNA binding pocket of ORF904 in structural superimpositions. Furthermore, R285 binds a sulfate anion, suggesting that R285 could interact with the phosphate backbone of template DNA and/or the primer and stabilize the growing primer.

https://doi.org/10.1021/acsomega.1c03881
ACS Omega 2021, 6, 28903−28911

Despite the impaired primer synthesis, both variants, RepB′ D238A and RepB′ R285A, retained the primase activity in the DNA replication assay (Figure 5B). The activity of RepB′ D238A was diminished compared to wild-type RepB′. These results further suggest that dinucleotides may be sufficient for initiating RSF1010 replication in our in vitro assay. Alternatively, it is also possible that low amounts of full-length primers have been produced, which our radioactive assay cannot detect.

Taken together, these results show that the helix bundle domain is required for dinucleotide synthesis and primer elongation.

DISCUSSION

Here, we have investigated primase RepB′ of the eubacterial IncQ plasmid RSF1010, which is exclusively replicated in the leading strand mode. Compared to known primases, RepB′ has two highly similar, unusually complex recognition sites that are required to initiate the synthesis of the two daughter DNA strands. The catalytic domain of RepB′ binds the first six nucleotides of ssiA at the 5′ end before the hairpin structure (nt 7−27). Here, we have shown that RepB′ synthesizes an 11−12 nucleotide-long DNA primer starting with dATP at thymine 32 on the 3′ end of ssiA. Our data indicate that W50 is involved in unwinding the hairpin during primer synthesis, which becomes necessary after the primer has exceeded a length of five nucleotides.

We show that the catalytic domain of RepB′ harbors a nucleotide binding site, which catalyzes the nucleotidyl transfer reaction. Furthermore, our mutational analysis of the helix bundle domain along with structural comparisons of archeal/eukaryotic primases indicates that the helix bundle domain of RepB′ has a dual function in binding dATP for primer initiation and in elongating the dinucleotide, possibly by stabilizing the initial dinucleotide primer/ssDNA template. Our results imply that RepB′ must adopt a closed active conformation, which brings the nucleotide binding sites of catalytic and helix bundle domains in proximity for the initial formation of a dinucleotide. We show that dinucleotide formation is considerably stimulated by dATP. Without dATP, primer synthesis is strongly reduced, showing that the incorporation of the first nucleotide is base-specific and not dependent on the sugar or tri-phosphate moiety. We also observe premature abortion of primer synthesis in the presence of excess dATP. Thus, the reoccupation of the dATP binding site triggers the formation of a new primer, indicating that the binding sites for primer initiation and elongation stay close together during primer synthesis.

Our observations are in agreement with the general model for primer synthesis put forward by Charles Richardson.[1] This model suggests that primases catalyze the formation of a phosphodiester bond between two adjacent nucleotides. Primer synthesis requires the hydrolysis of the nucleotide at the elongation site. The triphosphate moiety of the nucleotide at the initiation site is preserved, and this nucleotide becomes the 5′ end of the primer—in the case of RepB′ dATP.

After dinucleotide formation, RepB′ moves along the template DNA while nucleotides are being added to the 3′ hydroxyl group of the primer until an oligonucleotide primer of defined length is synthesized. Interestingly, we found that RepB′ variants with truncations in the linker still synthesize a full-length primer, although such truncations do not permit the interaction between catalytic and helix bundle domains. This is because α-helix 6 acts as a spacer between the two domains in the RepB′ crystal structure. Thus, our data suggest that α-helix 6 must be repositioned upon activation of RepB′ to bring catalytic and helix bundle domains in proximity, whereas the unstructured linker provides the flexibility for optimal interaction of the helix bundle domain with the growing primer. Based on previous *in vitro* replication data,[10] we can say that this has raised the possibility that the helix bundle domain could also act *in trans* together with the catalytic domain of another RepB′ molecule to synthesize a primer. The here-reported decreased primer synthesis of the truncated RepB′ linker variants indicates that the linker length plays an important role in efficient primer synthesis and that both RepB′ domains work more efficiently when they are coupled to each other. In line with this, we also observed the least efficient primer synthesis when isolated catalytic and helix bundle domains are combined. Therefore, it appears likely that RepB′ domains act *in cis* on ssiA DNA.

