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INITIATION OF DNA REPLICATION

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INTRODUCTION

Prior to dividing into two daughter cells, *E. coli* and *S. enterica* must duplicate their circular genomes, each encompassing about 4.7 megabase pairs of double-stranded DNA (25, 164). Successful completion of this critical step of cellular reproduction demands precise coordination between the nutritional state of the cell and the mechanism that triggers the start of new DNA polymerization. In recent years it has become clear that complex regulatory circuits control the initiation step of DNA replication by directing the assembly of a multi-component molecular machine (the orisome) that separates DNA strands and loads replicative helicase at *oriC*, the unique chromosomal origin of replication. Following assembly of the replisome and passage of new replication forks, the orisome must then be disassembled and its components inactivated to ensure that during a cell division cycle only one new round of DNA synthesis is triggered from every replication origin.

Although many details of the process still remain unclear, in this chapter we will discuss recent efforts to understand the regulated protein-DNA interactions that are responsible for properly timed initiation of chromosome replication. Information about newly identified nucleotide sequence features within *E. coli oriC*, as well as new structural and biochemical attributes of the bacterial initiator protein DnaA will be reviewed. We will also discuss the coordinated mechanisms that prevent improperly-timed DNA replication.

A number of recent and noteworthy reviews cover several topics presented here in greater detail (116, 122, 176, 180, 197, 267), and the reader is also referred to two new EcoSal chapters covering (4.4.2) DNA Replication Machinery, and (5.2.1) Replication, Segregation, and Cell Division, as well as a previous EcoSal chapter on timing of synthetic activities in the cell cycle (101). It is also important to note that two earlier monographs on initiation of DNA synthesis were written for EcoSal by Kaspar von Meyenburg and Flemming Hansen (249) and by Walter Messer and Christoph Weigel (169). The reader is referred to both of these excellent chapters for details that we are unable to include in this version.

We wish to dedicate this review to the memory of Walter Messer, not only to honor his considerable scientific contributions, but also to recognize the great kindness and support he

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bestowed on all who joined him in the quest to understand the regulation of bacterial cell growth.

AN ABBREVIATED HISTORY

Initiation of chromosome replication is precisely timed

Bacteria contain more DNA when growing rapidly and less when growing slowly, with the amount of DNA per cell varying continuously with growth rate. This observation, presented in 1958 by Schaechter et al. (214), raised questions about how growth rate-regulated expansion and contraction of DNA content was achieved. Although it is reasonable to expect that replication forks simply move more rapidly at ever faster growth rates, this is not the case. Studies done by Helmstetter and Cooper (47, 100), using synchronously-dividing *E. coli* B/r cultures, demonstrated that the average rate of DNA chain elongation is the same in cells with doubling times between 20–100 min, reviewed in (101). Over this range, approximately 40 min is required to duplicate the chromosome (termed the C period), and an additional 20 min (D period) is required for the cell to complete septum formation and divide (see Figure 1) (47, 100), although the duration of C and D period may be different in other strains, see for example (102). Exponentially-growing *E. coli* B/r will always divide 60 min after the onset of each round of chromosomal DNA synthesis. However, the time required to prepare to initiate a round of chromosome replication (I period) is not constant, but is strictly dependent on cellular growth rate. Based on this relationship of I and C+D, new rounds of DNA synthesis will initiate in the early, middle or late portion of the cell cycle, independent of the status of the cell's ongoing chromosome replication and septum formation (101) (see Figure 1).

One caveat of the constancy of C + D is that during rapid growth, at doubling times less than 60 min, there is insufficient time for cells to complete a round of chromosome replication and divide before new rounds of DNA synthesis must be triggered (Figure 1). Since replication forks progress bidirectionally from fixed replication origins on *Escherichia* and *Salmonella* chromosomes (78, 161, 162), newly divided daughter cells will inherit dichotomously branched chromosomes (termed theta structures, since they resemble the Greek letter θ). Chromosome configurations in *E coli* B/r cells growing with generation times of 20, 40, 50 and 80 minutes are shown in Figure 1. The reader is also referred to http://simon.bio.uva.nl/Object-Image/CellCycle/index.html (268) for an animated simulation of chromosome configurations at a variety of growth rates as well as different C + D periods. It is also worth noting that because of these overlapping rounds of DNA replication, cells must contain more replication origin copies at faster growth rates (Figure 1). However, all copies initiate replication synchronously during the cell division cycle (225).

The I+ C + D rule provides an explanation for the increased DNA content in rapidly growing bacteria and focuses attention on the mechanism that triggers initiation of DNA synthesis as a key regulatory step in the bacterial cell cycle.

