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Regulation of DnaA Assembly and Activity: Taking Directions From the Genome

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Abstract

To ensure proper timing of chromosome duplication during the cell cycle, bacteria must carefully regulate the activity of initiator protein, DnaA, and its interactions with the unique replication origin, *oriC*. Although several protein regulators of DnaA are known, recent evidence suggests that DnaA recognition sites, in multiple genomic locations, also play an important role in controlling assembly of pre-replication complexes. In *oriC*, closely spaced high and low affinity recognition sites direct DnaA-DnaA interactions and couple complex assembly to the availability of active DnaA-ATP. Additional recognition sites at loci distant from *oriC* modulate DnaA-ATP availability by repressing new synthesis, recharging inactive DnaA-ADP, or titrating DnaA. Relying on genomic DnaA binding sites, as well as protein regulators, to control DnaA function appears to provide the best combination of high precision and dynamic regulation necessary to couple DNA replication with cell growth over a range of nutritional conditions.

Keywords

oriC; pre-replication complex; cell cycle; chromosome replication; initiator protein

INTRODUCTION

All cells must duplicate their genomes before they divide, and, to ensure that chromosome replication is properly timed during the cell cycle, the initiation step of DNA synthesis is tightly regulated. Although different organisms use a variety of complex molecular mechanisms to precisely time replication fork assembly, all domains of life share basic regulatory circuitry comprising protein initiators that interact with specialized genomic regions, termed replication origins (40). The pre-replication complexes (pre-RC) formed from these components unwind double-stranded origin DNA and recruit the replication machinery (63, 78), and much of the regulation that controls initiation timing is focused on allowing, or preventing, pre-RC assembly (19, 66).

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It is not yet known how bacterial pre-RC are assembled with precise cell cycle timing. Details of initiator protein structure, DNA interactions, and regulation have emerged largely from studies in *Escherichia coli*, where pre-RC assemble from multiple copies of the highly conserved initiator protein, DnaA, interacting with the unique chromosomal replication origin, *oriC* (29). In this review we will evaluate our current understanding of how DnaA is regulated. Based on studies done over the last five years, two emerging themes will be presented. The first is that DnaA's ability to oligomerize is critical to its function, and is therefore subject to regulation. The second is that, in addition to protein factors, this regulation can be mediated through specific arrangements of 9-mer DnaA recognition sites in *oriC* and other genomic loci, which direct ordered assembly of DnaA complexes, as well regulating active DnaA availability. We shall integrate these themes and present a scheme for coordinating mechanisms that control DNA replication initiation. The reader is reminded that there are other reviews on the subject of DnaA and its role in initiating DNA synthesis (41, 63), which we recommend to those seeking additional details.

THE INITIATOR PROTEIN DNAA

DnaA is member of the AAA^+ family of proteins, whose activity is regulated by bound adenine nucleotide (79). Since DnaA-ATP is required for separation of *oriC* DNA strands (79), it is defined as the active form of the protein. The bound ATP is converted to ADP by an intrinsic ATPase activity (79), and, while DnaA-ADP is considered to be inactive, several studies have shown that the pre-RC can contain a combination of DnaA-ADP and DnaA-ATP monomers (34,92). Approximately twenty DnaA monomers are needed to form a pre-RC (17, 75), but *E. coli* cells contain many more molecules of the protein (about 1000-2000 monomers per cell) (80). Since the cellular amount of DnaA greatly exceeds the level needed to assemble pre-RCs, most of the DnaA cannot be active and available to bind *oriC*; if it were, then cells would initiate DNA replication continuously. Instead, precise replication initiation timing must be achieved by tight control of a very small pool of DnaA-ATP, using coordinated mechanisms that regulate synthesis, availability, and ATP hydrolysis during the cell cycle. These topics will be addressed throughout this article.

Although DnaA is a relatively abundant and stable protein, new DnaA synthesis is essential for initiation of DNA synthesis during each *E. coli* cell cycle (52). Newly made DnaA is bound to ATP, since, even though DnaA has a high affinity for both ATP and ADP (Kd=30 and 100 nM, respectively) (79), *E. coli* contain more ATP than ADP. Logically, the requirement for new DnaA synthesis indicates that the cellular DnaA-ATP levels must be increased before chromosome replication can begin, effectively coupling the rate of chromosome replication to the rate of cellular mass synthesis. Therefore, bacteria that accumulate mass rapidly also initiate DNA synthesis more frequently (that is, they have a shorter inter-initiation, or I period) (15). However, since the rate of DNA replication fork movement is constant over a variety of growth rates, rapidly growing *E. coli* initiate new rounds of replication on chromosomes that are already partially duplicated, and newly divided daughters inherit chromosomes with active replication forks and multiple replication origins (15, 82). All copies of *oriC* in these cells initiate DNA replication synchronously (82).

