SURVEY AND SUMMARY

Regulation of bacterial priming and daughter strand synthesis through helicase-primase interactions

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ABSTRACT

The replisome is a multi-component molecular machine responsible for rapidly and accurately copying the genome of an organism. A central member of the bacterial replisome is DnaB, the replicative helicase, which separates the parental duplex to provide templates for newly synthesized daughter strands. A unique RNA polymerase, the DnaG primase, associates with DnaB to repeatedly initiate thousands of Okazaki fragments per replication cycle on the lagging strand. A number of studies have shown that the stability and frequency of the interaction between DnaG and DnaB determines Okazaki fragment length. More recent work indicates that each DnaB hexamer associates with multiple DnaG molecules and that these primases can coordinate with one another to regulate their activities at a replication fork. Together, disparate lines of evidence are beginning to suggest that Okazaki fragment initiation may be controlled in part by crosstalk between multiple primases bound to the helicase.

INTRODUCTION

The replication of genomic DNA is a central event in the division and propagation of a bacterial cell. The nucleoprotein complex responsible for DNA replication is the replisome, which precisely coordinates the action of several discrete factors to efficiently and rapidly couple DNA unwinding with high-fidelity nucleic acid synthesis. Since the strands of template DNA are antiparallel, and because DNA polymerases are only capable of strand synthesis in the 5'–3' direction, genomic replication proceeds asymmetrically. One daughter strand, termed the leading strand, is continually synthesized in the same direction as the unwinding of the fork. The other, the lagging strand, is synthesized in the opposite direction in short bursts called Okazaki fragments (1).

Replication of the lagging strand is complicated by the inability of DNA polymerases to initiate strand synthesis. To overcome this challenge, cells utilize a special RNA polymerase, termed primase, to initiate each Okazaki fragment with a short oligoribonucleotide. The bacterial primase, DnaG, is recruited to the replication fork by an association with the replicative helicase, DnaB, where it synthesizes an RNA primer every 1.5–2 kb (2–4). Working together, the helicase and primase unwind the DNA template and initiate thousands of regularly-spaced Okazaki fragments to promote fork progression at a rate of ~1000 bases per second in rapidly dividing *Escherichia coli* cells.

The direct association of primase and helicase co-regulates their functions. For example, primase increases both the ATPase and helicase activities of DnaB (5). Similarly, DnaB can modulate the overall activity of DnaG, as well as the length of primers synthesized by the primase and its initiation specificity (6–8). Since isolated DnaG is weakly active *in vitro*, with a K_d of 1–10 µM for single-stranded template DNA, a K_M of 30–50 µM for rNTPs, and a maximum rate of approximately three primers per hour, activation of primase by DnaB is essential for replication to proceed at the rates observed *in vivo* (2,8–10). The importance of the helicase–primase interaction is underscored by certain viral systems, such as T7 bacteriophage gene product 4 (T7gp4), in which a DnaB-type helicase is fused with a DnaG-type primase in a single polypeptide to create a covalently tethered primase–helicase complex.

The physical mechanisms by which primase and helicase regulate each other's activity have been the focus of recent interest. Since enhancement of primase activity is unrelated to either the ATPase or helicase functions of DnaB (11), the simplest models posit that DnaB may activate DnaG merely by serving as a mobile docking station to increase the local concentration of single-stranded DNA template

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relative to primase. Such a structural role for the helicase is supported by a recent crystal structure of the T7gp4 primase–helicase, which shows that primase domains are loosely constrained to one side of the helicase ring, but that the structure of their catalytic center remains virtually unchanged from that determined in the absence of the helicase (12,13). However, this tethering model does not fully explain the more subtle aspects of the relationship between primase and helicase, such as how DnaB regulates the usage of primer initiation sites and alters the distribution of primer lengths synthesized by DnaG. This review will touch on a few recent developments that are beginning to shed light on this interplay and its importance to DNA replication.

