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Regulating DnaA complex assembly: it's time to fill the gaps

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Abstract

New rounds of bacterial chromosome replication are triggered during each cell division cycle by the initiator protein, DnaA. For precise timing, interactions of DnaA-ATP monomers with the replication origin, *oriC*, must be carefully regulated during formation of complexes that unwind origin DNA and load replicative helicase. Recent studies in *E. coli* suggest that high and low affinity DnaA recognition sites are positioned within *oriC* to direct staged assembly of bacterial pre-replication complexes, with DnaA contacting low affinity sites as it oligomerizes to “fill the gaps” between high affinity sites. The wide variability of *oriC* DnaA recognition site patterns seen in nature may reflect myriad gap-filling strategies needed to couple *oriC* function to the lifestyle of different bacterial types.

Introduction

During reproduction of all cell types, new rounds of chromosomal DNA synthesis are triggered with exquisite precision to ensure that completely copied genomes are available for each daughter cell. Regulation is focused on the assembly of initiator protein complexes that prepare chromosomes for new replication forks. In bacteria, pre-replication complexes (pre-RC), comprising multiple copies of initiator protein DnaA, unwind the unique chromosomal replication origin, *oriC*, and assist in the loading of DNA helicase onto the single strands. Although the mechanisms responsible for the timely and orderly completion of homo-multimeric DnaA complexes remain unknown, this review will focus on intriguing clues provided by the emerging relationship between DnaA's structural biochemistry and *oriC* recognition site interactions, with emphasis on the findings of the last four years. We examine growing evidence that arrangements of high and low affinity DnaA recognition sites in *oriC* direct formation of DnaA oligomers along the DNA, and we raise the possibility that oligomers spanning DNA gaps between high affinity recognition sites are important targets for regulatory factors that promote or repress pre-RC assembly. Finally, we will compare *E. coli*'s “gap-filling” strategy to other bacterial replication origins.

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Building a bacterial pre-RC

In *E. coli*, 20–30 monomers of DnaA bind specifically to *oriC* forming a large nucleoprotein complex that appears under the electron microscope as a ball of protein with DNA wrapped around the core [1,2]. The presence of *oriC* DNA stimulates DnaA monomers to form distinctive higher order complexes [3] and during the cell cycle, DnaA-*oriC* interactions are staged [4]. To achieve specific sub-complexes reproducibly during every cell cycle, an important relationship must exist between the structural and biochemical attributes of DnaA, and the arrangement of nucleotide sequences in *oriC* that bind to the protein.

Overview of DnaA attributes

DNA synthesis initiator proteins in all cell types generally share similar features, including the ability to bind DNA and membership in the AAA⁺ protein family, whose activity is regulated by binding and hydrolysis of ATP [5]. The bacterial initiator DnaA [6], is a modular protein (Figure 1) carrying three conserved domains (domains 1, 3, and 4) with specific roles in initiation, and a non-conserved flexible linker domain (domain 2), whose function is less clear.

Domain 1 is responsible for loading DNA helicase [7], and the same region interacts with DiaA, a protein that stimulates initiation by promoting DnaA oligomerization on *oriC* [8]. On the face opposite the helicase/DiaA interaction site, domain 1 carries a self-oligomerization region [9], which is required for initiation. A diverse number of other proteins, including RNA polymerase [10], HU [11], and Dps [12], also associate with DnaA predominantly through domain 1, although these interactions may not be directly related to the initiation step of DNA synthesis.

Domain 3 contains sites for ATP-binding and hydrolysis, as well as an independent oligomerization activity [6,13]. Based on structural information obtained from the hyperthermophile *Aquifex aeolicus*, the DnaA domain 3 oligomerization interface is proposed to switch between two conformations [13]. The ADP-bound form is modeled with a closed configuration, which blocks interaction of adjacent protomers. In contrast, DnaA-ATP protomers adopt an open configuration that allows head-to-tail interaction via a shared γ -phosphate, generating a right-handed helical filament [13]. This oligomeric organization, specific to DnaA-ATP, provides a mechanism for *oriC* DNA to guide ordered pre-RC assembly during the cell cycle (see below).