The regulation of new DnaA-ATP expression must be flexible in order to accommodate the differing number of origins/per cell that exist at different growth rates. This flexibility is achieved through auto-regulation (repression) of the *dnaA* gene, such that the amount of free DnaA-ATP available to bind and repress *dnaA*p helps determine the level of expression (2). This regulatory feature was demonstrated by studies in which the availability of free DnaA was reduced by adding extra DnaA binding sites on plasmids, causing a derepression of *dnaA*p activity and increasing expression levels up to 5-fold over normal (2, 38). Clearly, multiple copies of *oriC*, which provide additional DnaA binding sites, should increase DnaA expression. Less obvious, perhaps, is the role of other genomic sequences that can titrate DnaA, and thus decrease auto-repression. The *E. coli* genome contains a large number (over 300) of high affinity DnaA recognition sites (73), and depending on their location on the genome and the cellular growth rate, these sites can exist at a copy number of 2 or higher. Thus, at rapid growth rates, the increased copy number of *oriC* and other genomic loci can alter the availability of active DnaA-ATP (see further discussion below).

Based on structural and biochemical analyses, DnaA is divided into four distinct domains, and the reader is referred to two recent reviews (41, 53) for diagrams of the domain structure. Specific roles in DnaA function have been assigned to Domains III, IV, and I. Domain I (the N-terminal amino acids 1-90) is used primarily for protein-protein interactions (89). A hydrophobic face of this domain allows DnaA protomers to oligomerize, while the opposite side of the domain interacts with the replicative DNA helicase (DnaB) (1), as well as with DiaA, a recently identified positive modulator of pre-RC assembly (39).

Domain II (amino acids 90-130) is the only non-conserved region of the protein, and forms a flexible linker region whose length varies among the eubacteria (1). Deletions placed in domain II can repress initiation (61), but a GFP-tag can be inserted into this domain without loss of viability (6). While Domain II has not been assigned any specific role, the linker region may accommodate the need to position Domain I over the wide range of distances that separate DnaA recognition sites within *oriC*, or allow some flexibility in positioning DnaB on single-stranded DNA.

Domains III and IV are the most highly conserved among eubacteria, and also have structural motifs shared with archeal and eukaryotic initiator proteins (22). The functional roles of these two domains are well-characterized: Domain III (amino acids 131-346) carries motifs necessary for ATP binding/hydrolysis and oligomerization (22), and Domain IV (the C terminal amino acids 347-467) contains a helix-turn- helix motif for double-stranded DNA binding (22, 27), as well as an amphipathic region used for membrane interaction (30). Domain IV contacts both the major and minor grooves in 9mer nucleotide sequences termed R-boxes (27), with consensus 5’ TTA$_{A/T}$NCACA-3’ (77). DnaA interacts with consensus R-boxes with high affinity (Kd from 4 nM to 20nM) (77), and both the ATP-and ADP-forms of the protein bind equivalently (29, 59). DnaA is also able to contact 9mers with mismatches from consensus (37, 45, 59), but interacts less strongly (Kd > 100nM) (77) and requires cooperative interaction with a higher affinity site (60). Several versions of low affinity DnaA recognition sites have enhanced binding preference for DnaA-ATP compared to DnaA-ADP (45, 59).

The structure of Domains III/IV was solved using isolated fragments of DnaA from the thermophilic bacterium *Aquifex aeolicus* (22), and this analysis revealed conformational similarities among initiator proteins in eubacteria, archea, and eukaryotes. These domains of *Aa*DnaA, when bound to ATP, assemble into oligomers that take the form of a compact, right handed, helical filament (21). This configuration does not form with DnaA-ADP (20, 21), suggesting that, through the binding and hydrolysis of ATP, DnaA could switch between oligomer-promoting and oligomer-inhibiting states (21). This nucleotide switch feature of DnaA filament assembly may also influence the way that DnaA can interact with DNA. Double stranded DNA binding is possible with DnaA monomers, which make direct contact with a recognition site (1). However, DnaA helical filaments assembled in solution were recently reported to be incapable of interacting with double-stranded DNA, due to the flipping of domain IV into a compact arrangement with Domain III (20). Instead, the filament is proposed to form a channel in the Domain III interaction regions (20), which binds single stranded DNA (86). At *oriC*, this structure is proposed to engage and stabilize unwound DNA (20, 86) for delivery of helicase (64). Since DnaA-ATP binding to double-stranded DNA is required to build the complex used for origin DNA unwinding (51), the DnaA-ATP helical filament structure would be incompatible with pre-RC formation. Instead, it seems logical to propose that some intermediate or alternative oligomeric structure, in which the tendency of DnaA-ATP to compact is counterbalanced by specific positioning of 9-mer recognition sites (see below), is used to assemble pre-RC.

ORIC ENCODES DIRECTIONS FOR PRE-RC ASSEMBLY

Sequence organization of *oriC*

The minimal chromosomal DNA segment capable of autonomous replication in *E. coli* is 245 bp (90). Early sequence analysis of this *oriC* and five additional enterobacterial origins revealed regions of highly conserved nucleotide sequence regions separated by regions of variable sequence, but fixed length (94) (Figure 1A). The conserved regions were proposed to bind initiator protein and mediate DNA unwinding, and were used to identify a 9bp sequence (5'-TTATCCACA-3) that was termed the R-box (94), and was correctly believed to be a binding site for initiator DnaA (29). The less conserved regions were thought to be spacer DNA that carried little information (69). Over time, our understanding of the sequence elements in *oriC* has evolved (Figure 1), and we now consider the origin, including the "spacer elements" to be much more information-dense.