THE DnaB HELICASE AND DnaG PRIMASE

In its active state, DnaB oligomerizes into a homo-hexameric ring (14–17). Each subunit of the hexamer contains two domains important for function (Figure 1). The C-terminal domain of DnaB, which comprises two-thirds of the protein, contains the ATPase activity of the helicase and is the principal organizing factor for ring formation (Figure 2) (18). The N-terminal domain is an α-helical globule that is required for helicase activity (18–22). Together with the linker to the C-terminal region, the N-terminal domain of DnaB mediates the helicase's interactions with DnaG (Figure 1) (5,23).

DnaG is a similarly modular protein, but with three independent domains (Figure 1). An N-terminal Zinc Binding Domain, so named for its internal zinc-ribbon motif, has been proposed to recognize a trinucleotide initiation site on template DNA (Figure 2) (24–26). The Zinc Binding Domain is followed by the RNA Polymerase Domain, which carries out primer synthesis (27,28). The isolated polymerase domain of bacterial DnaG is capable of synthesizing primers *in vitro*; however, bacteriophage primases require both the Zinc Binding Domain and the RNA Polymerase Domain for activity either *in vitro* or *in vivo* (27,29,30). Recent structural and biochemical data from T7gp4 have suggested that the Zinc

Figure 1. Domain organization of DnaB/DnaG-type helicase/primase systems. Bacterial DnaB is a two-domain protein with an N-terminal interaction domain and a C-terminal helicase domain. DnaG is a three-domain protein with an N-terminal regulatory Zinc Binding Domain, central RNA Polymerase Domain and C-terminal Interaction Domain. Known areas of interaction between the two proteins are shown as arrows. The phage T4 gp41 helicase and gp61 primase are minimalized versions of DnaB and DnaG, respectively. The phage T7 gp4 protein, on the other hand, fuses DnaB- and DnaG-related domains into a single polypeptide.

Binding Domain can dissociate from its RNA Polymerase Domain, possibly acting as a tethered, but mobile, regulatory element (13,31).