Our data speak against the caliper model for primer termination[18] as linker truncations do not affect the RepB′ primer length. Unlike the steric clashes model,[21,22] our data support the idea that binding sites for primer initiation and elongation stay close together during primer synthesis. Instead, we speculate that primer synthesis is terminated at the hairpin tip, as the synthesis of a longer primer would require the primase to glide downstream ssiA, which might be prevented by the bound 5′-end of ssiA. A model for the primer synthesis of RepB′ is presented in Figure S5.

MATERIALS AND METHODS

Cloning. In order to generate truncations of the linker between catalytic and helix bundle domains, we amplified the DNA fragments encoding the catalytic domain (fragment nt 1−618) and fragments nt 642−969 und nt 657−969 encoding the C-terminal part of the protein including the linker and the helix bundle domain, respectively (Figure S2A). Fragment 1−618 was cloned into pET28b, using *Nde*I and *Eco*RI restriction enzyme sites. Fragments 639−969 and 657−969 were cleaved using *Eco*RI and *Hin*dIII first and then inserted into pET28b-encoding fragments 1−618 via *Eco*RI and *Hin*dIII, respectively. The phenylalanine triplet, introduced by the *Eco*RI site, was removed using the QuickChange Site-Directed Mutagenesis Kit (Agilent), producing constructs RepB′Δ6 (207−212) and RepB′Δ12 (207−218). Site-directed mutagenesis was used to generate RepB′ variants W50A, Y62F, Y162A, R234A, D238A, D281A, R285A, und E302A using the plasmid pET28b: repb' as the template and the primers listed in Table 1.

Protein Expression and Purification. RepB′ and its derivatives W50A, Y62F, Y162A, R234A, D238A, D281A, R285A, E302A, RepB′Δ6, and RepB′Δ12 were expressed and purified using the published protocol for RepB′[10] (Table 2). *E. coli* ER2566 was transformed with the respective plasmids. Bacteria were grown in LB medium to an optical density of 0.6 at 600 nm (OD_{600}). Overexpression was induced by the addition of 0.4 mM IPTG (isopropyl β-D-1-thiogalactopyranoside). After 3 h, cells were harvested by centrifugation (10 min, 5,000*g*, 4 °C) and resuspended in lysis buffer (50 mM Tris/HCl (pH 7), 150 mM $MgCl_2$, and 2.5 mM DTT). Cells were passed three times through a C5 EmulsiFlex homogenizer (Avestin). Cell debris was removed by centrifugation (30,000*g*, 30 min, 4 °C); the supernatant was diluted 1:2 with buffer A (20 mM Tris/HCl (pH 7), 50 mM $MgSO_4$, 1 mM DTT), then loaded on a HiTrap Heparin column (CV, column volume, 5

https://doi.org/10.1021/acsomega.1c03881
ACS Omega 2021, 6, 28903−28911

Table 1. Primers Used for Cloning and Mutagenesis

primers	
FW primer, run off sequencing	5′-gcctgccctggtagtggaaacc-3′
FW primer *Nde*I/cat, Δ6, Δ12	5′-tatctgcatatgaagaacgacaggactttgcagg-3′
RV pr. *Eco*RI/cat 207/Δ6,Δ12	5′-aatctggaattcgaggctggccagcctgcgggc-3′
FW primer *Eco*RI/hb 214/Δ6	5′-aatctggaattcagccgccaccggcgcacggcg-3′
FW primer *Eco*RI/hb 219/Δ12	5′-aatctggaattccggcgctggacgagtaccgc-3′
RV primer *Hin*dIII/hb, Δ6, Δ12	5′-aatctgaagcttctacatgctgaaatctggcccgc-3′
FW primer RepB′W50A	5′-gctccagaacacgccagcgctcaagcggatga-3′
RV primer RepB′W50A	5′-tcatccgcttgagcgctggcgtgttctggagc-3′
FW primer RepB′Y62F	5′-gggcaatgacgtgtttatcaggcccgccg-3′
RV primer RepB′Y62F	5′-cggcgggcctgataaacacgtcattgccc-3′
FW primer RepB′Y162A	5′-ccacccgcgccggtgctcagccgtgggt-3′
RV primer RepB′Y162A	5′-acccacggctgagcaccggcgcgggtgg-3′
FW primer RepB′R234A	5′-ccgggctggtcaaggccttcggtgatgacc-3′
RV primer RepB′R234A	5′-ggtcatcaccgaaggccttgaccagcccgg-3′
FW primer RepB′D238A	5′-gcgcttcggtgatgccctcagcaagtgcg-3′
RV primer RepB′D238A	5′-cgcacttgctgagggcatcaccgaagcgc-3′
FW primer RepB′D281A	5′-ggccacgaagcggcttacatcgagcgc-3′
RV primer RepB′D281A	5′-gcgctcgatgtaagccgcttcgtggcc-3′
FW primer RepB′R285A	5′-gcggattacatcgaggccaccgtcagcaaggt-3′
RV primer RepB′R285A	5′- accttgctgacggtggcctcgatgtaatccgc-3′
FW primer RepB′E302A	5′-gcgcgggccgcgctggcacgg-3′
RV primer RepB′E302A	5′-ccgtgccagcgcggcccgcgc-3′