Early evaluations of *oriC* suggested that there were five 9mers with similarity to the R-box sequence; these were named R1, R2, R3, R4, and R5M (also called the M box) (58, 94). R1, R2, and R4 are widely spaced, with R5M and R3 positioned in the gaps between them (Figure 1B). Subsequent biochemical studies revealed that R1, R2, and R4 are high affinity sites (Kd=5nM-50nM) (57, 77), while R5M and R3 show much lower affinities for DnaA (Kd>200nM) (57, 77). However, low affinity sites cannot be accurately predicted based on nucleotide sequence alone. Using direct measurements of DnaA binding as well as site directed mutagenesis (45, 59), additional low affinity 9mer sites were identified; these are τ1, τ2, I1, and I2, located between R1 and R2, and C3, C2, I3, and C1, found between R2 and R4 ((37), J. Grimwade and A. Leonard, unpublished observations). Low affinity

recognition sites are closely spaced, and arranged symmetrically into two arrays, one in each half of *oriC*, in regions considered previously to be fixed-length spacer DNA (Figure 1C). Within each array, two nucleotides separate each recognition site and all sites are oriented in the same direction. The array in the left half of *oriC* is oriented in the opposite direction of the sites in the right half. Several recognition sites in each array (I-sites and τ-sites) show a 4-fold preference for DnaA-ATP (45, 59). Several sites in the left array, and I3 in the right array, also contain or overlap a GATC sequence (66), the recognition site for DAM methylase and the sequestration protein, SeqA (55) (discussed in a later section). The role of these arrayed DnaA binding sites in pre-RC assembly will be discussed below.

While esthetically appealing, the symmetrical arrays of low affinity sites raise questions about the role of R3, whose spacing and orientation does not allow it to fit into the right array. Despite its similarity to consensus, R3 is the weakest among known R-boxes (57, 77) and was previously suggested to have an important function, since it was the last occupied site *in vivo* (10). However, re-evaluation of DnaA binding footprints (DMS (59), DNase I (29), and copper phananthroline (57)) in this region of *oriC* reveals patterns consistent with the occupation of C2 and C3, which overlap R3, rather than with R3 itself (J. Grimwade and A. Leonard, unpublished observations). The region of conserved nucleotides surrounding R3 is also broader than that associated with R1, R2 and R4 (Figure 1A, B). Thus we propose that R3 may be a “red herring” that has masked other low affinity sites used in pre-RC assembly, although it remains possible that R3 plays a role under certain (unidentified) physiological conditions.

Each array of low affinity DnaA recognition sites lies adjacent to a region of *oriC* that interacts with a DNA bending protein (Figure 1C). IHF binds to the left half, between R1 and R5M and is able to severely bend *oriC* (180 degrees) (74). In the right half, Fis binds between R2 and C3, and is reported to bend *oriC* greater than 50 degrees (32). These bending proteins are abundant and play diverse roles in gene regulation and recombination, in addition to a role at *oriC* (25, 72).

In addition to DnaA binding sites, all bacterial replication origins contain an A-T rich DUE (50). In *E. coli*, the DUE is a 70 bp region (Figure 1) containing three 13mer tandem repeats (5’-GATCTnTTnTTTT-3’) (7).

Pre-RC assembly is staged

High affinity recognition sites R1, R2, and R4 remain occupied by DnaA throughout the cell cycle (10, 66, 76), suggesting that the DnaA bound to these sites acts to mark the origin and forms a structural platform on which to assemble the pre-RC. This idea was confirmed by mutational analysis of *oriC*, which demonstrated that the high affinity sites are required to assist DnaA binding to their proximal lower affinity sites (60).

In rapidly growing *E. coli* cells, Fis also remains bound to its cognate *oriC* site throughout most of the cell cycle (10). Fis inhibits both IHF binding and low affinity DnaA interactions, and this inhibition is removed when DnaA displaces Fis near the time of initiation (75). Following this displacement, IHF binds, and stimulates additional DnaA-*oriC* interactions required to assemble pre-RC at low affinity sites (75). Synthesis of Fis, but not IHF, is

growth rate-regulated (4), suggesting that pre-RC assembly may be altered to accommodate fast and slow growth conditions.

The binding of DnaA to lower affinity recognition sites results in DNA strand separation, which is focused between the right and middle 13mer in the DUE (7). Once the DNA is unwound, DnaA-ATP binds to the top single strand of the DUE stabilizing the unwound complex (86), and, via Domain III and Domain I, interacts with the helicase loader, DnaC and DnaB helicase, respectively (64, 89).

Following initiating each new round of DNA synthesis, the pre-RC is disassembled and the origin is reset (66). For this to occur, some displaced DnaA must be allowed to re-associate with R1, R2 and R4 in the newly replicated copies of *oriC*, but the remaining lower affinity sites must be blocked, so that the pre-RC does not re-form. This blocking is performed by SeqA protein (66). SeqA interacts with hemimethylated GATCs, formed by new DNA synthesis (83). There are no GATCs in the high affinity R boxes of *oriC*, but GATCs are found in the DUE and the lower affinity sites R5M, τ1, τ2, I2 and I3. Thus, after replication, SeqA binding to hemimethylated GATCs in the lower affinity sites blocks pre-RC re-assembly, but allows rebinding of DnaA at R1, R2, and R4 (66). *OriC* is refractory to new initiations as well as adenosine remethylation of GATCs by DAM methylase for approximately 1/3 of the cell cycle, and this is referred to as the sequestration period (9). In SeqA-deletion strains, the released DnaA-ATP is able to rebind low affinity sites in *oriC*, and the resulting reassembly of the pre-RC causes over-initiation (66). SeqA also binds to the hemimethylated GATCs in the *dnaA* promoter, shutting down *dnaA* gene expression during the sequestration period (9).