Although the Zinc Binding and RNA Polymerase Domains are essentially conserved with phage primases, bacterial primases contain an extra element at their C-termini, termed the Interaction (or P16) Domain. This module binds to the N-terminus and linker region of DnaB (21,23,32–34), as well as to SSB (35). Many *dnaG* temperature sensitive replication mutations map to the Interaction Domain (36), underscoring the need for DnaG to interact with other proteins in the replisome. The Interaction Domain is principally composed of a helical bundle and terminates in a small helical hairpin that mediates its interaction with the replicative helicase (Figure 2) (21,32). Surprisingly, recent structural and biochemical studies have shown that the helical bundle of the DnaG Interaction Domain is related in fold to the N-terminus of DnaB and is required for co-activation of the helicase by primase (21,32).
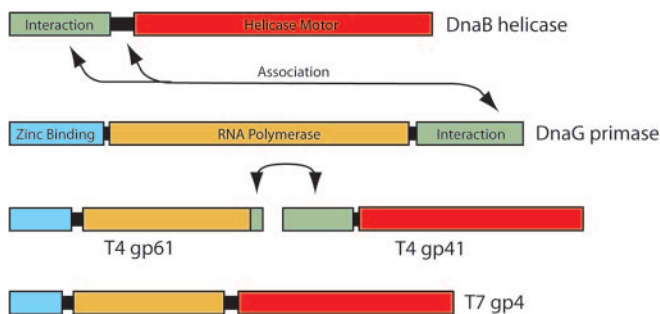
Several lines of evidence indicate that the interaction between primase and helicase has been fine-tuned among different organisms. To date, three distinct types of association have been observed. The first relies on non-covalent and relatively unstable contacts, as exemplified by *E.coli*, in which the primase–helicase complex can only be detected *in vitro* by relatively sensitive methods (7,18,21). The second class, which includes the *Bacillus stearothermophilus* and phage T4 primase–helicase complexes, also associates non-covalently, but forms stable interactions that can persist over a gel filtration column and may be visualized by electron and atomic force microscopy (5,37,38). The third class, typified by T7gp4, is distinguished by deletion of the interaction domain and the direct fusion of primase with the helicase into a single polypeptide chain (Figure 1).
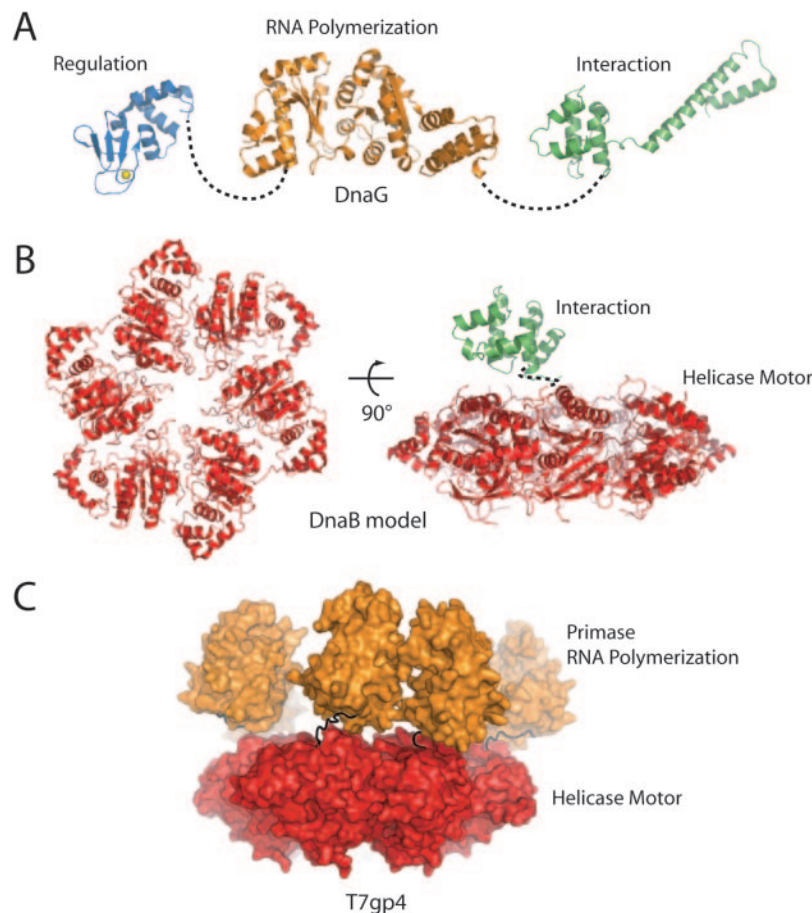
MULTIPLE PRIMASES BIND A SINGLE HELICASE RING

The covalent association between primase and helicase in T7gp4 directly facilitates the inclusion of up to six or seven primases per helicase ring (Figure 2). This assembly is complicated, however, by the expression *in vivo* of a truncated form of T7gp4 that both lacks the N-terminal primase Zinc Binding Domain and is incapable of primer synthesis (29). While these truncated monomers can assemble with full-length T7gp4 to alter the primase:helicase ratio *in vivo*, the functional significance of this mixture is currently unknown.

Even in systems with non-covalent primase/helicase associations, multiple primases are found to associate with a single helicase. For example, isothermal titration calorimetry (ITC) indicates that between four and six subunits of the T4 bacteriophage gp61 primase can bind the gp41 helicase *in vitro* (39). Similarly, analytical ultracentrifugation and gel filtration studies of the *B.stearothermophilus* primase–helicase complex shows that 2–3 primases bind each helicase (5), a value in excellent agreement with data obtained for the *E.coli* DnaB/G complex from fluorescence anisotropy and cross-linking/gel filtration analyses (40).

