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Replication Initiation in Bacteria

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Abstract

The initiation of chromosomal DNA replication starts at a replication origin, which in bacteria is a discrete locus that contains DNA sequence motifs recognized by an initiator protein whose role is to assemble the replication fork machinery at this site. In bacteria with a single chromosome, DnaA is the initiator and is highly conserved in all bacteria. As an adenine nucleotide binding protein, DnaA bound to ATP is active in the assembly of a DnaA oligomer onto these sites. Other proteins modulate DnaA oligomerization via their interaction with the N-terminal region of DnaA. Following the DnaA-dependent unwinding of an AT-rich region within the replication origin, DnaA then mediates the binding of DnaB, the replicative DNA helicase, in a complex with DnaC to form an intermediate named the prepriming complex. In the formation of this intermediate, the helicase is loaded onto the unwound region within the replication origin. As DnaC bound to DnaB inhibits its activity as a DNA helicase, DnaC must dissociate to activate DnaB. Apparently, the interaction of DnaB with primase (DnaG) and primer formation leads to the release of DnaC from DnaB, which is coordinated with or followed by translocation of DnaB to the junction of the replication fork. There, DnaB is able to coordinate its activity as a DNA helicase with the cellular replicase, DNA polymerase III holoenzyme, which uses the primers made by primase for leading strand DNA synthesis.

1. INTRODUCTION

DNA replication is a central process in all organisms. This biochemical pathway is divided into three stages: initiation, elongation, and termination. Individual proteins act in concert with others at each stage, performing specific functions that lead to the duplication of DNA. The study of DNA replication has its foundation in studies of *Escherichia coli* as a model system. Of interest, studies of other bacterial species, such as *Bacillus subtilis*, *Caulobacter crescentus*, and *Helicobacter pylori*, have revealed that the molecular mechanisms used among bacteria are remarkably similar. Moreover, the program of molecular events in these organisms at the stage of replication initiation is surprisingly like that of eukaryotes and archaebacteria. Together, these studies of bacteria may lead to the development of novel antibiotics that act to inhibit DNA replication in bacterial pathogens. These shared features include recognition of a replication origin by a DNA binding protein, or in some cases a complex of proteins, unwinding of the parental duplex DNA, loading of the replicative DNA helicase onto each strand of unwound DNA, and activation of the helicase. Recruitment of the cellular replicase follows, which leads to the assembly of the enzymatic machinery that

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acts at a replication fork to duplicate the chromosome. This chapter summarizes replication initiation in *E. coli*, and highlights similarities and important differences in the biochemical mechanisms of initiation in other bacterial species.

2. BACTERIAL REPLICATION ORIGINS

2.1 The Replication Origin of *E. coli*

DNA replication in bacteria starts at a unique locus called the replication origin, or *oriC*. Among bacteria, this site contains a variety of DNA sequence elements described in more detail below that are recognized by proteins that are either directly involved in replication initiation, or that regulate the frequency of this event to coordinate it with bacterial growth and cell division. In *E. coli oriC*, this 250 base pair (bp) sequence is located between the *gidA* and *mioC* genes at 84.3 min on the genetic map (reviewed in Ref. [1]). Originally, this site was identified by measuring the gene frequency in replicating cells [2,3], or in cells manipulated to initiate DNA replication synchronously. In the latter approach, a growing culture of a *dnaC*(Ts) mutant was transferred to an elevated temperature to block new rounds of DNA replication while permitting ongoing DNA replication to continue to completion [4]. A downshift to the permissive temperature leads to a new round of DNA replication and also an elevated ratio of *oriC* to distal sites, which were measured by radioactivity. Alternate methods rely on the ability of *oriC* to confer autonomous replication to derivatives of bacteriophage lambda under conditions in which the bacteriophage replication origin is inhibited [5,6], or to plasmids that confer antibiotic resistance [7–9]. For bacterial species that are not closely related evolutionarily to *E. coli*, the location of the respective replication origins is often found adjacent to the *dnaA* gene, but the length of *oriC* is variable, ranging from 100 to 1000 bp.

E. coli oriC has been the subject of molecular analyses by a variety of methods. One approach relies on comparative DNA sequence analysis of *E. coli oriC* and the replication origins of evolutionarily related bacteria to identify conserved DNA regions that presumably represent sequences recognized by specific proteins. Another is DNA footprinting to demonstrate that a protein is able to bind to a particular DNA sequence. A third relies on mutational analysis of minichromosomes wherein *oriC* joined to a drug resistance gene provides for maintenance of this DNA in bacterial cells. These approaches have led to the identification of five copies of a DNA motif named the DnaA box that is recognized by DnaA. To indicate the positions of these DnaA boxes, they have been named R1 through R5. These DnaA boxes are essential for *oriC* function on the basis that individual mutations in each cause impaired activity [10–12]. Although R1 and R4 are identical in DNA sequence, DnaA binds with higher affinity to R4 presumably because of its differential interaction with nucleotides that flank these sites [13–15]. Inasmuch as DnaA binds specifically to adenine-containing nucleotides [16], both DnaA-ATP and DnaA-ADP comparably recognize these DnaA boxes [16–18]. In contrast, DnaA-ATP binds with lesser affinity to R2 followed by R3, and with even lower affinity to sites named I1, I2, I3, R5, τ1, τ2, C1, C2, and C3 [17,18]. Of interest, recent studies suggest that DnaA-ATP may actually interact with C2 and C3 instead of R3, which are on either side and overlap R3 [19]. Hence, DnaA may not recognize the R3 DnaA box. These observations with DnaA-ATP differ from those with

DnaA complexed to ADP, which poorly recognizes the low-affinity sites. These differences in the adenine-bound forms of DnaA correlate with the activity of DnaA-ATP and not DnaA-ADP in replication initiation [16].

Considering that B-form DNA has about ten base pairs per turn of the DNA helix, other studies strongly suggest that the relative arrangement of DnaA molecules assembled at *oriC* is important. Phylogenetic comparison of the replication origins of Gram negative bacteria reveals conserved regions that are separated by DNA of fixed length whose sequences are not conserved, suggesting that spacing or phasing between the conserved regions is critical for *oriC* function [20]. Moreover, inverting the DnaA boxes R1, R2, or R4 [12], or changing the distance between R3 and R4 or R2 and R3 led to inactivation of *oriC* [21,22]. In contrast, insertion or deletion of 10–12 base pairs did not render *oriC* nonfunctional [21,23]. Together, these observations strongly suggest that the orientation of DnaA bound to a specific DnaA box relative to other DnaA-DnaA box complexes in *oriC* is essential.