Table 2. Constructs Used in This Study

construct	reference
RepB′ (1−323, full length)	10
RepB′ catalytic domain (1−206)	10
RepB′ helix bundle domain (206−323)	10
RepB′ Δ6 (Δ 207−212)	this study
RepB′ Δ12 (Δ 207−218)	this study
RepB′W50A	this study
RepB′Y62F	this study
RepB′D77A	10
RepB′D78A	10
RepB′D134A	10
RepB′R145A	10
RepB′Y162A	this study
RepB′R234A	this study
RepB′D238A	this study
RepB′D281A	this study
RepB′R285A	this study
RepB′E302A	this study

mL; GE Healthcare), and equilibrated with buffer A. The column was washed using a step gradient of 14% buffer B (20 mM Tris/HCl (pH 7), 50 mM $MgSO_4$, 2 M NaCl, and 1 mM DTT), followed by application of a linear gradient (14−21%, 5 CV). The eluted proteins were diluted 1:4 in buffer A and loaded on a 20 HS-column (CV: 7 mL, PerSeptive Biosystems), equilibrated with buffer A. Proteins were eluted over a linear gradient (0−20%, 5 CV) of buffer B. The purest fractions were pooled and separated on a HiLoad-16/60-Superdex75 column (GE Healthcare), equilibrated with buffer C (20 mM Tris/HCl (pH 7), 150 mM $MgSO_4$, and 1 mM DTT). All proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to confirm their purity (Figure S2B).

The catalytic domain and the helix bundle domain were expressed and purified as described in ref 10. Briefly, the two His-tagged proteins were expressed in *E. coli* ER 2566 (NEB) under the same conditions as described above for RepB′. Cell lysates were loaded on a HisTrap column (CV, 5 mL; GE Healthcare), equilibrated with buffer D (20 mM HEPES (pH 8), 200 mM $MgCl_2$, and 1 mM DTT), and eluted over a linear gradient of 0−50% (100 mL) buffer E (20 mM HEPES (pH 8), 200 mM $MgCl_2$, 1 M imidazole, and 1 mM DTT). The His-tag was cleaved off using thrombin and removed by dialysis against buffer C. The catalytic domain was further purified using the RepB′ purification protocol for cation exchange and size-exclusion chromatography (20 HS & S75), as described above (Figure S3).

Production of Circular Single- and Double-Stranded DNA of Phage M13mp18ssiA. The recombinant phage M13mp18ssiA (mp18, Max-Planck strain 18), which carries the ssiA sequence in the multicloning site, was generated in a previous study.[10] To produce ssDNA and dsDNA of M13mp18ssiA, the electrocompetent *E. coli* strain JM103 carrying the F′-plasmid was transformed with M13mp18ssiA dsDNA and grown at 37 °C in 400 μL LB medium for 1 h. A volume of 100 μL of indicator bacterium *E. coli* JM103 and 400 μL of *E. coli* transformed with M13mp18ssiA dsDNA were mixed with 3 mL of 0.7% soft agar and spread on X-Gal/IPTG agar plates containing 100 μg/mL ampicillin and incubated at 37 °C until blue pseudoplaques appeared on the bacterial lawn. A phage suspension was prepared by inoculation of 3 mL LBM medium (LB medium, supplemented with 5 mM $MgCl_2$) with a well-separated pseudoplaque.