Although primases from bacteria or T4 bacteriophage are monomers in the absence of substrate, the presence of

Figure 2. Structures of the DnaG primase and DnaB helicase. (**A**) Atomic structures of domains from DnaG (PDB codes 1D0Q, 1DDE, and 1T3W) (21,24,27) are colored as in Figure 1 and connections between domains are shown as dotted lines. (**B**) The N-terminal domain of DnaB has been solved by NMR and X-ray crystallography (PDB codes 1JWE and 1B79) (20,22), whereas the C-terminal region likely resembles that of the related T7gp4 helicase domain (PDB code 1E0J) (58). Domains are colored as in Figure 1 and for clarity the N-terminal domain has been omitted from the face-on view. Only one of six N-terminal domains is shown in the side view, and the two domains are shown separated from each other. Interestingly, the helical bundles of the N-terminus of DnaB and C-terminus of DnaG adopt very similar folds (21,32). (**C**) The crystal structure of the T7gp4 primase–helicase (lacking the N-terminal Zinc Binding Domain) illustrates the relative locations of the primase (orange) and helicase (red) modules (12). The linkers between the two domains are shown as black ribbons.

ssDNA template provided by the helicase may favor the multimerization of primase molecules. Early hints to this behavior derived from studies published over 20 years ago, which indicated that a 2:1 complex of DnaG to ssDNA containing a phage G4 replication origin was required for optimal primer synthesis *in vitro* (41). More recently, fluorescence anisotropy and cross-linking studies have indicated that *E.coli* primase binds single-stranded oligonucleotides cooperatively, with a Hill Coefficient between 1.5 and 2. Here, binding was determined to occur via a sequential dimerization pathway, in which one primase subunit first associates with template as a monomer, followed by the binding of a second primase to form a ternary complex (42). In phage T4, ITC and fluorescence anisotropy experiments indicate that four to six molecules of the gp61 primase bind a relatively short piece of single-stranded DNA (39,43), and electron micrographs taken in the presence of ssDNA reveal six-membered rings that are of approximately the same diameter as the DnaB-related T4 gp41 helicase (38).

MECHANISTIC IMPLICATIONS FOR A PRIMASE ARRAY

Two important ramifications stem from recent work on the association between primase and helicase. First, the interaction of these proteins increases the local concentration of ssDNA available to primase. Second, the arrangement co-localizes multiple primase subunits within close proximity of each other. Since binding of primase to ssDNA appears to induce multimerization of the protein, these properties may allow multiple primases arrayed upon the helicase to coordinate their activies.

In bacterial systems, multiple lines of evidence indirectly signify that primer synthesis is controlled by helicase-mediated primase oligomers. For example, *E.coli* primase associates similarly with both wild-type DnaB and an ATPase-/helicase-deficient mutant (11). In priming assays, binding to either the wild-type or mutant protein down-regulates primase processivity to yield a distribution of primer lengths that ranges from 8 to 30 nt. However, when

ATP-γ-S is substituted for ATP with either active or inactive DnaB, the average primer length shifts to 15–60 nt (11). Although ATP-γ-S is not known to affect primase, this nucleotide has been shown to cause the helicase to transition from a 6-fold symmetric state to a 3-fold symmetric structure (14,17). It thus appears that modifying the symmetry of the helicase, and thereby the spatial relationship of one primase to another, may be one factor that could influence the synthetic characteristics of primase. Consistent with this model, AFM studies have indicated that primase itself may induce a change in symmetry of the *B.stearothermophilus* helicase upon binding (37), although how this conformational change might affect the activity of primase has not yet been investigated in this system.

For bacteriophage primase/helicase complexes, productive crosstalk between primase subunits has been directly observed in both the T7 and T4 systems. In both viruses, only full-length primases containing the Zinc Binding and RNA Polymerase Domains are capable of primer synthesis. However, truncated primases containing only the RNA Polymerase Domain can be complemented *in vitro* by catalytically inactive, full-length primase mutants (43,44). These data provide concrete evidence that priming can be regulated by communication between primases tethered to the same helicase. Such an event may occur by interactions that allow the regulatory Zinc Binding Domain of one subunit to cooperate with the catalytic RNA Polymerase Domain of another *in trans*. Indeed, recent structural and biochemical studies indicate that bacterial primases can modulate their activity and processivity through crosstalk between the Zinc Binding and RNA Polymerase domains (45). Thus, in addition to increasing the local concentration of the ssDNA template, the association of primase with the helicase ring may also increase the likelihood that intermolecular primase contacts will occur, thereby modulating primer synthesis.