As will be described in more detail below, DnaA self-oligomerizes via sites in its N-terminal domain (domain I), and its ATP binding module (domain III). In view of observations that DnaA oligomerization is required for replication initiation [24–26], these findings support a model in which DnaA complexed to the individual DnaA boxes at *oriC* forms a specific nucleoprotein complex. An attractive idea is that the DnaA boxes serve as sites for nucleation of DnaA-ATP. These DnaA molecules bound in an ordered manner at these sites serve to recruit additional DnaA molecules to the nearby lower affinity sites, which presumably stabilizes a DnaA oligomer. On the basis of the orientation of the DnaA boxes, τ-, I-, and C-sites [17–19], Leonard and Grimwade proposed that *oriC* is a bipartite structure, and suggested that DnaA assembles on the left and right portions of *oriC* as separate opposing oligomers [1,19]. In support, alterations in the DNA sequence, orientation, or spacing of the low-affinity sites in the left or right portions of *oriC* disrupted DnaA binding, and caused impaired function of *oriC* in DNA replication [19].

The work summarized above relies on the analysis of plasmids that carry *oriC*. Of interest, studies of these DNA motifs when *oriC* is in its natural environment of the *E. coli* chromosome revealed important differences. An early study showed that insertion of a two kilobase pair DNA fragment between R3 and R4 inactivates *oriC* when borne by a plasmid, but not when this insertion mutation was placed into the *E. coli* chromosome [27]. This work also showed that deletion of R4 in an *oriC*-containing plasmid blocks origin function, but its removal from the chromosome did not interfere with viability. However, initiation in the R4 deletion mutant under culture conditions that lead to rapid growth and multiple initiations was no longer synchronous. [a] More recent studies showed that deletion of the region that removes R4, the DNA motifs C3, C2, C1, I3, and R3, like the R4 deletion mutation

[a]Although bacteria are considered to be haploid, individual cells in rapidly growing cultures initiate new rounds of DNA replication prior to cell division and before the previous cycle has been completed [28]. Depending on the culture conditions, such cells bear chromosomes that are partially diploid, partially tetraploid, or even partially octaploid. As measured by flow cytometry under conditions in which cell division and new initiations are blocked, but ongoing DNA replication can proceed to completion, cells normally carry 2^n chromosomes where $n \geq 0$ and is an integer [29–31]. Hence, cells growing quickly initiate DNA replication synchronously and at a particular time in the bacterial cell cycle. In contrast, asynchronous DNA replication leads to odd numbers of chromosomes as observed in mutants carrying *dnaA*(Ts) alleles defective in ATP binding, suggesting that ATP binding by DnaA is necessary for proper regulation of initiation [32]. Other mutations (*fis*, *himA*, *seqA*, *dam*, *hupA*, and *hda*) also cause asynchronous initiations because the respective mutations perturb the initiation process by direct or indirect effects.

described above, did not impair viability [33]. Physiological characterization of this mutant revealed a decreased rate of growth, fewer origins per cell, and asynchronous initiations. These defects were exacerbated in rich growth media but were suppressed in poor media. These findings suggest that the segment at the right in *oriC* is uniquely required when the conditions call for multiple initiations within a single cell. Hence, the DNA elements within *oriC* needed for its function are not fixed but depend on physiological conditions. Bearing in mind the idea that R4 serves as a DnaA nucleation site that directs its cooperative binding to the C3, C2, C1, and I3 sites nearby, the formation of this DnaA oligomer may be needed under the conditions of multiple initiations.

In addition to the DNA motifs recognized by DnaA, *E. coli oriC* has an unusual abundance of 11 GATC sequences that are targeted by DNA adenine methylase (reviewed in Ref. [28]). For simplicity, Fig. 1 omits these sites, which are also preferentially recognized by SeqA when they exist in the hemi-methylated state. Shortly after initiation, SeqA complexed to the resultant hemi-methylated DNA appears to occlude *oriC* from DnaA and other proteins needed for replisome assembly. After SeqA dissociates from *oriC*, DNA adenine methylase is able to convert the hemi-methylated sites in *oriC* to the fully methylated form to permit another cycle of initiation.

2.1.1 The DNA Unwinding Element of oriC and DnaA—Replication initiation from *oriC* in vitro requires its presence in a super-coiled DNA. Assembled at *oriC*, DnaA complexed to ATP or ATPγS, an analogue that is poorly hydrolyzed by most ATPases, induces the unwinding of an AT-rich region located near the left border of *oriC*. This region, which is sometimes described as a DNA unwinding element or DUE [39], contains three 13mer repeats that when unwound at an optimal temperature of 38°C are sensitive to nucleases or chemicals that are specific for single-stranded DNA [40]. Presumably, this condition aids in the disruption of base pairs. An interesting conundrum that is not understood, DnaA binds with relatively high affinity to ATP (K_D = 0.03 μM), yet unwinding requires much higher concentrations of ~5 mM ATP [16]. Despite this lack of understanding, ATPγS in place of ATP but not ADP supports unwinding. Hence, ATP hydrolysis is not required. Apparently, DnaA complexed to ATP or ATPγS is in a conformation that is active in unwinding whereas DnaA-ADP is not. As evidence that the conformation of DnaA complexed to ADP is different from DnaA-ATP, X-ray crystallographic analysis of domains III and IV of *Aquifex aeolicus* DnaA complexed to ADP revealed a different structure in comparison with DnaA bound to AMP-PCP, an ATP analogue (see later) [41,42]. The co-crystal containing ADP supports a model of a closed ring composed of six molecules of DnaA, each arranged with ADP at the interface separating adjacent protomers. The co-crystal containing AMP-PCP underpins a model of a right-handed helical filament. Each model has hydrophobic and positively charged amino acids in the interior channel. Evidence presented below supports a model that the unwound DNA interacts with accessible amino acids of the channel's interior.

To test the role in DNA binding of specific hydrophobic and positively charged amino acids that line the inner channel, mutant DnaA proteins bearing alanine substitutions for Val221 and Arg245 were shown to be impaired in unwinding of *oriC* contained in a linear duplex DNA [43]. Under comparable conditions, wild-type DnaA complexed to ATP was active but

DnaA-ADP was not. An independent study showed that wild-type DnaA is active in strand displacement of a duplex oligonucleotide of 15 base pairs, but not of a 30 base pair duplex DNA, suggesting that DnaA exploits the relative thermodynamic instability of the DUE by binding to the transiently unwound DNA [44]. These biochemical observations correlate with the X-ray crystallographic structure of domains III and IV of *A. aeolicus* DnaA complexed to AMP-PCP and polydA$_{12}$ [44], supporting the model that unwinding of *oriC* involves the binding of the unwound DNA to the inner cavity of DnaA assembled as a helical filament.