In order to produce M13mp18ssiA dsDNA, a 3 mL pre-culture of indicator bacterium *E. coli* JM103 was grown in LBM medium at 37 °C for 3 h (250 rpm) and then diluted 1:10 with LBM medium in a 250 mL Erlenmeyer flask (250 rpm). 30 mL of main culture was then infected with 3 mL of phage suspension. The infected culture was grown for 5 h at 37 °C (500 rpm). Bacteria were harvested (5 min, 16,000 g, 4 °C) and the M13mp18ssiA RF-DNA extracted.

To produce M13mp18ssiA ssDNA, a pre-culture of *E. coli* JM103 (M13mp18ssiA$^+$) was diluted 1:50 in TY medium. A volume of 6 mL of diluted culture was mixed with 1.5 mL of phage suspension and incubated for 5 h at 37 °C in a 250 mL Erlenmeyer flask under vigorous shaking (450 rpm). Bacteria were pelleted by centrifugation (5 min, 16,000*g*, 4 °C) and the phage containing supernatant was transferred into a 2 mL Eppendorf tube and centrifuged again (5 min, 16,000*g*, 4 °C) to remove residual bacteria. Finally, a volume of 1 mL of phage suspension was transferred into a new 1.5 mL Eppendorf tube, mixed with 200 μL of ice-cold PEG−NaCl solution (20% PEG 6000 and 2.5 M NaCl) and precipitated on ice for an hour. The phages were pelleted (5 min, 16,000 g, 4 °C) and resuspended in 10 μL of M13 buffer (20 mM Tris/HCl (pH 7.6), 10 mM NaCl, and 0.1 mM EDTA).

The phage suspension was heated to 60 °C and mixed thoroughly with 50 μL of hot phenol (60 °C). The mixture was cooled down to RT, vortexed, and centrifuged (3 min, 16,000*g*). The aqueous phase was taken off, mixed with 100 μL of chloroform, centrifuged (16,000*g*, RT), and the supernatant was transferred to a 1.5 mL Eppendorf tube. The DNA was mixed with 1/10 volume of Na acetate (3 M, pH 5.2) and 2.5 volumes of ethanol (100%) and precipitated for 2 h at −20 °C. After centrifugation (15 min, 16,000*g*, 4 °C), the supernatant was discarded and the DNA pellet was washed with 70% ethanol, centrifuged (1 min, 16,000*g*, 4 °C), and the washing step repeated with 100% ice-cold ethanol. The DNA pellet was

https://doi.org/10.1021/acsomega.1c03881
ACS Omega 2021, 6, 28903−28911

dried at RT, redissolved in 100 μL TS buffer (1 mM Tris/HCl (pH 8.0) and 1 mM NaCl), heated for 10 min at 65 °C), and cooled down to RT.

DNA Replication Assay. The initiation of complementary DNA strand synthesis was tested *in vitro* using a published protocol.[10] Briefly, 0.5 units Vent DNA polymerase, 50 nM primase RepB′, 30 ng M13mp18 ssDNA, and 375 μM dNTPs were incubated in 1×NEB buffer for 10 min at 37 °C, followed by 8 min of incubation at 72 °C. The reaction was stopped by the addition of 0.5% SDS. Samples were separated by electrophoresis on 1% TAE agarose gels and the DNA stained with 0.5% ethidium bromide.

Primer Synthesis Assay. The reaction contained 3 μM primase, 240 ng M13mp18ssiA ssDNA, 10 μM dCTP, 90 μM [$^{32}\alpha$P] dCTP (3,000 Ci/mmol), 100 μM dGTP, 100 μM dTTP, and 1 mM dATP in 1×NEB-2 buffer. After incubation for 1 h at 37 °C, formaldehyde loading buffer (95% formaldehyde, 18 mM EDTA, 0.025% SDS, 0.25% xylene cyanol, and 0.25% bromophenol blue) was added and heated for 2 min at 95 °C to stop the reaction. Samples were cooled down on ice before separation by vertical electrophoresis under denaturing conditions on a 20% polyacrylamide gel (28 cm × 16 cm × 0.2 cm) containing 8 M urea and 1 × TBE buffer (pH 8).