Domain 4 confers the DNA binding capability of DnaA [13,14]. Two motifs (helix-turn-helix and basic loop) are responsible for multiple major and minor groove DNA contacts along the consensus 9 mer recognition sequence 5'-TTA/TTNCACA-3'. DnaA is also able to contact variations of this sequence, albeit with lower affinity [14]. Interactions with single-stranded DNA are also detected, but are less well characterized [15], and appear to require regions from both domain 4 and domain 3 [16*][17*]. Additionally, a short amphipathic helix, spanning the region connecting domains 3 and 4, is the site where DnaA interacts with membrane lipids [18].

Regulating DnaA-ATP availability in cells

In *E. coli* and other bacterial species, DnaA-ATP is required for initiation of DNA replication and is considered to be the active form of the protein (e.g. the “active initiator”) [19]. DnaA-ATP availability must be stringently regulated, and it is not surprising that levels fluctuate during the cell cycle, reaching a maximum near the time of initiation [20]. DnaA-ATP synthesis is required during each cell cycle to assemble pre-RC. However, recent studies indicate that new synthesis may not provide sufficient initiator [21**], and mechanisms promoting nucleotide exchange are needed to recycle inactive DnaA-ADP. One

exchange mechanism requires that DnaA-ADP interact with membrane acidic phospholipids [18], and a second mechanism is dependent on DnaA-ADP interacting with two chromosomal DNA loci known as DARS (DnaA-reactivating sequences)[21**]. Both DARS carry a novel arrangement of back-to-back DnaA recognition sites that is required to promote nucleotide exchange.

A variety of post-initiation regulatory mechanisms also exist in bacteria to lower DnaA-ATP levels and prohibit inappropriate access to *oriC* during the cell cycle. These mechanisms are coupled to DNA replication, and include activities that stimulate DnaA-ATP hydrolysis, titrate available DnaA away from *oriC*, or block newly replicated DnaA recognition sites in *oriC* [22](Figure 2). The Hda protein (identified in *E. coli*) is associated with the β -clamp of the replisome, and as the replication fork encounters bound DnaA-ATP, Hda stimulates DnaA's weak self-ATP-hydrolysis activity [23]. Chromosome replication also results in duplication of DnaA titration sites on the chromosome. In *E. coli*, the predominant site for DnaA titration is *datA*, a DNA locus with a high DnaA binding capacity [24]. DnaA titration sites have also been identified in *S. coelicolor* and *B. subtilis* genomes[25], and YabA is a β -clamp-associated protein in *B. subtilis* that also titrates DnaA away from *oriC* [26*] [27**]. N⁶-adenine methylation within GATC sites in *E. coli oriC* and *dnaA* promoter also regulates properly timed and once-per cycle initiation activity. In *oriC* and *dnaA* regions, SeqA [28] binds specifically to newly replicated hemimethylated GATC blocking inappropriate pre-RC re-assembly as well as *dnaA* transcription for at least 1/3 of the cell cycle prior to remethylation by Dam methyltransferase [4]. Lack of mRNA inhibits new DnaA-ATP synthesis, and this, combined with the Hda-mediated hydrolysis, effectively lowers the cellular levels of DnaA-ATP after initiation [29]. Additional factors have been identified in *B. subtilis* (Spo0A [30], SirA [31], and ParA/Soj [32**]), *C. crescentus* (CtrA) [33], and *M. tuberculosis* (MtrA) [34] that regulate initiation of DNA synthesis at *oriC* by binding directly to DnaA or to *oriC*.

Origin recognition in bacteria and the transition to a pre-RC

In living *E. coli* cells, naked *oriC* does not exist. Instead, DnaA occupies three high affinity 9 mer recognition sites (R boxes 1, 2, and 4) throughout the cell cycle, forming a nucleoprotein complex analogous to the origin recognition complex (ORC) associated with eukaryotic replication origins [4]. R1, R2, and R4 bind active DnaA-ATP and inactive DnaA-ADP with similar affinities, and it is not known which form is bound in living cells. The ORC-like complex in *E. coli* rapidly transitions to a fully-assembled pre-RC at the time of initiation, when extra copies of DnaA-ATP occupy lower affinity 9 mer sites (R5M, I sites, and τ sites) [4], dispersed in each half of *oriC* [35]. Unlike high affinity R boxes, I and τ sites bind preferentially to DnaA-ATP [36]. High affinity DnaA binding in *E. coli oriC* is essential for pre-RC assembly [37**], since physiological concentrations of DnaA cannot occupy any low affinity recognition sites in *oriC* without cooperative assistance from DnaA bound to a proximal high affinity site [37**]. However, it is not yet known whether the assistance is limited to the DnaA-ATP form bound to the high affinity site.