Initiation of bacterial DNA replication requires supercoiled *oriC* DNA, and changes in the level of DNA supercoiling or transcriptional activity near *oriC* alter origin function and initiation timing (3, 81). For this reason, both DNA topoisomerases and RNA polymerase must be considered regulators of *oriC* activity. DnaA was also recently shown to interact directly with RNA polymerase (26) as well as several proteins associated with bacterial chromatin (13, 14), and we await further studies to increase our understanding of the role of these interactions in pre-RC assembly.

Filling the Gaps: A New Pre-RC Assembly Model

Both Domain I and Domain III of DnaA have self-oligomerization activity (1, 21, 91), and studies using oligomerization-defective mutants (23, 24) indicate that both domains are needed for initiation of chromosome replication. We propose a simple model for ordered pre-RC assembly whereby DnaA oligomers “fill the gaps” by extending along the DNA between two high affinity anchor sites. (53). Placement of DnaA protomers and their assembly into an oligomeric structure is guided by DnaA binding to the closely-spaced arrayed low affinity recognition sites. Thus, “instructive” DNA that carries the information for positioning DnaA into oligomers during pre-RC assembly replaces the notion of “spacer” DNA. This model adds several steps where regulatory factors might either block or permit the extension of a growing DnaA oligomer. The first step is DnaA extension from the high affinity anchor site to the proximal weak site (60), and the second step is extension of a DnaA filament associated with the arrays. The distance from each high affinity site to the

proximal low affinity site ranges from 3bp (R4 to C1) to 45bp (R1 to R5M) (52). This spacing differs from the close 2bp between arrayed low affinity sites, and suggests that different oligomerization strategies are used in the two steps. In the first step, extension between a strong and weak site may require DnaA protomers to interact by their Domain I regions, so that the Domain II linker can be used to extend a DnaA molecule over the distance separating the two sites. Additional factors may also be required to stabilize the interaction. For example a recently identified protein, DiaA, binds to DnaA Domain I and has a stimulatory role on pre-RC assembly (47), and so would be an ideal candidate to stabilize the first extension of DnaA in the growing oligomer.

Once a bond is formed between DnaA bound at a high and low affinity sites, the second step of low affinity site occupation can begin. In this step, DnaA molecules must progressively fill the arrayed weak sites, and this filling directs the formation of a filament. It seems logical to propose that the close spacing of the arrayed sites, as well as the presence of sites that prefer DnaA-ATP, suggests that filament assembly requires Domain III interactions, which form only with DnaA-ATP (53). However, the oligomers associating with a double-stranded DNA array may be structurally different than the compact helical filament formed in solution, in which Domain IV is flipped up and not available to bind DNA (20).

When low affinity site occupation is completed, oligomers assembled in each half of *oriC* are expected to be anchored between the pairs of high affinity sites, and these oligomers may connect to form a contiguous filament that extends the entire length of *oriC* between R1 and R4. Although the arrayed binding sites do not extend over the entire DNA gap between R1 and R2 or R2 and R4 (37, J. Grimwade and A. Leonard, unpublished observation), the bending proteins that also bind in these gap regions could help with filament extension. For example, DNA bending by IHF, which is known to promote pre-RC assembly (75), would bring R1 and R5M into proximity to anchor the leftward oligomer extending from R2. However, the exact positioning of DnaA oligomers in *oriC* remains to be determined.

It is also unclear how filament formation helps mediate the ability of the pre-RC to unwind *oriC*, and whether or not the filament must achieve a minimum length for this purpose. Only the left half of *oriC* is required for viability under slow growth conditions (87), but both halves are needed for rapid growth, and cells with origins lacking R4 have severe perturbations in their initiation timing (70). It is likely that for normal, cell cycle-regulated origin activity, all DnaA binding elements in *oriC* are required to correctly direct pre-RC assembly.

REGULATING DNAA ACTIVITY AND AVAILABILITY

In order to precisely time pre-RC assembly in the cell cycle, the amount of DnaA, particularly DnaA-ATP, available to bind to *oriC* must be tightly regulated. This regulation appears to be mediated through a coordinated series of mechanisms that are linked to replication fork movement and DNA sequence elements. During the sequestration period, when initiation and DnaA expression are blocked, new replication forks continue to move around the chromosome (9). Fork movement plays an important role in regulating DnaA activity in three different ways: 1) DnaA-ATP bound to the chromosome is inactivated by a

mechanism termed RIDA (43); 2) duplication of genomic DnaA recognition sites, including a high capacity site termed *datA*, titrates free DnaA and reduces DnaA availability (49); and 3) duplication of specialized chromosomal regions, termed DARS, stimulate recharging of DnaA-ADP back into DnaA-ATP (28). All three regulatory features are likely to be interconnected and this will be discussed in a later section. Here we will provide an overview of each mechanism separately.