INITIATING AN OKAZAKI FRAGMENT WITH MULTIPLE PRIMASES

An emerging picture of the primase–helicase complex is that of a highly mobile and modular priming factory. One model for the control of Okazaki fragment initiation posits that monomeric primase is relatively inactive in isolation, but that an association between the Zinc Binding Domain of one primase may activate and regulate the RNA Polymerase Domain of a second primase *in trans* (Figure 3). Under this model, primase would be specifically activated at a replication fork by the helicase, which is known to localize multiple primases to newly unwound lagging strands and to leading strand gaps occurring at sites of DNA damage (46).

Practical reasons may have led to the evolution of such a mechanism. Although cells require the relatively non-specific *de novo* synthesis activity of primase for DNA replication, they must also strictly limit primase action to the replication fork. Failing to do so could result in the synthesis of RNA primers at non-replisomal single-stranded regions within the genome, possibly to deleterious effect. Although these regions would likely be coated with SSB, an unregulated association between SSB and an active primase could lead to RNA incorporation within the genome. Indeed, bacteriophage G4 primes its own replication by recruiting bacterial DnaG to a specific location via carefully spaced SSB molecules and stem–loop structures (47,48). In bacteria, the low intrinsic activity of primase likely prevents the enzyme from inadvertently synthesizing primers at random locations,
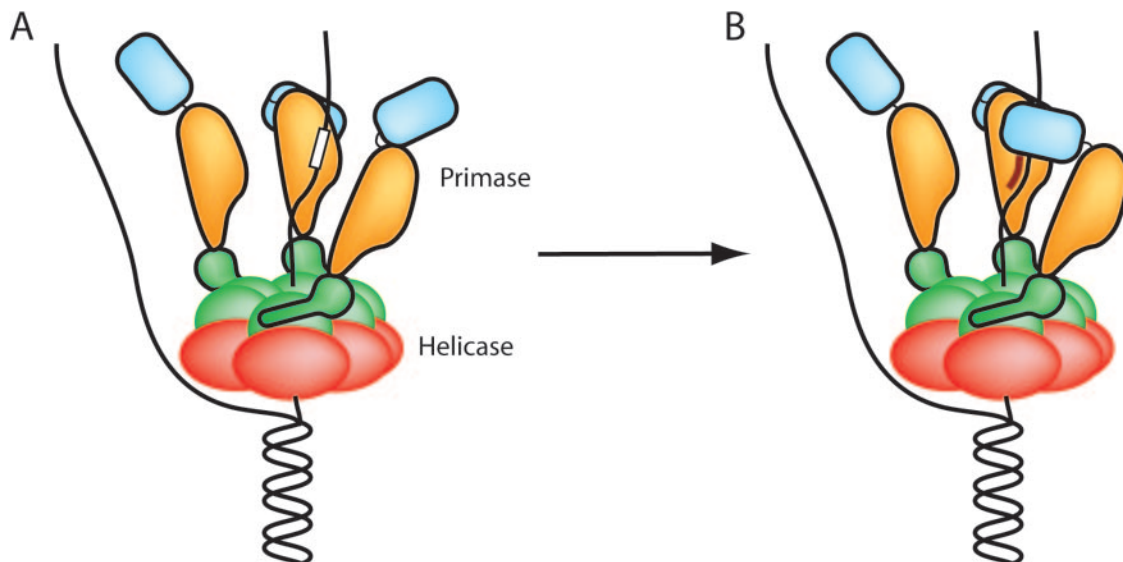
Figure 3. Model for the functional coordination of multiple primases bound to the replicative helicase. Domains of the DnaG primase are outlined in black, while those of the DnaB helicase are not. Domains are colored as in Figure 1. **(A)** As the helicase unwinds the parental strands, single-stranded DNA containing a trinucleotide primer initiation site (white rectangle) is extruded from the helicase ring and non-specifically passes through the catalytic center of a primase's RNA Polymerase Domain. **(B)** The Zinc Binding Domain of one primase molecule may form a ternary complex with a second primase's RNA Polymerase Domain and the DNA template, forming an active primase unit to allow the recognition of the primer initiation site and the activation of primer synthesis.

either alone or as part of a fortuitous complex with SSB, whereas its association with DnaB constrains primase to act on the proper template for appropriate synthesis at a replication fork. It is tempting to speculate that in bacteria, a helicase-mediated association between multiple primases may serve to further regulate function, since DnaG oligomers would only efficiently form *in vivo* upon binding to DnaB.