Two DNA bending proteins, IHF and Fis, also interact at specific sites in the left half and right half of *oriC* respectively, and both play a role regulating low affinity site occupation [38]. Fis associates with *oriC* throughout most of the cell cycle, repressing pre-RC assembly, but is displaced as DnaA levels increase near the time of initiation [38]. Displacement of Fis promotes binding of IHF [38], stimulating DnaA binding at low affinity sites [39]. In addition to IHF, DiaA [40] also stimulates timely pre-RC assembly in *E. coli* by binding directly to a subset of DnaA molecules at *oriC* [41*] and aiding cooperative DnaA interactions. DiaA competes with DnaB helicase for binding to DnaA [41*], suggesting this regulator may act at two different steps during pre-RC assembly.

Filling the gap: a model for regulation of pre-RC assembly in bacteria

Based on the observation that a single high affinity DnaA binding site assists loading of only nearby, proximal weaker sites [37^{**}], we propose a model whereby each high affinity DnaA interaction with *oriC* serves as a nucleator and anchor for oligomers extending along the DNA. During pre-RC assembly, a DnaA structure that spans DNA in the gaps between R1 and R2, and between R2 and R4, would be formed (Figure 3). The DNA sequences contained in these two gaps encode the regulatory features (described previously) that are expected to promote or prohibit stable formation and attachment of emanating DnaA oligomers. For example, I sites and τ sites in the gap regions would establish specific DNA-based requirements for DnaA-ATP during assembly, and the multiple GATC sites in the gap regions provide targets for post-initiation oligomer blocking agents, like SeqA (Figure 3). DNA bending proteins, also binding in a gap, could prohibit oligomer extension between high affinity sites (Fis) or bend the DNA into a configuration that promotes gap-filling (IHF). Additionally, oligomers assembled in each half of *oriC* may play distinct roles in proper growth-rate regulated initiation control [42], since the left region of *E. coli oriC* is sufficient for viability when cells are growing slowly in nutrient-poor media, but the right region is required for multi-fork replication and rapid growth rate in rich media. Based on this observation, it is likely that intact *oriC* is required for synchronous initiations under conditions when multiple *oriC* copies are present.

The DnaA binding sequences within each gap are also positioned such that they would act to promote oligomer assembly during the transition to the pre-RC. In the left half of *oriC*, the gap between R1 and R2 contains 4 closely spaced low affinity binding sites (R5M, τ 2, I1, and I2) that are observed to be bound *in vivo* at the time DNA replication initiates [4]. Mutations that reduce binding to these sites also reduce *in vivo* origin activity [36], indicating that sequence-specific DnaA binding at low affinity sites in this gap region is required. Re-examination of the *E. coli oriC* nucleotide sequence between R2 and R4 suggests that additional closely spaced low affinity DnaA recognition sites also exist ([43] and unpublished observations). An array of low affinity sites would facilitate ordered and stable oligomer assembly in the gaps by placing DnaA-ATP protomers close enough to stimulate domain 3 interactions (Figure 3). The model proposed here also raises questions about how anchoring DnaA at high affinity R boxes connects with the DnaA-ATP oligomers that “fill the gap”. For DnaA to span the distance between high and low affinity recognition sites, the flexible domain 2 linker [44] may be required to extend DnaA protomers attached by domain 1 interactions (Figure 3). Interestingly, it was recently shown that deletions in domain 2 reduce initiation efficiency [45]. Regulators such as DiaA may also promote pre-RC assembly at this stage [41^{*}] by stabilizing the interaction between the anchoring and extended DnaA (Figure 3).