As noted above, the DUE of *E. coli oriC* contains three 13mer motifs. Within each 13mer, a 6mer sequence (consensus: AGATCT) is present that is specifically recognized by DnaA-ATP when this DNA is single-stranded [45,46]. Preferential binding to the 6mers in the top strand of the DUE is thought to require the initial binding of DnaA to the DnaA box named R1 that is proximal to the DUE as well as to DNA to the right up to and including the DnaA box named R5 [43,47,48]. Presumably, the interaction between adjacent DnaA monomers stabilizes their binding to this region of duplex DNA. Single-molecule fluorescence assays showed that DnaA assembles one monomer at a time in the 3′-to-5′ direction as a dynamic filament on single-stranded DNA [49]. Confirming results from the X-ray structure of the truncated form of *A. aeolicus* DnaA bound to polydA$_{12}$ [44], each monomer binds to three nucleotides of ssDNA [49].

2.1.2 Binding Sites for Fis and IHF Within E. coli oriC—*E. coli oriC* also carries binding sites for Fis (factor for inversion stimulation), and IHF (integration host factor) [50,51]. Fis was originally discovered as a required factor for Hin and Gin site-specific DNA recombinases of *Salmonella* and bacteriophage Mu, respectively [52,53]. IHF was identified by its involvement in the integration of bacteriophage λDNA during lysogeny [54]. Despite the different locations of their binding sites, a common feature of both proteins is their ability to bend DNA. Mutation of their respective binding sites in *oriC* disrupts origin function [55], which correlates with the asynchronous initiations observed in mutants that lack *fis* or *ihfB* [56,57]. The latter gene encodes one of the two subunits of IHF [58]. Other work showed that these DNA binding proteins act during the initiation stage of DNA replication. One study showed that IHF stimulates the DnaA-dependent unwinding of the 13mer region of *oriC* [59], which correlates with in vitro footprinting studies in which IHF apparently leads to the redistribution of DnaA to R2, R3, R5, and to low-affinity sites recognized by DnaA-ATP [60]. These findings complement in vivo footprinting results, which showed that both R3 and the IHF site became protected at the time of initiation in synchronized cells [61]. Other studies suggest that IHF and Fis have opposing effects on the binding of DnaA to *oriC*. Low levels of Fis apparently inhibit the unwinding of *oriC* by interfering with the binding of IHF and DnaA [62,63]. However, higher levels of DnaA augmented by the presence of IHF overcome this inhibition. These events correlate with the lack of occlusion of the Fis site at initiation in vivo whereas this site is protected throughout other periods of the cell cycle [61]. Evidently, the dynamic influence of Fis and IHF on the binding of DnaA to R3 suggests that these proteins modulate the frequency of initiation (reviewed in Ref. [1]).

2.2 Replication Origins of Other Bacteria

The replication origins of bacteria closely related to *E. coli* are similar in both DNA sequence and the relative organization of the conserved DNA sequence motifs described above [20,64] (reviewed in Refs. [1,65]). These observations suggest that the mechanism of replication initiation is similar in evolutionarily related bacteria. As evidence, one of its two chromosomes named chromosome 1 of *Vibrio cholerae*[b] has a single replication origin (*oriC1*) that is flanked by *gidA* and *mioC*, and is similar in DNA sequence and the organization of essential DNA motifs compared with *E. coli oriC*. As *oriC1* can functionally replace *E. coli oriC* [66], *E. coli* DnaA is capable of recognizing this DNA, and can direct the assembly of the replication fork machinery at this site to duplicate the chromosome. However, the *V. cholerae* origin is a less effective substitute for *E. coli oriC* as indicated by less frequent initiations.

E. coli oriC contains sequence motifs that are bound with varying affinities by DnaA. Likewise, the replication origin of *Caulobacter crescentus* has DNA motifs named G1 and G2 that are bound by DnaA with moderate affinity, whereas DnaA has a weak affinity for sites named W1–5 [69]. Presumably, these differences in affinity reflect the need for the ordered assembly of DnaA molecules at the *C. crescentus* origin, leading to the expectation that the chromosomal origins of other bacteria also contain sites that vary in their affinity for DnaA. If so, assembly of a DnaA oligomer may be a common requirement at all bacterial origins despite their diverse DNA sequences, the variation in the relative positions and orientations of DNA motifs within including the number and spacing of DnaA boxes, their locations in the chromosome relative to flanking genes, and their different lengths.

The *C. crescentus* origin also contains sites that are recognized by CtrA, which negatively regulates initiation to coordinate duplication of the chromosome with cellular development [69,70]. Biochemical studies reveal that CtrA, which is regulated by phosphorylation, inhibits the binding of DnaA to the *C. crescentus* origin [70,71]. Of interest, a protein analogous to *E. coli* IHF overlaps a binding site for CtrA. Occupancy of the IHF site is thought to displace CtrA, leading to initiation [72]. A DNA methyltransferase, CcrM, methylates the adenine base in the sequence GANTC [73]. Unlike the indirect role of DNA adenine methylase in controlling the frequency of initiation in *E. coli* [28], CcrM-dependent methylation is not involved in the control of replication initiation in *C. crescentus*. However, methylation of promoter regions by CcrM is required for the efficient transcription of many genes that are essential for cell cycle progression [74,75].

Compared with the replication origins summarized above, which are contiguous DNA sequences, the origins of some bacteria are bipartite. As represented by the replication origins of *B. subtilis* and *H. pylori*, a gene (*dnaA* for these but not all other organisms with bipartite origins) separates two essential regions named *oriC1* and *oriC2* (Fig. 1). These

[b] In bacteria with divided genomes, a protein not related to DnaA recognizes a specific replication origin of a chromosome. For example, the second chromosome of *V. cholerae* contains a single replication origin named *oriC2* located between two genes, *rctA* and *rctB* (Fig. 1). *oriC2* contains an array of tandem repeats named the 11mer and 12mer that are bound by monomers and dimers of RctB [37,38]. Another DNA motif named the 39mer is specifically bound by monomeric RctB. The methylation status of GATC sites that are recognized by DNA adenine methylase influences the binding of RctB to these sites wherein the dimer of RctB negatively regulates the frequency of initiation from *oriC2* by binding to the 39mers [66]. DnaA is essential for initiation from *oriC2*, but apparently performs an auxiliary role [67,68].

regions bear clusters of DnaA boxes, but *oriC2* also contains the DUE. For the *B. subtilis* origin, deletion analysis of the intervening *dnaA* gene revealed that much of it could be removed without affecting the function of the remaining DNA as a replication origin when carried in a plasmid [76]. It has not been determined if the *B. subtilis* origin can tolerate the removal of all of *dnaA*, or if a minimal length of spacer DNA is required. The latter result suggests that pairing of the left and right portions is essential for replication initiation, which also requires two additional proteins named DnaD and DnaB [77,78]. Homologues of *B. subtilis* DnaB or DnaD are not present in *E. coli*. Note that *E. coli* DnaB is unrelated to *B. subtilis* DnaB. DnaD is recruited to the origin through an interaction with DnaA.