In addition to restricting the location of primer synthesis, the *E.coli* replisome also reduces the frequency of priming so as to ignore ∼19 out of every 20 possible initiation sites, thereby yielding the 1.5–2 kb Okazaki fragments observed *in vivo* (49,50). Interestingly, in T7 bacteriophage the isolated RNA Polymerase Domain can bind single-stranded DNA almost as well as the entire primase fragment, but lacks sequence specificity (31). This may indicate that the catalytic core of primase constantly binds to and dissociates from its template, but that it does so non-productively. If an interaction between adjacent primases bound to the replicative helicase were required to recognize and act upon a trinucleotide initiation site (Figure 3B), a productive association between the Zinc Binding and RNA Polymerase Domains would occur less frequently, greatly reducing the frequency of priming. The Zinc Binding Domain of one primase might thus enforce sequence specificity by recognizing an initiation site within single-stranded DNA that is bound to the RNA Polymerase Domain of another primase. Since the rate-limiting step of primer synthesis appears to be at or before the formation of the first phosphodiester bond (9), it is possible that the Zinc Binding Domain increases activity and enforces specificity by stabilizing two incoming ribonucleotides within the active site of a second primase, thereby favoring primer initiation.

PREVENTING OVER-PRIMING AFTER EACH PRIMING EVENT

Since there are only 50–100 primase molecules per *E.coli* cell (2), but thousands of Okazaki fragments, DnaG must be recycled from each primer for successive rounds of primer synthesis. It has been shown that a dissociative interaction between the primase and helicase determines the frequency of Okazaki fragment synthesis and thereby adjusts this 'replication fork clock' (51,52). In bacteria, binding of the Interaction Domain to the Single-Stranded DNA Binding protein forces a primase–primer/template complex to dissociate from the helicase (53,54), while in phage T7, ^{1}H-^{15}N HSQC NMR experiments have revealed that the gp4 Zinc Binding Domain can stably interact with primed RNA/DNA heteroduplexes (13,31). Recent single molecule experiments with the T7 replication system have also shown that the primase's Zinc Binding Domain is necessary to cause pausing of the entire replication fork after each priming event (55). These findings suggest that bacterial primase remains associated with the newly synthesized primer/template, possibly as a means to stabilize the relatively short RNA/DNA heteroduplex and to ensure appropriate transfer to the replicative polymerase. It also appears that the dissociation of primase from the helicase in bacteria may be a controlled process that contributes to the timing of Okazaki fragment synthesis by rendering the primosome inactive after each priming event (56).

If a stable primase–primer/template interaction exists both to remove a primase subunit from the helicase and to help protect the nascent heteroduplex, this complex might also be an effective signal that one round of primer synthesis has been successfully completed. Since multiple primases normally bind the replicative helicase, the dissociation of one subunit would leave a lower concentration of primase at the replication fork. If primase crosstalk helps favor Okazaki fragment initiation, the probability that any remaining primase(s) could associate with each other would be reduced, thereby further decreasing the frequency of priming. The controlled, post-synthesis dissociation of DnaG from DnaB, coupled with a propensity for multiple primases to coordinate initiation site selection and primer synthesis, might therefore be an effective mechanism to 'inform' the replisome that the lagging strand has been successfully primed. This could in turn prevent immediate re-priming, an event that would lead to abnormally short Okazaki fragments. In contrast, excess levels of DnaG would serve to increase the occupancy of primase bound to DnaB and decrease Okazaki fragment length, a phenomenon observed *in vitro* (51,52,57). Future work to investigate how helicase-directed primase oligomers regulate primer synthesis both *in vitro* and *in vivo* should shed light upon this poorly understood process and the role it plays in appropriately regulating DNA replication.

Conflict of interest statement. None declared.

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