Early models for initiation control circuitry

What is the nature of a regulatory mechanism that triggers new rounds of DNA synthesis at the correct time during the cell cycle? A simple regulatory circuit to control the initiation

step, referred to as the replicon model, was described in 1963 by Jacob, Brenner, and Cuzin (114) and is considered to be the first formal conceptualization of a defined genetic site from which DNA synthesis begins (originally called the replicator, and now referred to as the replication origin), and replicon-encoded, origin activating factors, called initiators. The concept that the initiator was a protein in *E. coli* was supported by early studies demonstrating that new DNA synthesis is sensitive to chloramphenicol because ongoing protein synthesis is required for initiation (142, 159, 213). Additional studies demonstrated that cells always initiate DNA replication at a constant mass per chromosomal origin (57), and led to the conclusion that a threshold level of one or more initiator proteins triggers new rounds of DNA synthesis.

Identification of the genes that encoded the initiators came from studies on temperature-sensitive, conditional-lethal mutants of *E. coli*, in which two DNA replication-defective phenotypes were identified (132, 133). One class, “immediate stop” mutants, halted DNA synthesis immediately upon shift to non-permissive temperature, indicating that the mutated gene products were required for the elongation phase of DNA replication. The “delayed stop” mutants, in contrast, continued to make DNA after a shift to non-permissive temperature, gradually stopping after a reproducible period of time (132, 133, 169). The kinetics of the delayed stop mutants suggested that the defective gene products were required specifically for the initiation step of DNA synthesis, and subsequently, two genes, *dnaA* and *dnaC*, were identified, (reviewed in (169, 249)). As described in more detail below, the DnaA protein is the bacterial initiator, and in *E. coli*, DnaC protein is required to load replicative helicase.

The first step towards identifying the replicator came from studies in which the position of the unique *E. coli* replication origin was mapped by marker frequency analysis to a chromosomal site near *ilv* (22, 23). Hiraga then demonstrated that this region of chromosomal DNA, when carried on F’ plasmids, was sufficient to suppress incompatibility of F plasmids to replicate in Hfr strains (105). He first called this region *poh*$^{+}$, (permissive on Hfr) and then named the genetic locus “*oriC*” to designate the **ori**gin of **c**hromosome replication (105). *OriC* was more precisely mapped to be in, or near a 1.3 kb Hind III fragment located at 84.3 minutes on the *E. coli* chromosome (161). A 9 kilobase pair Eco RI fragment from this region of the chromosome was ultimately found to be capable of directing autonomous extrachromosomal replication when circularized into a plasmid (250, 262).

Several modifications to the replicon model were made to account for constancy of initiation mass (202, 230), and these models enforced the importance of having both positive and negative regulatory features to ensure both proper timing and only once-per-cell cycle initiations. As described in detail in the following sections, the present view is that in *E. coli and S. enterica*, DnaA protein is the positive regulator whose threshold level triggers new rounds of DNA replication and determines initiation mass (116, 154), with a variety of post-initiation regulators quickly repressing the potential to re-initiate. It is also clear that in order to ensure that initiations are precisely timed, it is necessary to assemble multi-component complexes to activate replication origins (61); these complexes have been termed “orisomes” (49, 146, 269). Regulation of DnaA accessibility to *oriC*, the ordered assembly

and disassembly of a multi-DnaA complex at *oriC*, and the means by which DnaA unwinds *oriC* remain important questions to be answered and we will discuss the current state of knowledge on these topics in the following sections.

FACTORS INVOLVED IN INITIATION OF CHROMOSOME REPLICATION

DnaA, DnaB, and DnaC Proteins: primary components of the orisome

Prior to assembly of bidirectional replication forks, a replication origin is unwound and two molecules of replicative DNA helicase are loaded onto available single-strands (136). In *Escherichia* and *Salmonella*, three proteins: DnaA, DnaB, and DnaC are sufficient to perform these activities. DnaA is responsible for site-specific unwinding of *oriC* and assists DnaC, a dedicated helicase loading protein, in positioning DnaB, the replicative DNA helicase, onto available single stranded DNA (136). All three components are members of the AAA^+ family (**A**TPases **A**ssociated with various cellular **A**ctivities), reviewed in (244), whose activity is regulated by the binding and hydrolysis of ATP.

DnaA is the initiator protein—DnaA is a highly conserved 52 kDa protein that has been identified as the primary initiator protein in eubacteria (154, 176, 272). DnaA levels are the critical factor in determining the cellular mass at initiation (154), and over-production of wild-type DnaA results in increased initiations from *oriC in vivo* (11) and abolition of cell cycle-specific initiation timing (199). These extra initiations do not necessarily increase intracellular DNA content due to replication fork collapse near *oriC* (10, 222, 226).