A phosphor screen (Type MS, PerkinElmer) was exposed to the gel overnight at −80 °C and then read out using a phosphor scanner (Cyclone, PerkinElmer). Oligonucleotides of 6, 8, 10, 12, and 14 nucleotides in length (TIB MOLBIOL, Berlin) were radioactively phosphorylated with [$^{32}\gamma$P] dATP at their 5′ ends using phosphokinase (NEB) following the manufacturer's protocol and used as length standards.

Run Off DNA Sequencing Reaction. A DNA replication assay was carried out as described earlier. The DNA sequencing was carried out by AGOWA Genomics (Berlin). Primers are listed in Table 1..

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c03881.

> Structurally conserved amino acids in the catalytic domains of RepB′ and Pri-type primases; RSF1010 replication and RepB′ recognition sequences; RepB′ primer synthesis in the presence of various dNTP compositions; sequence and structural comparison of RepB′ with IncQ and Pri-type primases; structural comparison of helix bundle domains of RepB′ and Pri-type primases; and schematic of the proposed RepB′ primase mechanism (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Sebastian Geibel − *Institute for Molecular Infection Biology & Rudolf Virchow Center for Integrative and Translational Bioimaging, Julius-Maximilians-Universität Würzburg, 97080 Würzburg, Germany;* orcid.org/0000-0003-0068-680X; Email: sebastian.geibel@uni-wuerzburg.de

Authors

Sofia Banchenko − *Charité—Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, Institute of Medical Physics and Biophysics, 10117 Berlin, Germany;* orcid.org/0000-0003-2166-1222

Christoph Weise − *Freie Universität Berlin, Institute for Chemistry and Biochemistry, 14195 Berlin, Germany*

Erich Lanka − *Max-Planck-Institut für Molekulare Genetik, D-14195 Berlin, Germany*

Wolfram Saenger − *Freie Universität Berlin, Institute for Chemistry, Biochemistry and Structural Biochemistry, 14195 Berlin, Germany*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.1c03881

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Claudia Alings and Clemens Langner for technical support in the lab and Dr. Eberhardt Scherzinger for advice. This work was supported by the German Research Foundation (SA196/43-1), bayresq.net, and the Elite Network of Bavaria (N-BM-2013-246).

■ REFERENCES

(1) Frick, D. N.; Richardson, C. C. DNA primases. *Annu. Rev. Biochem.* **2001**, *70*, 39−80.

(2) Lin, L.-S.; Meyer, R. J. DNA synthesis is initiated at two positions within the origin of replication of plasmid R1162. *Nucleic Acids Res.* **1987**, *15*, 8319−8331.

(3) Tanaka, K.; Kino, K.; Taguchi, Y.; Miao, D.-M.; Honda, Y.; Sakai, H.; Komano, T.; Bagdasarian, M. Functional difference between the two oppositely oriented priming signals essential for the initiation of the broad host-range plasmid RSF1010 DNA replication. *Nucleic Acids Res.* **1994**, *22*, 767−772.

(4) Scherzinger, E.; Bagdasarian, M. M.; Scholz, P.; Lurz, R.; Ruckert, B.; Bagdasarian, M. Replication of the broad host range plasmid RSF1010: requirement for three plasmid-encoded proteins. *Proc. Natl. Acad. Sci. U. S. A.* **1984**, *81*, 654−658.

(5) Scherzinger, E.; Haring, V.; Lurz, R.; Otto, S. Plasmid RSF1010 DNA replicationin vitropromoted by purified RSF1010 RepA, RepB and RepC proteins. *Nucleic Acids Res.* **1991**, *19*, 1203−1211.

(6) Miao, D.-M.; Sakai, H.; Okamoto, S.; Tanaka, K.; Okuda, M.; Honda, Y.; Komano, T.; Bagdasarian, M. The interaction of RepC initiator with iterons in the replication of the broad host-range plasmid RSF1010. *Nucleic Acids Res.* **1995**, *23*, 3295−3300.

(7) Scherzinger, E.; Ziegelin, G.; Bárcena, M.; Carazo, J. M.; Lurz, R.; Lanka, E. The RepA protein of plasmid RSF1010 is a replicative DNA helicase. *J. Biol. Chem.* **1997**, *272*, 30228−30236.

