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Replication initiation at the *Escherichia coli* chromosomal origin

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Abstract

To initiate DNA replication, DnaA recognizes and binds to specific sequences within the *Escherichia coli* chromosomal origin (*oriC*), and then unwinds a region within *oriC*. Next, DnaA interacts with DnaB helicase in loading the DnaB-DnaC complex on each separated strand. Primer formation by primase (DnaG) induces the dissociation of DnaC from DnaB, which involves the hydrolysis of ATP bound to DnaC. Recent evidence indicates that DnaC acts as a checkpoint in the transition from initiation to the elongation stage of DNA replication. Freed from DnaC, DnaB helicase unwinds the parental duplex DNA while interacting the cellular replicase, DNA polymerase III holoenzyme, and primase as it intermittently forms primers that are extended by the replicase in duplicating the chromosome.

Introduction

In free-living organisms, the replisome is a multi-protein molecular machine that duplicates the genome. Replisome assembly occurs at replication origins, and is a highly regulated event that is strictly coordinated with the cell cycle. For *Escherichia coli*, replisome assembly occurs at its single unique replication origin (*oriC*) of its circular genome, and can be divided into defined steps. During these steps before even a single deoxyribonucleotide is incorporated by the cellular replicase, proteins act in a dynamic manner as molecular machines, inducing movement as they perform work. These steps and the role of respective proteins are summarized below.

The structure of *oriC*

The study of *E. coli oriC* has revealed a variety of DNA sequence motifs that are functionally important (Figure 1; see refs [1,2] for reviews of *E. coli oriC*). One is the DnaA box, a nine base pair DNA motif that is recognized by DnaA protein, which is responsible for directing events that lead to the assembly of two replisomes for bidirectional fork movement (reviewed in ref [3]). Five DnaA boxes of similar DNA sequence are in *oriC* that vary in their affinity of binding by DnaA. DnaA binds to the high affinity sites (R1, R2 and R4) throughout most of the cell cycle [4,5]. At initiation, DnaA complexed to ATP additionally recognizes DnaA boxes R5 and R3, which are intermediate in affinity, and also specifically recognizes low affinity DNA motifs named τ - and I-sites within *oriC* [6,7]. Mutational analysis validates the functional importance of the DnaA boxes and I-sites,

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complementing several independent studies that show that the ordered assembly of DnaA and its oligomerization by interacting with these sites are essential for initiation and its coordination with the cell cycle [6,8–13]. Near the left border of *oriC*, an AT-rich region carries three 13-mer motifs that become unwound by DnaA complexed to ATP [14]. Binding sites in *oriC* recognized by IHF, Fis and SeqA correlate with their roles in initiation (see below). Surprisingly, removing the right half of *oriC* in the chromosome does not inactivate its function, but initiation is severely asynchronous [15]. Evidently, sequences in this region that includes a DnaA box named R4 are needed for the temporal regulation of initiation. Furthermore, the spatial organization of the following DNA motifs is critical for *oriC* function. The spacing between R3 and R4, or R2 and R3 must be one helical turn of DNA as a 5 base pair insertion inactivates *oriC* [16,17]. However a 10 base pair insertion does not [16,18]. Also, the spacing between R1 and the 13mer region cannot tolerate a single base pair addition or deletion [19].

Proteins that act at the stage of initiation

DnaA

Biochemical and structural studies combined with molecular genetic approaches indicate that DnaA contains several domains (Figure 2; reviewed in ref [20]. Domain I (amino acids 1–90) functions to interact with several proteins [21,22], including DnaB helicase in the recruitment of the DnaB-DnaC complex to *oriC* [23–26], and also acts in DnaA oligomerization [10,12,27]. These interactions appear to require distinct regions within domain I [12,26]. Domain II (90–130) may function as a flexible linker based on the variability in length of this region among *dnaA* homologues, and the lack of amino acid sequence conservation. In support that this region does not have a discrete function, ablation of contiguous segments within domain II did not affect cell viability [28,29]. However, deletion of residues 96–120 within domain II reduces the activity of DnaA in initiation [30,31], so this region is not entirely dispensable. Domain III (130–347) contains the sensor I, II (box VIII), and box VII motifs of the AAA⁺ protein family (reviewed in [32]), and functions in ATP binding, ATP hydrolysis, and in DnaA oligomerization [11,33]. Moreover by analogy with other AAA⁺ proteins, a conserved arginine in box VII is thought to be the “arginine finger” residue that interacts with the γ -phosphate of ATP, and coordinates a conformational change with ATP hydrolysis [33]. In addition, a portion near the beginning of domain IIIa interacts with DnaB [23,25]. Domain IV (347–467) acts to recognize the DnaA box motif [9,34–37]. A region involved in membrane binding is located between domains III and IV that is postulated to induce the dissociation of ADP from DnaA in vivo so that DnaA can bind ATP to support initiation (reviewed in [38]). These functional domains correlate well with structures of the C-terminal two-thirds of *Aquifex aeolicus* DnaA (domain III–IV), and the N-terminal region of *E. coli* DnaA (domain I) that were solved by X-ray crystallography and NMR spectroscopy, respectively [26,37].