Similar deletion analysis of the *dnaA* gene that separates the two parts of the *H. pylori* origin has not been performed. However, the binding of DnaA to *oriC2* requires that it is supercoiled, which is essential for DnaA to unwind the DUE [79,80]. Only two of the three DnaA boxes display this requirement for supercoiling. By comparison, supercoiling does not affect the binding of *E. coli* DnaA to the DnaA boxes of *E. coli oriC* [14,81]. For the *H. pylori* origin, the region at the left (*oriC1*) affects the amount of unwound DNA produced. In its absence, about 20 base pairs is unwound compared with as much as 52 base pairs when *oriC1* is present, suggesting that *oriC1* and *oriC2* interact, but are separated by a loop containing the *dnaA* gene [79].

3. MECHANISM OF INITIATION

3.1 Replication Initiation is a Stepwise Process that Begins with the Formation of a Complex of DnaA Assembled at oriC

DnaA protein initiates DNA replication by forming a specific DnaA-*oriC* complex. The types of high and low-affinity sites, the influence of the adenine nucleotide bound to DnaA on recognition of these sites, and evidence that describes the structure of this complex have been summarized above. Structure–function studies of DnaA indicate that it has four functional domains (Fig. 2). Domain IV is necessary and sufficient for binding to the DnaA box motif. Specific residues that confer DNA binding activity as well as specificity in DNA binding have been identified by molecular genetic methods [84,85]. Structures of this domain bound to the DnaA box complement the analyses described above [15,42], and also identify amino acid residues that contact nucleotides flanking the DnaA box sequence. As mentioned above, domains I and III contribute to DNA binding by promoting cooperative interactions between neighboring DnaA molecules assembled at *oriC* [24,25,86].

3.2 DnaA: Domain I Interacts with Other Proteins that Affect Self-Oligomerization of DnaA at oriC

Following the assembly of DnaA-ATP at *oriC*, DnaA induces strand opening of the AT-rich region, creating an intermediate named the open complex [40]. Early studies showed that HU, a protein that binds to DNA nonspecifically and is also a major component of the bacterial nucleoid, or IHF stimulate the formation of this intermediate [54,59,87]. More recent studies have identified a protein named DiaA that, like HU and IHF, stimulates the DnaA-dependent unwinding of *oriC* [88]. The underlying biochemical mechanism for DiaA and HU appears to involve their ability to interact with domain I of DnaA to facilitate DnaA

oligomeriza-tion or to stabilize the DnaA oligomer after it has formed [89,90]. The evidence that DiaA interacts with domain I is based on the observation that the substitution of phenylalanine with alanine at residue 46 of DnaA inhibits its interaction with DiaA [90], and on reports that DiaA, a structural homologue of *H. pylori* HobA [91], apparently interacts with the same surface in domain I of DnaA as *H. pylori* HobA [92]. In contrast with these observations and as summarized earlier, IHF is suggested to reorganize individual DnaA molecules bound to respective low- and high-affinity DnaA sites within *oriC* [60]. Assuming that IHF operates at *oriC* by a single biochemical pathway, it appears that this protein uses a different mechanism than DiaA and HU to stimulate unwinding by DnaA.

Domain I of *E. coli* DnaA also interacts with Dps [93], ribosomal protein L2 [94], and Hda [95]. Dps is a stress-induced protein that protects the bacterial chromosome from DNA damage by sequestering and oxidizing Fe^{2+} [96,97] (reviewed in Refs. [98,99]). It also inhibits strand opening of *oriC* in vitro, which correlates with in vivo evidence that Dps reduces the frequency of initiation [93]. Together, these observations suggest that Dps may act as a checkpoint to inhibit initiation during oxidative stress so that the cell has an opportunity to repair its DNA. Dps may inhibit initiation by interfering with DnaA oligomerization at *oriC*.

By interacting with the N-terminal region of DnaA, ribosomal protein L2 as well as a truncated form lacking 59 N-terminal residues inhibits initiation of *oriC*-containing plasmids in vitro by disrupting the ability of DnaA to self-oligomerize at *oriC* [94]. Like Dps, L2 obstructs the DnaA-dependent unwinding of *oriC*. In free-living organisms, both replication initiation and ribosome biogenesis are coordinated with cell growth but the mechanism underlying this coordination is not understood (reviewed in Ref. [100]). L2 is encoded by *rplB*, which is in the S10 ribosomal operon that is autoregulated by ribosomal protein L4 at the transcriptional and translational level [101]. As a possible explanation, when ribosome-free L2 is in excess, such as during the transition from rapid to slow cell growth, it may interact with DnaA to inhibit initiation while L4 that is also in oversupply due to expression of the S10 operon inhibits expression of the operon. This model provides a feedback mechanism to coordinate the control of ribosome biogenesis with DNA replication.

In a pathway in which Hda complexed to the β clamp stimulates the hydrolysis of ATP bound to DnaA to regulate its activity (reviewed in Ref. [102]), Hda interacts with the AAA + domain of DnaA to stimulate ATP hydrolysis, and also with domain I [95,103]. Deletion analysis revealed that the N-terminal 56 residues of DnaA are involved in this interaction [95]. An N44A substitution in DnaA caused a small reduction in the physical interaction between DnaA and Hda in a pull-down assay. It is not known if this substitution affects DnaA oligomerization.