DnaA binds to DNA at a 9-mer sequence with the consensus 5’-TTA/TTNCACA-3’ (24, 215) although lower affinity binding sites that deviate from the consensus in one or more base pairs have been identified (87, 95, 124). As described in more detail in the following sections, both high and low affinity DnaA recognition sites exist in *oriC* and become occupied prior to DnaA-catalyzed unwinding (146). Approximately 300 additional consensus recognition sites for DnaA are found distributed throughout the chromosome (207).

DnaA has a high affinity for ATP (K_D=30 nM) and ADP (K_D=100nM) (218). Since the ATP levels in the cell are higher than ADP, newly synthesized DnaA is commonly believed to be in the ATP form. DnaA-ATP is the active form of the protein, and, *in vitro*, DnaA-ATP is necessary for both *oriC* unwinding and initiation of DNA replication (218). The activity conferred by ATP is allosteric, since DnaA bound to a poorly hydrolysable analog of ATP is also active for unwinding and replication *in vitro* (218). An intrinsic ATPase activity converts DnaA-ATP to inactive DnaA-ADP, but this activity is too slow to be effective in cells, and so must be stimulated in a post-initiation, DNA replication-coupled mechanism called RIDA (**R**egulatory **I**nactivation of **D**na**A**) (117) (see section on Hda, below). Hydrolysis of DnaA-ATP is critical, since over-initiation, fork collapse, and cell death is observed in cells carrying mutations, for example *dnaAcos*, that cannot down-regulate DnaA by hydrolysis (36, 123, 127, 182, 222).

Structural properties of DnaA—DnaA is divided into four functional domains (238) (Figure 2). Domain I (N-terminus amino acids 1–90) interacts at separate locations with

DnaB (217, 237) and other regulatory proteins, such as DiaA (113), Hda (236), HU (44), and Dps (45) (see below). Domain I also plays a role in DnaA self-oligomerization (68, 256). Several laboratories have performed deletion and site-directed mutagenesis of DnaA Domain I, and these mutants reveal critical regions needed for protein-protein interactions (1, 68, 223, 256).

Domain II (amino acids 90–130) is a flexible linker region without a clearly defined role in initiation of DNA replication. Domain II is the least conserved domain among eubacterial DnaAs, but the length of the linker appears to be important for function (184), since only some deletions in this region are tolerated, and one viable deletion mutant is reported to have an under-initiating phenotype (172). Domain II appears to be the only region in which large insertions can be made in DnaA, a feature that has been used to construct a functional GFP-tagged DnaA (27).

Domain III (amino acids 130–347) is the region that establishes DnaA as a member of the AAA^+ family of ATPases (60, 118). Domain III can be further divided into smaller subdomains that are common among AAA^+ proteins (Figure 2). Most of the N-terminal and central region of domain III (termed the Base), is an αβα-nucleotide-binding fold that carries highly conserved Walker A and B motifs involved in adenine nucleotide binding and magnesium ion interaction, reviewed in (116, 118, 167). This region also contains Sensor I (86) and Box VII motifs, which site-directed mutational analyses have shown to be important for γ-phosphate hydrolysis and DnaA oligmerization (67, 124). Box VII contains a conserved arginine (Arg 285) that is required for DnaA function (67, 124). The remaining C-terminal region of Domain III is an alpha helical bundle (termed the Lid) that carries the conserved AAA^+ motif Sensor II, also involved in monitoring nucleotide binding (118). An invariant arginine critical for DnaA function is also found at the tip of a helix within the Sensor II motif (182). Properly folded DnaA produces a nucleotide binding pocket at the juncture between Base and Lid that is accessible to all of the aforementioned motifs simultaneously.

Domain IV (amino acids 347–467) is the DNA-binding domain that interacts with 9-mer recognition sites (74, 206, 239), and also contains amino acids that are responsible for membrane interaction (82).

Structural studies of DnaA domain III and IV in the thermophilic bacterium *Aquifex aeolicus* (64, 65) reveal a novel basis for ATP-dependent DnaA assembly into an oligomer. DnaA-DnaA interactions are modeled within a bipartite nucleotide binding pocket comprising Sensor II residues of one protomer and the Box VII residues of its neighbor. An adjacent Box VII arginine finger becomes accessible to its ATP-bound neighbor due to an open configuration of the Base and Lid that does not exist in DnaA-ADP (64) (Figure 2). Through the binding and hydrolysis of ATP, DnaA switches back and forth between oligomer-promoting and oligomer-inhibiting states. The assembled DnaA oligomer exists as an extended right handed superhelical filament rather than the ring shape often observed for AAActhat form a V-shaped steric wedge protruding from Domain III in such a way as to prevent a flat ring assembly (64).