DnaB and the DnaB-DnaC complex

DnaB is the replicative DNA helicase that separates the DNA strands of the *E. coli* chromosome to support replication fork movement. This enzyme consists of six protomers aligned side-by-side into a ring-like structure through which one strand of the parental DNA passes while also excluding the other DNA strand [39–41]. At the apex of the replication fork, the single-stranded DNA that passes through the central channel of DnaB serves as the template for the synthesis of the lagging strand in the form of Okazaki fragments. As DnaB unwinds DNA, it moves in the 5'-to-3' direction on this parental DNA strand. Structural studies reveal that the DnaB protomer has a bi-lobed shape composed of a larger C-terminal domain bearing a RecA-like fold joined by a linker to a smaller N-terminal domain (Figure 2; [42–44]). In the DnaB hexamer, the larger lobe is closest to the apex of the replication

fork. A model describes a rotary mechanism for how members of this helicase family (helicase superfamily 4) of molecular motors couple ATP hydrolysis to translocation and DNA unwinding [45–48]. In this model, a hairpin loop from each protomer contacts a nucleotide of the single stranded DNA during translocation that is coordinated with the hydrolysis of ATP bound at the interface between protomers where the Walker A and B motifs in the RecA-like fold are from one protomer and the arginine finger (arginine 409) is from the neighboring protomer.

For the *E. coli* chromosome, and many episomes that use *E. coli* as a host, DnaB must be complexed with DnaC for the helicase to be able to bind to the respective replication origin (reviewed in ref [49]). However bound to DnaB, DnaC inhibits its helicase and ATPase activity, so DnaC must dissociate for DnaB to be active. Most studies of DnaB complexed with DnaC have assumed that the DnaB₆-DnaC₆ complex is the functional form. However, recent results suggest that the DnaB-DnaC complex assembled at *oriC* contains three DnaC monomers per DnaB hexamer [50]. Interestingly, the N-terminal domains of DnaC and bacteriophage λ P protein are similar in amino acid sequence [51]. For DnaC, its N-terminal region appears to interact with DnaB [52]. Bacteriophage λ uses P protein presumably complexed via its N-terminal region to engage DnaB. As the viral analogue of DnaC, λ P protein displaces DnaC from the DnaB-DnaC complex and also prevails over DnaC in binding to DnaB [53]. Like the DnaB₆-DnaC₃ complex, three λ P monomers are in the DnaB₆-[λ P protein]₃ complex. For either complex, its formation is necessary in order for DnaB to load at the respective replication origin.

DnaC

DnaC is the partner for DnaB, and is essential for DnaB to function in initiation. Because of this partnership, DnaC has been described as the “helicase loader” [54–57]. This term is a misnomer because it implies an active role for DnaC; the evidence thus far indicates that DnaA is the helicase loader, and DnaC has a passive but essential role in the delivery of DnaB at *oriC*.

Bound to the larger domain of DnaB to form the DnaB-DnaC complex [58,59], DnaC apparently interacts via an N-terminal region to inhibit the ATPase and helicase activity of DnaB (Figure 2; [52]). As the DnaB-DnaC complex is essential for initiation, negative regulation of DnaB function by DnaC is required at the step of helicase loading. Once the helicase has bound to *oriC*, dissociation of DnaC from DnaB is required for subsequent DNA replication. Hence, DnaC is the regulator of DnaB.