Extending the model: bacterial *oriC* diversity

Prokaryotic replication origins vary greatly with regard to size (ranging from 180–3800 bp) and number of DnaA consensus sites (ranging from 1 to 30) [46]. Can the gap-filling model we propose for *E. coli oriC* accommodate this high degree of variability? One intriguing possibility is that the number of gaps (determined by the placement of high affinity recognition sites) might vary among organisms, depending on the degree of regulation required over a range of growth conditions. In origins with few gaps, such as the asymmetrically dividing *C. crescentus*, one regulator is able to play a significant role in repressing pre-RC assembly [47]. In *C. crescentus*, the global regulator CtrA, which blocks initiation of DNA replication only in the flagellated daughter, binds proximally to the single high affinity DnaA recognition site found in *Cori* [48], producing a simple on/off switch that would block extension of a DnaA oligomer. In the case of the slow growing *Mycobacteria*

or *Streptomyces* genera, larger numbers of high affinity sites would not only increase the number of DnaA-ATP molecules required to assemble pre-RC, but would also provide greater opportunities for regulators to act within each gap [49]. Thus, we suggest that gap-filling features may be optimized to the life style of the host bacterium, in the same way that DnaA structure is likely to be optimized for its respective *oriC* [50].

Conclusions

Bacterial replication origins carry densely packed information that directs the orderly formation of pre-RC assembly during the cell cycle. This information is, in part, expressed by the relative positions and orientations of high and low affinity DnaA recognition sites that promote formation of oligomers, as well as the interspersed recognition sites for regulators of oligomer assembly. If the model we have proposed is viable, we expect that all bacterial origins will contain more sites for DnaA interaction than previously predicted based on the canonical 9 mer sequence. Future comparative analysis among different bacterial types should establish new rules for DnaA-DNA recognition and pre-RC assembly, and mutational analysis of weak DnaA-*oriC* interactions should aid our efforts to understand how pre-RC assembly is regulated over wide ranges of nutritional conditions.

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Figure 1. Structure of DnaA

DnaA contains four domains, labeled with Roman numerals. Key amino acids, responsible for the indicated functions, are marked (details and references included in text).

Figure 2. Mechanisms that prohibit DnaA-ATP access to *oriC* after initiation

Three mechanisms, coupled to DNA replication, prevent untimely re-initiation by decreasing levels of available DnaA-ATP or by blocking DnaA binding sites in *oriC*. **A)** In *E. coli*, hydrolysis of DnaA-ATP bound to the chromosome is assisted by Hda associated with the β -clamp. The nascent DNA is red, and the parental strand is blue. **B)** Duplication of chromosomal loci, such as *datA* in *E. coli*, creates additional DnaA binding sites (marked by black rectangles) that titrate DnaA and decrease the level of available protein. In *B. subtilis*, YabA associated with the clamp plays a similar titrating role, by binding DnaA and limiting its access to *oriC* (not shown). **C)** Chromosome replication creates hemimethylated GATC sequences (small black circles), some of which are located in low affinity DnaA binding sites (black rectangles) in *oriC* or the *dnaA* promoter. SeqA binding to the hemimethylated GATCs blocks DnaA from rebinding to these sites, while DnaA can rebind to sites that do not contain a GATC.

Figure 3. Model of pre-RC assembly by filling gaps between occupied high affinity DnaA binding sites

After initiation of chromosome replication, DnaA rebinds to high affinity R1, R2 and R4 sites. Extension of DnaA oligomers from these anchor sites to weak sites in the gap regions is prevented (indicated by blocked arrows) by inhibitors SeqA and Fis (top cartoon). In early pre-RC assembly (stage 1), after sequestration ends, DnaA bound to R1, R2 and R4 recruits additional DnaA protomers via domain 1 interactions, stabilized by binding to weak sites in *oriC* and by association with DiaA. As DnaA-ATP levels rise in the cell (stage 2), closely spaced low affinity DnaA binding sites position DnaA protomers, facilitating domain 3 interactions (lines joining DnaA molecules) and stable gap-filling. Extension from R2 and R4 into the right gap region causes Fis displacement, allowing IHF to bind and bend *oriC* so that R1 and R5 interact, completing pre-RC assembly and strand separation by filling the left gap. DnaA-ATP binds to the single-stranded unwound region via domain 3 and domain 4 interactions.