Regulatory Inactivation of DnaA

Although sequestration prevents immediate pre-RC reassembly, the levels of DnaA-ATP must also be reduced following initiation to ensure that chromosome replication starts only once per cell cycle. In *E. coli*, DnaA-ATP is converted into DnaA-ADP by a process termed RIDA (42). RIDA is mediated by Hda protein, stably associated with the sliding clamp of the DNA polymerase holoenzyme (44). A conserved motif (arginine finger) in Hda interacts directly with the ATPase region of DnaA to stimulate hydrolysis (65). ADP binding to Hda may also be important for *in vivo* activity (88). Hda must associate with DNA-loaded clamps to stimulate hydrolysis (46), and since clamps co-localize with replisomes, DnaA-ATP inactivation is expected to take place when ongoing replication forks encounter DNA-bound DnaA-ATP. Loss of Hda activity produces a variety of phenotypes in *E. coli*, ranging from lethality caused by excessive over-initiation (8, 44) to modest overinitiation and asynchrony (71). However, compensatory mutations identified in Hda-defective strains appear to be responsible for less severe phenotypes (71). In some Hda mutants, the levels of DnaA-ADP are near those seen in wild-type cells (28), suggesting that there may be other, not yet unidentified, mechanisms that stimulate DnaA-ATP hydrolysis in these strains.

DnaA Reactivation Sequences

While DnaA-ATP produced by new synthesis is required for initiation in *E. coli*, additional DnaA-ATP for pre-RC assembly is also supplied by mechanisms that recharge DnaA-ADP. One recently discovered recharging mechanism is mediated by specific genomic sequences, termed DARS (28), found in two widely separated chromosomal DNA regions. DARS1 and 2 map near *bioD* and *mutH*, respectively (28). In each DARS, recharging requires three closely spaced DnaA recognition sites, with two adjacent sites positioned in opposite orientations. While the mechanism is not yet clear, this configuration promotes removal of nucleotide from bound DnaA-ADP, and the apo-DnaA then associates with ATP due to the higher cellular ATP levels (28). Loss of DARS delays initiation during the cell cycle (28), suggesting that these sequences normally contribute to the threshold level of DnaA-ATP required for initiation. A soluble factor, not yet identified, is also implicated in the regulation of DARS2 activity (28).

DnaA-ADP can also be recharged via interaction with membrane acidic phospholipids (31). While it is not known how much DnaA-ATP is supplied by membrane recharging, *E. coli* that are unable to make acidic phospholipids are growth arrested (5). Both DnaA and hemimethylated *oriC* interact with membranes (6, 68), and these interactions may facilitate DnaA recharging at a particular time during the cell cycle.

DnaA Titration

There are more than 300 consensus 9mer DnaA recognition sites positioned around the *E. coli* genome (73), and the majority of these sites appear to serve no role other than to regulate DnaA availability by titration. Although the majority of DnaA binding sites are dispersed, a few regions of the genome contain clusters of recognition sites that might act to direct assembly of higher order DnaA structures, similarly to *oriC* (73). One cluster, called *datA*, has a particularly high capacity for DnaA (60-300 molecules) (49), and is suggested to be the major DnaA titration locus on the chromosome. *DatA* is located about 450 kb away from *oriC* (48), and is duplicated near the end of the sequestration period in rapidly growing cells. The 950 bp *datA* region contains only five DnaA recognition sites with the nucleotide sequence necessary to bind DnaA with high affinity (48). Since one DnaA recognition site appears to be capable of binding a single DnaA molecule (60, 77), additional DnaA interaction sites must exist in *datA* in order for the locus to reach its full binding capacity. Interestingly, sequence analysis reveals that there are potential arrays of closely spaced low affinity sites in the *datA* DNA between strong sites (37), suggesting that *datA*'s high titration capacity may be due to DNA-directed assembly of DnaA-ATP oligomers, similar to DnaA complex assembly in *oriC* (37, 53). DnaA-ADP should not be capable of equivalent high capacity interactions (21). Inactivation of *datA* causes early initiation during the cell cycle (67), and *E. coli* does not tolerate many extra copies of *datA* (62). Additional positions of closely spaced recognition sites with the capacity to direct formation of DnaA-ATP oligomers include *mutH*, *mioCp*, *dnaA*, and *nrdD* (37, 73), although these smaller clusters of DnaA binding sites would not be expected to have the same capacity as *datA*.

IS DNAA DYNAMICALLY DISTRIBUTED AROUND THE GENOME?

Pre-RC assembly, which requires only a few molecules of DnaA-ATP be available to *oriC*, is timed with exquisite precision each and every cell cycle. However, mechanisms that produce, titrate, and inactivate DnaA-ATP must act concurrently for at least a portion of the cell cycle. How, then, do these mechanism provide the correct amount of DnaA-ATP to *oriC* at the correct time?