In *B. subtilis*, SirA and other proteins have been identified that interact with DnaA. Studies have shown that SirA, which is under SpoOA-P regulation, interacts with domain I of *B. subtilis* DnaA to inhibit replication initiation in diploid cells committed to the formation of dormant spores [104–106]. Both *B. subtilis* SirA and *H. pylori* HobA bind to the same surface within domain I of their respective DnaAs [106]. However unlike HobA, which is essential for self-oligomerization of *H. pylori* DnaA [107], SirA inhibits DnaA in initiation

probably by destabilizing the DnaA oligomer or by interfering with its assembly at *oriC* [92,108]. (In contrast, YabA, DnaD, and Soj of *B. subtilis* interact with domain III [109–115].) These and other observations suggest that proteins of respective bacteria bind to the same or other sites in domain I to affect either formation of the DnaA oligomer at *oriC* or its stability. A model has been proposed that domain I acts as a sensor by its interaction with other proteins in order to adjust the frequency of initiation with changes in cell physiology, or the cell's environment [94].

3.3 DnaB

DNA helicases are enzymes that coordinate nucleotide hydrolysis with their translocation and unwinding of duplex nucleic acids. Of the six superfamilies of these enzymes, DnaB is in Superfamily 4 that is categorized by the presence of a RecA-type domain (Fig. 2) [116,117]. Whereas other members of this superfamily are assemblies of both hexameric and heptameric subunits, DnaB is a hexamer of identical subunits; a DnaB heptamer has not been observed. Each of the six protomers of DnaB has an N-terminal domain joined by a linker to the C-terminal RecA domain, which functions to coordinate nucleoside triphosphate hydrolysis with both unwinding of duplex DNA and translocation of the enzyme in the 5′-to-3′ direction relative to the single-stranded DNA to which the enzyme is bound [118–124]. Both early cryo-electron microscopy of *E. coli* DnaB [125,126], and X-ray crystallographic analysis of DnaB from thermophilic bacteria revealed that DnaB is a toroid [82,83,127]. These and other X-ray structures integrated into the framework of cryo-electron microscopic structures support a model that the form of DnaB complexed with DnaC is an open ring with a right-handed turn, which contrasts with the closed ring structure of DnaB in the absence of DnaC [128,129].

X-ray structures of *A. aeolicus* DnaB in the absence of DNA revealed constricted and dilated conformations of the portion of the DnaB ring formed by the N-terminal domains of neighboring DnaB protomers [128]. These observations strongly suggest dynamic movement of its domains, which have been corroborated in hydrogen-deuterium exchange experiments discussed below. With DnaB bound to an artificial replication fork, fluorescence energy transfer experiments indicated that the larger C-terminal domain is oriented nearest the fork junction [130]. The DNA strand bound by DnaB passes through its central cavity. A mechanism has been proposed in which this single-stranded DNA interacts with specific amino acids in the central cavity while the other DNA strand of the replication fork is excluded during translocation [116,131–133].

3.4 The DnaB–DnaC Complex and Its Formation

Studies characterizing the DnaB–DnaC complex revealed that as many as six DnaC molecules bind to DnaB [134–136]. Although early studies suggested that ATP is needed for DnaC to form a complex with DnaB [137], later studies showed that ATP or ADP is not required [136,138,139]. These nucleotides do not substantially affect binding affinity. Several lines of evidence show that DnaC interacts via residues near its N-terminus with specific residues near the C-terminus of DnaB. Genetic and biochemical studies demonstrated that amino acid substitutions within residues 8–11 and 31–44 of DnaC abrogate its interaction with DnaB and the function of DnaC in replication initiation [140].

In hydrogen-deuterium exchange experiments, which measures protein dynamics by the relative ability of amide hydrogens in a protein to exchange with deuterium in a buffer made with heavy water, peptides corresponding to these segments of DnaC were found to become occluded when DnaC is complexed to DnaB [141]. Together, these results indicate that these regions of DnaC interact directly with DnaB.

Other evidence showed that mutant DnaBs bearing I297A, L304A, or E435A substitutions are defective in complementing a temperature-sensitive *dnaB* mutant at nonpermissive temperature [141]. To substantiate that the E435A substitution interferes with DnaB function, biochemical experiments were performed, which showed that this protein is inactive in DNA replication of an *oriC*-containing plasmid. In addition, this substitution blocks the interaction of DnaB with DnaC. These results are complemented by hydrogen-deuterium exchange experiments, which indicated that peptides bearing residues 295–304 and 431–435 display greatly reduced rates of exchange when DnaB is complexed to DnaC, but not when the proteins were analyzed separately [141]. Hence, these regions of DnaB interact with DnaC in formation of the DnaB–DnaC complex.

3.5 Formation of the Prepriming Complex

After formation of the open complex, the DnaB–DnaC complex binds to the unwound region of *oriC* to form an intermediate named the prepriming complex (Fig. 3). Quantitative analysis combined with permanganate footprinting experiments indicated that each separated strand is bound by a single DnaB–DnaC complex [142–144]. The one bound to the top strand is near the left border of *oriC* [142]. The complex on the bottom strand is near the DnaA box named R1. On the basis that DnaB and not DnaC interacts directly with DnaA in *E. coli*, it is thought that DnaA directs the loading of the DnaB–DnaC complex on each unwound strand [145,146]. Two regions of DnaA interact with DnaB. Deletion mutants lacking domain I or portions of it as well as E21A or F46A substitutions of DnaA are defective in interacting with DnaB, indicating a requirement for domain I [90,146]. A second interacting region in domain III is indicated by evidence that a monoclonal antibody that recognizes a conformational epitope within amino acids 111–148 of DnaA inhibits its interaction with DnaB [145]. Deletion analysis restricted this interacting region to residues 135–148 [147]. Formation of a DnaA oligomer is also required to load the DnaB–DnaC complex at *oriC*. As evidence, a mutant DnaA bearing a W6A substitution is unable to form a DnaA oligomer at *oriC*, and fails to load the DnaB–DnaC complex [25]. Thus, DnaA must be able to interact with DnaB and also form a DnaA oligomer to load the DnaB–DnaC complex at *oriC*.

In contrast with the results summarized above, DnaC from *A. aeolicus* is able to interact physically with DnaA from this bacterium. These results suggest that DnaC assists in helicase loading in this organism, raising the possibility of a similar mechanism in *E. coli*. However, enzyme-linked immunosorbent assays to measure an interaction between DnaA and DnaC of *E. coli* were unsuccessful under conditions that were able to detect an interaction between DnaA and DnaB, or DnaB and DnaC [145]. Considering that the *dnaC* gene is proposed to have arisen by gene duplication of *dnaA* followed by the separate evolution of *dnaA* and *dnaC* so that the respective proteins perform separate functions in

initiation, an alternate possibility is that the respective DnaCs have evolved to differ in their ability to interact with DnaA.