As an AAA+ protein [60,61], DnaC binds ATP albeit with low affinity ($K_D \sim 8 \mu\text{M}$), and is a weak ATPase [54,62]. It is purified as a monomer whereas many other AAA+ proteins like DnaB are ring-shaped oligomers [63]. To validate DnaC as an AAA+ protein, substitution of the conserved lysine with arginine in the Walker A box causes undetectable ATP binding and inactivity in DNA replication of an *oriC*-containing plasmid [54]. Also, in vivo studies show that missense mutations in each AAA+ motif impair DnaC function [52,64]. As described in more detail below, the motif named box VII is also essential.

Measured by a variety of methods, DnaC also binds weakly to single-stranded DNA [50,54,65,66]. This activity that is stimulated by ATP may help to load DnaB at *oriC* (see below).

Steps during Initiation

DnaA-dependent unwinding of *oriC*

DnaA assembled at *oriC*, and complexed to ATP or ATP γ S unwinds the AT-rich region carrying the 13-mer motifs (Figure 1; [14]). In comparison, ADP is much less effective. Hence, ATP binding but not its hydrolysis by DnaA is required for strand opening [67]. ATP apparently induces a conformational change in DnaA or supports the formation of a structure needed for strand opening. To support the latter possibility, DnaA complexed with the ATP analog, AMP-PCP, forms a right-handed helical filament that has a central channel as determined by X-ray crystallography [68]. Biochemical evidence to support the functional importance of this structure is based in part on the properties of a mutant DnaA bearing an alanine substitution for tryptophan 6, which resides in a hydrophobic patch. The substitution inhibits DnaA oligomer formation, presumably as this filament, by apparently impairing the interaction between adjacent protomers [12,26]. Other evidence suggests that DnaA interacts directly with the top strand of the unwound region [26,69]. Specifically, alanine substitutions for valine 211 or arginine 245 lead to inactivity in unwinding, implicating the involvement of these positively charged residues that occupy the central channel [70]. Together, these results support the proposal that residues in the interior channel of the DnaA filament interact electrostatically with the single-stranded DNA [68]. Somehow, this model must accommodate observations that a mutant DnaA bearing an R281A substitution is defective in DNA replication due to impaired DnaA oligomerization at *oriC* but is active in unwinding [7,11]. Hence in contradiction with the model, a mutant DnaA defective in self-oligomerization is active in unwinding. More experiments are needed to understand the relationship between unwinding and DnaA oligomer/filament formation.

Recent studies show that other proteins influence the unwinding of *oriC* by DnaA. One is HU, a dimer composed of α_2 or the $\alpha\beta$ subunits that are abundant during early to midlog phase, or the β_2 dimer of stationary phase cells [71]. Its role in initiation was originally discovered by its stimulation of *oriC* unwinding by DnaA [72]. Another is a protein named DiaA [73]. The *diaA* gene was initially identified by the ability of a *diaA* mutation disrupted by insertion of Tn5 to suppress the cold-sensitive phenotype of a *dnaAcos* mutant. HU (α_2 or the $\alpha\beta$ dimer), or DiaA stimulates unwinding of *oriC* by interacting with the N-terminal region of DnaA [21,74]. This interaction appears either to facilitate DnaA oligomer formation, or to stabilize the DnaA oligomer after it has formed at *oriC*. Supporting these in vitro results, physiological studies show that strains lacking *hupA* that encodes the α subunit of HU or *diaA* initiate at inappropriate times in the cell cycle [73,75]. Apparently, the action of these proteins during unwinding is important for the proper timing of initiation. In contrast, Dps or both a truncated or full-length form of ribosomal protein L2 antagonizes unwinding and formation of the DnaA oligomer in vitro [21,76]. Corresponding in vivo evidence shows that initiation is less frequent in *dps*⁺ strains compared with mutants lacking Dps to support the conclusion that Dps negatively regulates DnaA. Because the ribosomal protein is essential, similar in vivo experiments cannot be performed. Together, these observations suggest that the N-terminal region (domain I) of DnaA acts as a sensor for these and perhaps other proteins yet to be identified to modulate the activity of DnaA and the initiation process under certain conditions.