An intriguing possibility is that, due to interplay between regulatory mechanisms, DnaA molecules are dynamically displaced around the genome during the cell cycle (Figure 2). This would start when DnaA-ATP is removed from *oriC* immediately after initiation. RIDA does not appear to inactivate this DnaA, since, in the absence of SeqA, the pre-RC is immediately reassembled (66). Therefore, the DnaA-ATP that is displaced from *oriC* must be relocated. The most obvious relocation sites are nearby titration loci such as *datA*, where the DnaA-ATP would form oligomeric filaments (21, 37). The DnaA-ATP occupying the titration loci would be hydrolyzed by RIDA when the loci were replicated. Some of the DnaA-ADP that is produced must associate with the newly duplicated high affinity binding sites in the titration loci, since there is no obvious mechanism to prevent it from doing so. However, since DnaA-ADP cannot be used to reform filaments (21), the titration loci would be expected to release more DnaA-ADP than could be rebound. This excess DnaA-ADP is now available to interact with DARS loci, where it is recharged and used to supplement new DnaA-ATP for the next round pre-RC assembly.

It is suggested that precise timing of pre-RC formation requires that DnaA-ATP fill all available chromosomal titration sites before the arrays of low affinity sites in *oriC* become occupied (36). If this were the case, then DnaA-ATP displaced from *oriC* would bind only to newly duplicated titration loci, since all others would be completely occupied. In this scenario, the DnaA-ATP oligomers would then remain bound to the loci up to a full generation until replication forks passed through the region.

It is possible that the number of DnaA molecules displaced from *oriC* after each round of initiation is not constant over all growth conditions. A requirement for rapid *E. coli* growth is synchronous initiation of DNA synthesis from multiple copies of *oriC* (82). To ensure that all copies of *oriC* fire, an initiation cascade of DnaA is proposed to provide initiation potential to every origin once a single copy triggers new DNA synthesis (35, 54). Although the cellular DnaA concentration is constant at all growth rates (35), at least one growth rate regulated protein, Fis (4), is associated with *oriC* (10, 32). Since the role of Fis is to repress DnaA binding to low affinity sites in *oriC* (75), the number of DnaA molecules required at *oriC* to displace Fis and form the pre-RC may be larger than the number that is needed to form pre-RC in the absence of Fis. This Fis switch may be abrupt or gradual as growth rates are reduced. In support of this model, the right half of *oriC* (where Fis binding occurs) is required for rapid *E. coli* growth, but is less important for viability during slow growth (87).

COMPARING OTHER BACTERIA AND EUKARYOTES

Although DnaA is highly conserved among bacterial types, comparison of *oriC*s from different bacteria reveals widely differing arrangements of high affinity DnaA recognition sites, with regard to both the number of sites and the distance that sites are separated from one another (93). It remains difficult to determine the position of low affinity 9mer sites in different bacteria, since, as is the case for *E. coli*, identification of these sites is likely to require direct assays of DnaA-*oriC* interactions (56). However, while the organization of most sequenced bacterial origins seems to be compatible with the model of DNA-directed DnaA-ATP oligomerization, the differences in DnaA binding site placement in bacterial origins suggest that variations in bacterial life-styles must be accommodated by different sets of directions for pre-RC assembly encoded by *oriC*. We must await future studies to tell which features of pre-RC assembly in *E. coli* are conserved and essential for all bacterial origin activity.

The use of DnaA titration and elongation-coupled mechanisms to down-regulate DnaA activity appears to be conserved among many bacterial types, but direct analogs of SeqA, Hda, *datA*, and DARS do not necessarily exist. For example, although *B. subtilis* does not retain analogs of SeqA or Hda, similar regulators are found. For example, YabA protein, like Hda, is associated with the replisome's sliding clamp (12). However, unlike Hda, YabA appears to prevent reinitiation by acting as a fork-associated DnaA titration mechanism rather than a stimulator of DnaA-ATP hydrolysis (33, 85). DnaA in *B. subtilis* is also shown to interact with multiple genomic "titration" sites, but in this case, the bound DnaA is associated with the primosomal proteins DnaB and DnaD (84). As more bacterial organisms are examined, it is likely that many more variations of the *E. coli* regulatory systems will be revealed.

Comparison of modes of pre-RC assembly in prokaryotes and eukaryotes must be done with caution, but there are temporal and functional similarities in initiator protein-origin complex assembly. The persistent complex formed by DnaA monomers occupying R1, R2, and R4 serves the same role in prokaryotic cells that the hexameric ORC serves in lower eukaryotes. Both mark replication origins (19, 66), and serve as a scaffold for recruitment of more initiator protein to the growing pre-RC (18, 60). However, in eukaryotes the ORC subunits assemble prior to interacting with chromosomal replication origins (11), and for this reason eukaryotic replication origins do not require specific arrangements of multiple initiator protein binding sites to direct pre-RC assembly (16). It appears the need to replicate genomes of increasing size led to a switch from DNA-directed assembly of initiator proteins into pre-RC (prokaryotic timing mechanism) to the use of preassembled initiator protein complexes. Eliminating the need for DNA-based assembly instructions would permit both shorter origin nucleotide sequences and the higher degree of origin degeneracy necessary for multi-origin replication.