As genetic evidence indicates that DnaC is required for replication initiation but not at the elongation stage during which DNA is duplicated [148–151], what is its role? Initial biochemical characterization of DnaC showed that its binding to DnaB attenuates its ATPase and helicase activities [152]. As additional support of the inhibitory effect of DnaC on DnaB, elevated ratios of DnaC to DnaB beyond the optimum for in vitro DNA replication of an *oriC*-containing plasmid is inhibitory [153]. Likewise, overproduction of DnaC in vivo causes inviability [140,153], and inhibits replication fork movement [154]. Hence, DnaC negatively regulates the helicase activity of DnaB, but DnaC evidently provides an essential function at an event prior to the binding of DnaB to DNA.

Other studies demonstrate that DnaC binds adenine nucleotides, which modulate its function [137,138,155]. Specifically, ATP stimulates its binding to single-stranded DNA [138, 139, 144, 156, 157]. However, ATP or ADP is not needed for DnaC to interact with DnaB [138,139], nor does a nucleotide bound to DnaB dramatically affect the strength of this interaction [136].

Despite the work summarized above, ATP bound to DnaC is essential for DnaC function at *oriC* [136,138,139]. For example, an arginine substitution of the conserved lysine in the Walker A box caused undetectable ATP binding and inactivity in DNA replication of an *oriC*-containing plasmid [138]. In support, genetic evidence showed that missense mutations in each AAA+ motif interfere with DnaC function [140,158]. Together with the results of the preceding paragraph, these findings strongly suggest that ATP binding by DnaC is essential at a step after formation of the DnaB–DnaC complex.

Considering that the structure of DnaB is a closed ring, how does ring opening occur so that the DnaB–DnaC complex can bind to the unwound region of *oriC*, and is ATP binding by DnaC required at the step of helicase loading? Insight into the role of ATP is suggested by the X-ray crystallographic structure of the ATP binding domain of *A. aeolicus* DnaC boundto ADP-BeF_3, an ATP mimetic [139]. The results support a model of DnaCself-oligomerized as a right-handed helical filament. 3D reconstruction of electron microscopic images of negatively stained DnaB compared with the DnaB–DnaC complex and biochemical studies suggest that DnaC assembled as a helical filament onto DnaB opens the DnaB ring [129]. When the DnaB–DnaC complex binds to the unwound DNA within *oriC*, it is attractive to consider that the single-stranded DNA passes through the gap in the ring. In contrast, hydrogen-deuterium exchange studies of the DnaB–DnaC complex in comparison with these proteins alone support a different conclusion [141]. Specific predictions can be made for DnaB and the DnaB–DnaC complex. If DnaB is a closed ring, the interface between its protomers is expected to be occluded. In the open ring form of DnaB in the DnaB–DnaC complex, one interface relative to the remainder should exchange more readily. However, results from hydrogen-deuterium exchange experiments indicated that the interface between protomers of the DnaB hexamer opens and closes spontaneously, and that DnaC traps DnaB in the open ring conformation [141].

3.6 Helicase Activation Requires the Dissociation of DnaC from DnaB

A proposed mechanism for loading of the DnaB–DnaC complex at *oriC* involves occupancy of the interior channel of the helical DnaC filament by the unwound DNA of *oriC* [129]. An interaction between DnaA oligomerized at *oriC* and protomers of DnaC in the helical filament is suggested to mediate helicase loading [139]. DnaC must then dissociate from DnaB to reveal its helicase activity. Apparently, the interaction of the N-terminal domain of DnaB with primase and primer formation induces a conformational change in the C-terminal domain of DnaB where DnaC is bound, leading to the dissociation of DnaC from DnaB [144]. This dissociation appears to be coordinated with hydrolysis of ATP bound to DnaC on the basis of the following evidence. With wild-type DnaC bound to ATP but not ATPγS, DnaC is able to dissociate from DnaB, indicating that ATP hydrolysis is required for its release [144]. Like other members of the AAA+ superfamily of ATPases, DnaC contains a highly conserved arginine named the arginine finger in a conserved amino acid motif named box VII. This residue is proposed to coordinate ATP hydrolysis with a conformational change. In experiments that characterize a mutant protein bearing an alanine substitution for the arginine finger residue, it unlike wild-type DnaC failed to dissociate from DnaB under conditions that support the interaction of primase with DnaB and primer formation. Hence, this mutant is either unable to hydrolyze ATP or sense whether ATP has been hydrolyzed, so one or both activities are evidently required for DnaC to dissociate from DnaB.

Whereas DnaC is essential in *E. coli* for the DnaB–DnaC complex to be placed at *oriC*, not all bacteria carry a *dnaC* gene. As an example, *H. pylori* does not encode *dnaC*. Like the replicative helicases found in some archaea, eukaryotic cells, and some viruses, the helicase of *H. pylori* is a dodecamer formed by the arrangement of two hexamers via their respective N-terminal domains [159,160]. Using an *E. coli dnaC*(Ts) mutant, a plasmid encoding *H. pylori dnaB* but not one bearing *E. coli dnaB* was able to complement the *dnaC* mutant at nonpermissive temperature, suggesting that this heterologous DnaB can bypass the requirement for DnaC in vivo [161,162]. Presumably, the interaction of *E. coli* DnaA with *H. pylori* DnaB mediates loading of the helicase at *oriC*. Recent experiments showed that the binding of *H. pylori* DnaB to single-stranded DNA requires ATP, but that ATP hydrolysis and ssDNA binding are not sufficient to separate the hexamers of the dodecamer [160]. Instead, its interaction with *H. pylori* primase causes the dodecamer to dissociate into individual hexamers as measured by multiangle light scattering and small angle X-ray scattering. Of interest, the interface joining the two hexamers in the X-ray crystallographic structure of *H. pylori* DnaB partially obscures the primase interaction site described in *G. stearothermophilus* DnaB [82]. Somehow, this interface becomes available for primase to interact, leading to dissociation of the dodecamer.

3.7 Events Leading to Assembly of the Replisome

In the model described in Fig. 3, DnaA assembled at *oriC* loads a single DnaB–DnaC complex on each strand of the unwound region. The interaction of primase with DnaB and primer formation then leads to the dissociation of DnaC and activation of DnaB. To elaborate on the interaction of primase with DnaB, this process is transient in that primase remains associated with DnaB during primer synthesis but then dissociates after primer synthesis is complete [163–166]. The 3′-end of the primer, now available after primase has

left, is extended by DNA polymerase III holoenzyme as it synthesizes the leading strand in concert with the translocation of DnaB as it unwinds the parental duplex DNA (reviewed in Refs. [167,168]). Positioned at the apex of each replication fork, DnaB moves in the 5′-to-3′ direction relative to the DNA strand to which it is bound. DnaB is subsequently recognized by primase, leading to the synthesis of another primer. This primer and those synthesized later are used for the synthesis of Okazaki fragments.