DnaA loads DnaB helicase at *oriC*

After the unwinding of *oriC*, DnaA loads one DnaB-DnaC complex on the top strand near the left border of *oriC* and a second DnaB-DnaC complex on the lower strand next to DnaA box R1 (Figure 1; [77,78]). As *oriC* plasmid replication is bidirectional in vitro even when DnaB is at a subsaturating level [79], helicase loading is concerted. The loading process can be measured with an *oriC*-containing plasmid, or with a single-stranded DNA named M13

A-site that carries a DnaA box in a hairpin structure [50,80]. DNA replication of this SSB-covered single-stranded DNA requires the binding of DnaA to the hairpin followed by the DnaB-DnaC complex. The complex assembled with either this DNA or a supercoiled plasmid carrying *oriC* can be isolated and analyzed by quantitative immunoblotting to determine the stoichiometry of DnaA, DnaB and DnaC relative to the amount of either DNA, which is determined in ethidium bromide-stained agarose gels relative to known amounts of the same DNA. Once DnaB has bound to the DNA, the activity of the intermediate named the prepriming complex can be measured by the addition of primase that interacts with DnaB to synthesize primers, and of DNA polymerase III holoenzyme that extends these primers during DNA synthesis. For the *oriC*-containing plasmid, DNA gyrase is also required at this stage to remove the positive superhelicity that would otherwise accumulate ahead of the replication fork and impede fork movement.

Several studies support the conclusion that DnaA loads DnaB at *oriC*. First, a monoclonal antibody inhibits the interaction between DnaA and DnaB in solid phase binding assays, the retention of DnaB in the prepriming complex, and DNA replication of the single-stranded DNA or of the *oriC*-containing plasmid [23,24]. This antibody binds to an epitope within residues 111–148 that contains domain II and overlaps domain III of DnaA. By deletion analysis, this interacting region has been localized to residues 130–148 near the N-terminus of domain III [25]. That consecutive stretches of 30–36 residues from amino acid 79 to 135 in domain II can be removed without loss of DnaA function suggests that domain II is not required to load DnaB [29], and that the interacting region is within residues 135–148. Deletion of residues 24–86 within domain I also reduces the interaction with DnaB, implicating a second interacting region [25]. Interestingly, mutant DnaAs carrying E21A or F46A substitutions are active in all other activities of DnaA that do not involve the DnaB-DnaC complex [74]. However, these mutants are essentially inactive in DNA replication of M13 A-site ssDNA or of an *oriC*-containing plasmid. Direct evidence shows that the F46A substitution impairs this interaction with DnaB. In the NMR structure of domain I [26], glutamate 21 and phenylalanine 46 are in the second and third α helices of a solvent-exposed surface, suggesting that this region of DnaA interacts with DnaB in the process of helicase loading. In summary, two regions of DnaA appear to interact with DnaB. It is not clear how the loading of two helicases on each strand within the unwound region of *oriC* is coupled.

Stimulated by ATP, DnaC binds weakly to single-stranded DNA as described above. In addition, DnaA assisted by HU opens two of the three 13mers whereupon the addition of the DnaB-DnaC complex enlarges this unwound region [54,81]. These results raise the possibility that single-stranded DNA binding by DnaC helps to load DnaB at *oriC*. DnaC also interacts with DnaA [57], suggesting that DnaC cooperates with DnaA to load DnaB. If DnaC is necessary and sufficient for helicase loading, it should substitute for mutant DnaAs that are able to unwind *oriC* but fail to support initiation due to another biochemical defect. However, DnaC was unable to escort DnaB at *oriC* unwound by mutant DnaAs that are defective in self-oligomerization [11,12]. If DnaC acts directly in helicase, loading, it apparently cannot do so without wild type DnaA. In restarting replication forks by comparison, the DnaB-DnaC complex requires either the prior assembly of PriA, PriB and DnaT, or PriC and PriA or Rep at stalled forks, suggesting a separate loading mechanism (reviewed in ref [82,83]).