CONCLUDING REMARKS

To achieve a cell cycle timing mechanism with high precision, bacteria devote multiple interrelated mechanisms to control availability of a very small population of initiator DnaA-ATP molecules. Mechanisms capable of raising the levels of DnaA-ATP are counterbalanced by inactivation and titration mechanisms. By using a combination of protein regulators and genomic sites, bacteria are able to expand or contract DnaA-ATP availability to provide the precise amount of DnaA-ATP required to support pre-RC assembly over a wide variety of growth conditions, a feat that would not be not possible using a single regulator. Surprisingly, the replication origin sequence also includes an instruction set for DnaA-ATP interactions, to ensure pre-RC assembly is ordered and reproducible for every consecutive cell cycle. Additionally, these instructions, by directing staged pre-RC assembly, provide for modulation by a variety of regulators, some of which are growth rate dependent. Further studies will be required to determine the role of each DNA and protein component that couples initiation of DNA replication to the bacterial cell cycle, and these will need to be evaluated under different growth conditions. Despite the complexity, the workings of the timing mechanism are within our grasp, as are opportunities to identify more novel targets for the development of antibiotics.

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TERMS

Origin recognition complex (ORC)	The complex of initiator proteins and replication origins that exists throughout the majority of the cell cycle.
Pre-replication complex (pre-RC)	The complex required to prepare DNA for new replication forks by unwinding the replication origin and loading the replicative DNA helicase.
R-box	DnaA recognition site with consensus 5'-TT$_{A/T}$TNCACA-3'.
I-site	Low affinity DnaA binding sites in *oriC* that show a 4-fold preference for DnaA-ATP. Binding to I-sites is stimulated when IHF binds to *oriC*.
τ-site	Additional low affinity binding sites in oriC that show preference for DnaA-ATP.

C-site	cryptic low affinity DnaA binding sites that cannot be predicted based on sequence, but must be identified based on direct binding measurments.

ACRONYMS

DUE	DNA Unwinding Element
RIDA	Regulatory inactivation of DnaA
AAA$^+$	ATPases associated with a variety of cellular activities
DARS	DnaA recharging site
IHF	Integration host factor
Fis	Factor for inversion stimulation

SUMMARY POINTS

1. Regulation of bacterial pre-RC assembly is dependent on mechanisms that precisely control the availability of active DnaA as well as its staged oligomerization into complexes at *oriC*.
2. The instructions for ordered assembly of bacterial pre-replication complexes are encoded within the nucleotide sequence of *oriC* as a specific arrangement of high and low affinity DnaA recognition sites.
3. A “filling the gaps” model places greater focus on low affinity DnaA interaction in *oriC* and raises new questions about DNA-directed pre-RC assembly and the role of DNA bending proteins as growth rate-modulators of initiation timing.
4. Specialized arrangements of DnaA recognition sites strategically positioned around the bacterial genome modulate the availability of DnaA-ATP by directing formation of higher order DnaA-ATP complexes at titration loci, and, at DARS, positioning DnaA-ADP in a specific configuration that allows recharging.
5. In *E. coli*, precise regulation of available DnaA-ATP may be mediated by interplay between RIDA and DARS, caused by dynamic displacement of DnaA from *oriC*, to titration loci, to DARS. This interplay would allow more precise control over DnaA activity than can be accomplished by regulating DnaA at the level of gene expression alone.

FUTURE ISSUES

1. What is the role of DnaA domain I in the assembly of bacterial pre-RC?
2. Why don’t all bacteria use the same mechanisms to inactivate DnaA?
3. What are the particular arrangements of low affinity DnaA recognition used by different bacterial types and will these arrangements correlate with any particular aspect of cell growth?
4. How do DnaA oligomers impart the torsional stress needed to unwind *oriC*?
5. Do bacterial pre-RC change composition as a function of growth rate or particular physiological state?

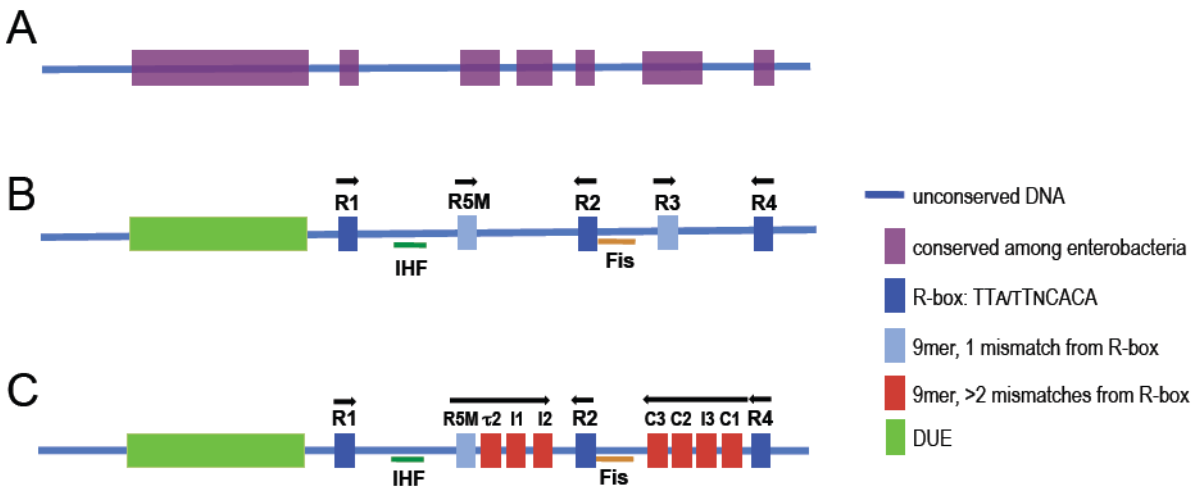

Figure 1.