(8) Honda, Y.; Nakamura, T.; Tanaka, K.; Higashi, A.; Sakai, H.; Komano, T.; Bagdasarian, M. DnaG-dependent priming signals can substitute for the two essential DNA initiation signals inoriVof the broad host-range plasmid RSF1010. *Nucleic Acids Res.* **1992**, *20*, 1733−1737.

(9) Honda, Y.; Sakai, H.; Hiasa, H.; Tanaka, K.; Komano, T.; Bagdasarian, M. Functional division and reconstruction of a plasmid replication origin: molecular dissection of the oriV of the broad-host-range plasmid RSF1010. *Proc. Natl. Acad. Sci. U. S. A.* **1991**, *88*, 179−183.

(10) Geibel, S.; Banchenko, S.; Engel, M.; Lanka, E.; Saenger, W. Structure and function of primase RepB' encoded by broad-host-range plasmid RSF1010 that replicates exclusively in leading-strand mode. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 7810−7815.

(11) Miao, D.-M.; Honda, Y.; Tanaka, K.; Higashi, A.; Nakamura, T.; Taguchi, Y.; Sakai, H.; Komano, T.; Bagdasarian, M. A base-paired hairpin structure essential for the functional priming signal for DNA replication of the broad host range plasmid RSF1010. *Nucleic Acids Res.* **1993**, *21*, 4900−4903.

(12) Keck, J. L.; Roche, D. D.; Lynch, A. S.; Berger, J. M. Structure of the RNA Polymerase Domain of E. coli Primase. *Science* **2000**, *287*, 2482−2486.
(13) Augustin, M. A.; Huber, R.; Kaiser, J. T. Crystal structure of a DNA-dependent RNA polymerase (DNA primase). *Nat. Struct. Biol.* **2001**, *8*, 57−61.
(14) Steitz, T. A. A mechanism for all polymerases. *Nature* **1998**, *391*, 231−232.
(15) Bell, S. D. Initiating DNA replication: a matter of prime importance. *Biochem. Soc. Trans.* **2019**, *47*, 351−356.
(16) Holzer, S.; Yan, J.; Kilkenny, M. L.; Bell, S. D.; Pellegrini, L. Primer synthesis by a eukaryotic-like archaeal primase is independent of its Fe-S cluster. *Nat. Commun.* **2017**, *8*, 1718.
(17) Boudet, J.; Devillier, J. C.; Wiegand, T.; Salmon, L.; Meier, B. H.; Lipps, G.; Allain, F. H. A Small Helical Bundle Prepares Primer Synthesis by Binding Two Nucleotides that Enhance Sequence-Specific Recognition of the DNA Template. *Cell* **2019**, *176*, 154−e13.
(18) Yan, J.; Holzer, S.; Pellegrini, L.; Bell, S. D. An archaeal primase functions as a nanoscale caliper to define primer length. *Proc. Natl. Acad. Sci. U. S. A.* **2018**, *115*, 6697−6702.
(19) Qimron, U.; Lee, S.-J.; Hamdan, S. M.; Richardson, C. C. Primer initiation and extension by T7 DNA primase. *EMBO J.* **2006**, *25*, 2199−2208.
(20) Corn, J. E.; Pease, P. J.; Hura, G. L.; Berger, J. M. Crosstalk between primase subunits can act to regulate primer synthesis in trans. *Mol. Cell* **2005**, *20*, 391−401.
(21) Bergsch, J.; Devillier, J.-C.; Jeschke, G.; Lipps, G. Stringent Primer Termination by an Archaeo-Eukaryotic DNA Primase. *Front. Microbiol.* **2021**, *12*, 652928.
(22) Baranovskiy, A. G.; Babayeva, N. D.; Zhang, Y.; Gu, J.; Suwa, Y.; Pavlov, Y. I.; Tahirov, T. H. Mechanism of Concerted RNA-DNA Primer Synthesis by the Human Primosome. *J. Biol. Chem.* **2016**, *291*, 10006−10020.

https://doi.org/10.1021/acsomega.1c03881
ACS Omega 2021, 6, 28903−28911