4. SUMMARY

Several lines of evidence indicate that DnaA assembles as a self-oligomer. In addition, the adenine nucleotide bound to DnaA, the interaction between and possibly among DnaA protomers of the oligomer, and DNA motifs to which these protomers are bound critically affect the assembly process. IHF bound to its cognate recognition site and Fis presumably at its binding site in *oriC* as well as other proteins that interact with DnaA via its N-terminal domain I also affect DnaA assembly at *oriC*, which is required for DnaA to unwind the AT-rich DUE element. DnaA then places the DnaB–DnaC complex onto each unwound strand within *oriC* and models have been proposed to explain the biochemical mechanism. Following helicase loading, activation of DnaB involves the dissociation of DnaC that appears to be induced by the interaction of primase with DnaB and primer formation. As primase and DnaC interact with the N-terminal and C-terminal domains of DnaB, respectively, a presumed conformational change in DnaB induced by primase binding apparently causes the dissociation of DnaC. Although structures of these proteins from thermophilic bacteria are known, the conformational change in DnaB has not been determined.

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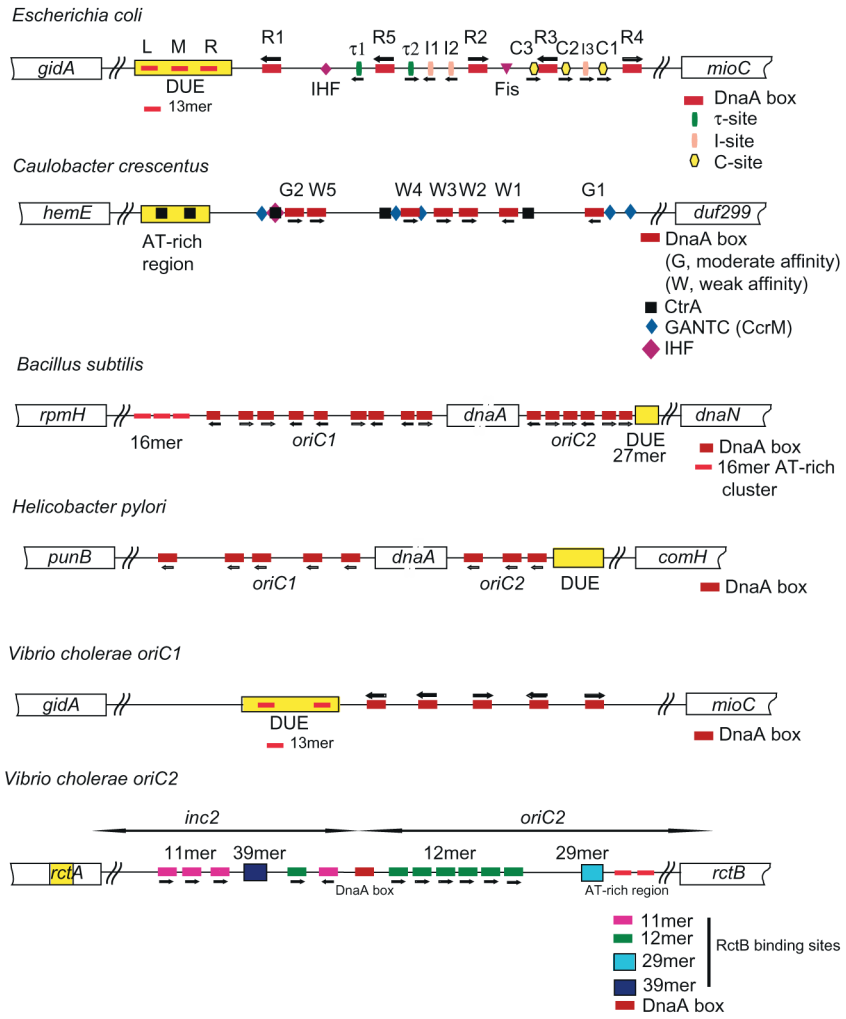

Fig. 1.

Bacterial replication origins carry DNA sequence motifs recognized by DnaA and other proteins, and an AT-rich region named the DNA unwinding element (DUE). Among bacteria however, the presence, relative arrangement, and position of these DNA motifs are not conserved. For *E. coli*, DnaA in a complex with either ATP or ADP recognizes a specific DnaA box with comparable affinity, but the relative affinity among the individual DnaA boxes varies. In comparison, DnaA-ATP and not DnaA-ADP specifically binds to I-, τ-, and C-sites. The affinities of DnaA-ATP to these sites are less than to the DnaA boxes. *E. coli oriC* also carries binding sites for Fis and IHF, whereas the replication origin of *C. crescentus* contains a binding site for IHF but not Fis. In contrast with these replication origins, which are contiguous DNA sequences, the replication origins of *B. subtilis* and *H. pylori* are bipartite in which *oriC1* and *oriC2* are separated by the *dnaA* gene. *B. subtilis oriC1* has three AT-rich 16mer repeats upstream of *dnaA* [34,35]. A 27mer AT-rich cluster in the *oriC2* region is unwound by DnaA [36]. *V. cholerae* carries two chromosomes. Initiation from *oriC1* appears to be similar to *E. coli oriC*. Initiation from *oriC2* requires RctB, which recognizes 11mer and 12mer iterons, and also a 39mer and a truncated form of the 39mer named the 29mer. The 39mer in the *inc2* region and the 29mer bound by the monomeric

form of RctB together with the DnaA box bound by *V. cholerae* DnaA regulate the frequency of initiation from *oriC2* [37]. *Adapted from Refs.* [36–38].