Evolution of *oriC* map. Our understanding of the information encoded in the 245 bp *E. coli oriC* has evolved over the years, revealing the origin is much more information dense than was previously thought. A) Comparison of sequences from six different enterobacerial origins of replication revealed regions of high conservation (94). B) Repeated 9mer sequences within the conserved regions were used to predict five recognition sites, called R-boxes, for the initiator protein, DnaA (58,94). Additional repeats in an AT-rich conserved region was demonstrated to be the DNA unwinding element (50) where the double stranded DNA strands separate (7). C) Recent analyses, based on direct assays of DnaA binding to *oriC* reveal that each half of *oriC* contains an array of closely spaced low affinity DnaA binding sites that deviate from R-boxes by 3 or more bp and have preference for binding DnaA-ATP (37, J. Grimwade and A. Leonard, unpublished observations). These two arrays replace sequence that was previously considered to be “spacer” DNA, and lead to a model where an assembly of an oligomeric DnaA complex is directed by precise placement of DnaA protomers between high affinity anchors in *oriC* (see (53) and text for details of the model).

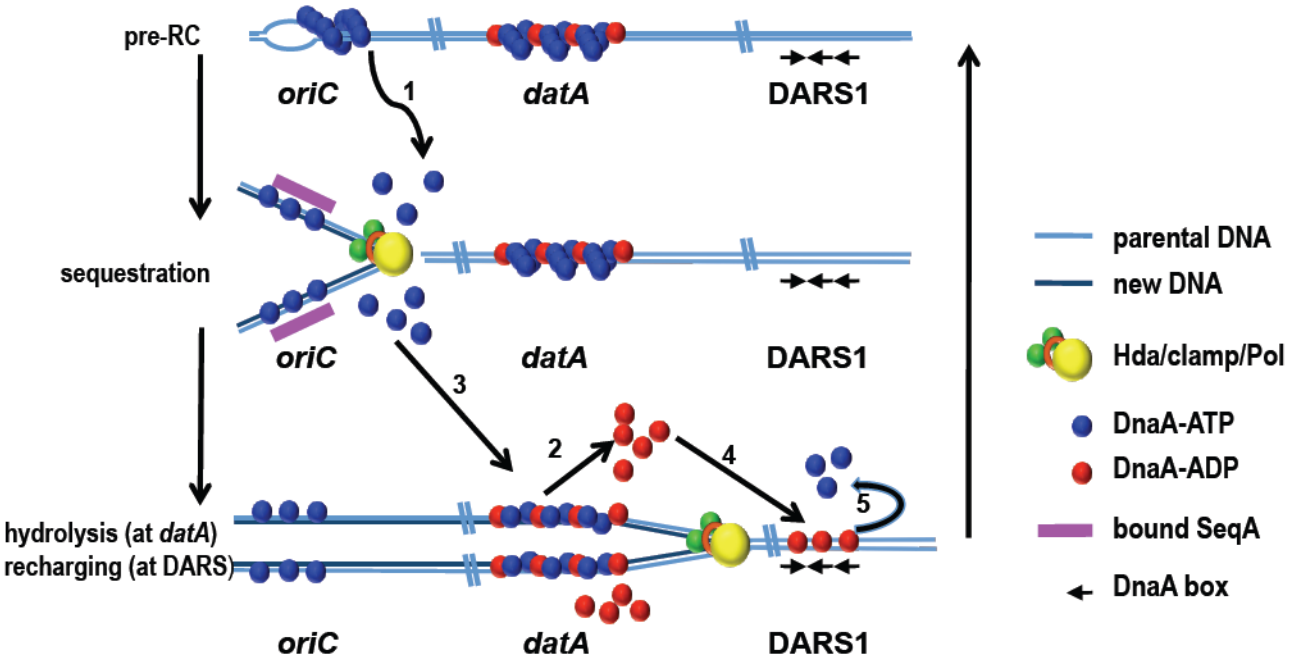

Figure 2.

Model for dynamic displacement of DnaA around the *E. coli* chromosome. Top panel) In the pre-RC, chromosomal DnaA titration sites represented by *datA*, and *oriC*, are completely occupied by DnaA-ATP. DARS is largely inactive, since there is little free DNA-ADP available to it. Only one of the two DARS loci is shown. Middle panel) DnaA-ATP is displaced from *oriC* (stage 1), and cannot rebind because the origin is sequestered by SeqA. The Hda protein associated with the Pol/clamp does not act on this DnaA-ATP (66), which remains free until titration loci are duplicated. Only one fork and one replisome are shown. Bottom panel) When a titration locus is duplicated, bound DnaA-ATP is displaced. Hda stimulates hydrolysis of this displaced DnaA-ATP (stage 2), and some of the DnaA-ADP rebinds at available high affinity titration sites. The DnaA-ATP displaced from *oriC* will then fill the available lower affinity binding sites in the titration loci (stage 3). The DnaA-ADP that was generated by Hda feeds the DARS locus (stage 4), where DnaA-ADP recharged to DnaA-ATP (stage 5). The recycled DnaA-ATP is then available to fill remaining sites in titration loci, and can be used to form the pre-RC for the next generation.