DnaC acts as a checkpoint in the transition from initiation to the elongation stage of DNA replication

After DnaB loads at *oriC*, DnaC must dissociate so that DnaB can unwind the parental duplex DNA. Recent evidence indicates that primer formation by primase, which interacts with the smaller domain of DnaB during primer synthesis [42,84,85], causes the dissociation of DnaC bound to the larger C-terminal domain of DnaB (Figure 1). Apparently, the

interaction of primase with DnaB induces a conformational change in the helicase that causes DnaC to dissociate [50]. By analogy with other AAA+ proteins, conserved arginines in the box VII motif (RVMPRM) of DnaC presumably coordinate ATP hydrolysis with a conformational change needed for dissociation. Alanine substitutions for the conserved arginines render the mutant proteins unresponsive to the signal that involves primase-dependent primer formation.

Events after initiation

Replication fork movement from *oriC* is bidirectional. Once freed of DnaC, DnaB translocates in the 5'-to-3' direction on the lagging DNA strand as it unwinds the parental DNA (Figure 1). For DnaB moving either the rightward or leftward, the interaction of primase with DnaB leads to primer formation, which is followed by the binding to the primer end by one unit of a dimer of DNA polymerase III holoenzyme. This unit of the dimer synthesizes the leading strand as it interacts via its τ subunit with DnaB helicase as it moves (reviewed in ref [86,87]). The subsequent interaction of primase with DnaB leads to the synthesis of primers for Okazaki fragment synthesis [84,85,88]. Models that incorporate biochemical observations with the structures of bacterial DnaB and primase suggest how the helicase and primase coordinate their functions [42,89]. At each replication fork, the ensemble of DnaB, the dimeric DNA polymerase III holoenzyme concurrently synthesizing both leading and lagging strands, and primase that transiently interacts with DnaB to synthesize primers for Okazaki fragment synthesis represents the enzymatic machinery that duplicates the *E. coli* chromosome.

Conclusions

The *E. coli* replication origin serves as a site to assemble the replication fork machinery for bidirectional fork movement. DnaA initiates this process by binding to *oriC* and then unwinds a region within it. The critical event that leads to the assembly of the replication fork machinery involves the DnaA-dependent loading of the DnaB-DnaC complex, followed by the release of DnaC. This process of assembly is dynamic, and involves the binding and release of these proteins in a specific order.

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Highlights

DnaA interacts with DnaA boxes, I-sites and τ -sites in *oriC*.

At *oriC*, DnaA loads DnaB helicase from the DnaB-DnaC complex.

Assembly of the replication fork machinery is a step-wise process.

Primer formation by primase (DnaG) induces the dissociation of DnaC from DnaB.

DnaC acts as a checkpoint in initiation.

Figure 1.

Escherichia coli oriC carries recognition sites for DnaA, including DnaA boxes, I-sites and τ -sites. Also shown are binding sites for IHF and Fis, and the 13-mer sequences that become unwound by DnaA complexed to ATP. Following the binding of DnaA to the sites described above and unwinding, DnaA loads one DnaB₆-DnaC₃ complex each on the top and bottom strands of the unwound region. The first primers synthesized by primase on the top and bottom strands both induce the dissociation of DnaC from DnaB so that DnaB can act as a DNA helicase to unwind the parental duplex DNA, and prime the synthesis of each leading strand for the rightward and leftward progressing replication forks. The subsequent occasional interaction of primase with DnaB leads to primers (not shown) for Okazaki fragment synthesis.

Figure 2.

Functional domains of DnaA, DnaB, and DnaC protein. (A) In the domain organization of DnaA, a region near the N-terminus is involved in the interaction between DnaA and DnaB, HU, Dps, DiaA, or ribosomal protein L2 (see *DnaA-dependent unwinding of oriC*). A region within this domain is also required for DnaA oligomerization. Domain II functions as a flexible linker joining domain I and IIIa and IIIb, but this domain is not entirely dispensable. Domain III is involved in ATP binding and carries the amino acid sequence motifs shared among AAA+ family members. Domain IV is involved in DNA binding. (B) Domains of DnaB identified by trypsin digestion correlate with the X-ray crystallographic structure of *Bacillus stearothermophilus* DnaB [42,90,91]. Regions of DnaB that interact with primase, DnaC and ATP, which include the Walker A and Walker B box and the arginine finger (arginine 409 of *E. coli* DnaB) are shown. (C) The interacting domains of DnaC with DnaB and with ATP, including the AAA+ motifs and the conserved arginines in box VII are shown. The numbers in the respective lines refer to the coordinates for *E. coli* DnaA, DnaB and DnaC protein.