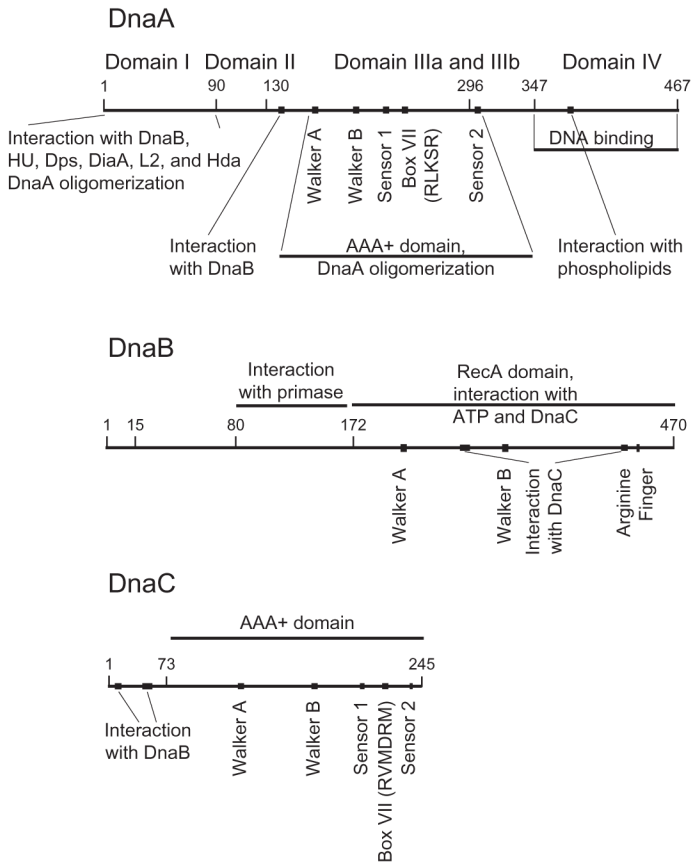

Fig. 2.

Domain organization of DnaA, DnaB, and DnaC protein. The *numbers* in the respective lines refer to the coordinates for *E. coli* DnaA, DnaB, and DnaC protein. DnaA: Domain I interacts with DnaB, HU, Dps, DiaA, Hda, and ribosomal protein L2, and is also required for DnaA oligomerization. Domain II may function as a flexible linker to join domain I and III. Domain III carries the amino acid sequence motifs shared among AAA+ family members that act in ATP binding and its hydrolysis. This domain also functions in DnaA oligomerization, and appears to carry a site denoted by a *filled square* that interacts with DnaB. Domain IIIa carries an abbreviated RecA-type fold. Domain IIIb contains a three-helix bundle. Domain IV binds to the DnaA box and presumably also to I-, τ-, and C-sites. A region that interacts with acidic phospholipids is in domain IV. The borders separating the domains have been determined by functional analysis of DnaA together with a homology model based on the X-ray crystal structure of domain III and IV of *A. aeolicus* DnaA. DnaB: Its N-terminal domain interacts with primase and its larger C-terminal domain functions in ATP binding and hydrolysis. On the basis of the X-ray crystallographic structures of *Geobacillus kaustophilus* and *Geobacillus stearothermophilus* DnaB [82,83], this C-terminal domain that also interacts with DnaC is similar in structure to RecA. The filled symbols represent the Walker A and B boxes and the arginine finger (arginine 442 of *E. coli* DnaB), and the DnaC-interacting domains. DnaC: The interacting domains of DnaC with DnaB and with ATP, including the AAA+ motifs and the conserved arginines in box VII are shown. *Reviewed in Ref.* [42].

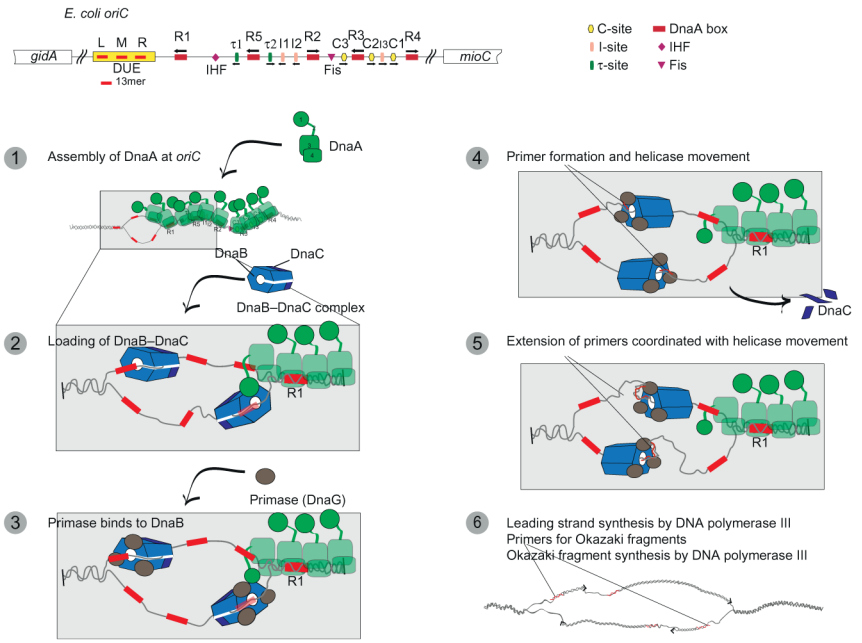

Fig. 3.

Assembly of DnaA, and the DnaB–DnaC complex at *E. coli oriC*, and activation of DnaB is a step-wise process. Step 1: DnaA recognizes specific DNA elements in *E. coli oriC* (see Fig. 1 and the text) to assemble a DnaA oligomer at this site. The numbers in the figure of DnaA represent its functional domains. Upon assembly of DnaA-ATP as a self-oligomer, it induces unwinding of a region containing the 13mers by binding to the top strand. At this step, domain IV of DnaA binds to the DnaA boxes and presumably to C-, I-, and τ-sites whereas domain I and III of a DnaA monomer interacts with another to assemble the DnaA oligomer. The interaction of HU with domain I of DnaA stabilizes the DnaA oligomer, but this interaction is not shown for simplicity. Step 2: Domain I of DnaA interacts with the N-terminal domain of DnaB in the $DnaB_6$–$DnaC_3$ complex to load this complex onto each DNA strand of the unwound region. Step 3: Primase interacts with the N-terminal domain of DnaB, which is required for primer synthesis. Step 4: As primase synthesizes a primer (shown as *red* (*dark gray* in the print version) *squiggles*) on the top and bottom strands, this event is coupled with the translocation of DnaB and the dissociation of DnaC from DnaB. Step 5: Primase completes primer synthesis and will then dissociate from DnaB after the primer is transferred to DNA poly-merase III holoenzyme. DnaB moves on each single-stranded DNA toward the junction of each replication fork. Step 6: DNA polymerase III holoenzyme (not shown) extends these primers for the synthesis of each leading strand. During this elongation stage of DNA replication, DnaB at the junction of each replication fork will act as a DNA helicase to unwind the parental duplex DNA. The translocation of DnaB and its transient interaction with primase leads to the synthesis of subsequent primers on the top and bottom DNA strands. These primers are used by DNA polymerase III holoenzyme for the synthesis of Okazaki fragments.