Structural studies have also revealed two modes for DnaA interaction with DNA. The axial channel produced by the assembly of the DnaA-ATP helical filament has been proposed to directly engage unwound single stranded DNA in the origin (64, 196) and provide a structure to assist in the delivery of DnaB-C complex (177). Domain IV contains a helix-turn-helix (HTH) motif for double-stranded DNA binding termed the DnaA signature sequence (KDHTTVI), as well as a proximal region termed the Basic loop (65). Both major and minor grooves of an *oriC* R box interact with DnaA (74). One helix and one loop of the HTH insert into the major groove and an arginine in the Basic loop is recognized by bases in the minor groove (65, 74). DNA is bent by 28 degrees when bound to DnaA (74).

***dnaA* mutants—**DnaA is an essential protein, but a variety of conditional lethal (temperature sensitive) mutants have been isolated, for example see (39, 132). DnaA(ts) mutants include alleles with defects in Domain I (*dnaA*508), Domain III (*dnaA*5, *dnaA*46, *dnaA*601/602, and *dnaA*604/605) and Domain IV (*dnaA*203/204) (97), although most also contain an additional amino substitution (97). Excellent reviews of these *dnaA*(ts) alleles are available (169, 224) and the reader is referred to them for more details. It should be noted that the Domain III mutants all share the same amino acid substitution in the ATP binding cleft, which helped identify the importance of this region of DnaA for nucleotide binding and activity. More recently, mutagenesis of DnaA has been driven by structural studies, to identify amino acid residues critical for domain-specific functions, for examples, see (68, 124, 125, 239).

Regulation of *dnaA* expression—The *dnaA* gene, located 42 kilobase pairs counterclockwise to *oriC* (25), is the first gene in an operon that also contains *dnaN* and *recF* (92, 96, 192). The *dnaN* gene encodes the β-clamp of DNA polymerase III holoenzyme and the *recF* gene encodes a DNA recombination protein. The entire operon is transcribed from two promoters upstream of the *dnaA* gene, termed *dnaA*1p and *dnaA*2p, although *dnaN* and *recF* also appear to have separate promoters (4). The two *dnaA* promoters are separated by approximately 80 bp, and this region of DNA contains a consensus DnaA box and one box deviating from consensus in one position (96), separated by a putative third box, deviating from consensus at two positions (95). There are also three ATP-DnaA boxes (Speck-Messer sites) flanking the DnaA boxes (232). Both *dnaA* promoters are negatively regulated by the DnaA protein (9, 33, 138, 169), and it was proposed that a DnaA oligomer formed around the consensus DnaA box, and blocked RNA polymerase from binding to either *dnaA*1p or *dnaA*2p (144). Autorepression appears to be largely mediated by DnaA-ATP binding to the promoter region, with DnaA-ADP having less of an effect on transcription (232). Other negative regulators, including the DNA bending protein Fis , and the stringent response nucleotide ppGpp , also have been reported to bind to the promoter region, causing growth-rate regulation of transcription (43, 73, 201), although there are conflicting data as to whether or not the cellular levels of DnaA protein are also growth-rate regulated (43, 93). The promoter region also contains several GATC sequences, which, as described below, are involved in binding the SeqA protein immediately after the gene is duplicated (38, 138, 158); this binding represses transcription (38).

Transcription of the *dnaA* gene fluctuates during the cell cycle, due primarily to SeqA blocking the hemimethylated promoter region (38, 241) (described in more detail below). It seems less likely that cell cycle-specific *dnaA* expression results from autorepression, since cellular DnaA-ATP levels are highest immediately before initiation (140), and transcription of the *dnaA* gene is not decreased until after initiation of new DNA synthesis (28). Rather, it appears that autoregulation may be more important in allowing the expression level of *dnaA* to increase when additional copies of *oriC*, or other DnaA titrating sites, are present in the cell. Addition of DnaA titrating sites to *E. coli* causes an increase in expression from the *dnaA* gene, proportional to the number of DnaA molecules bound by the titrating site (98, 174). This de-repression would allow rapidly growing cells to accommodate additional copies of *oriC* without changing the cellular age at initiation.

DnaB and DnaC—DnaB helicase (50 kDa) oligomerizes into a hexomeric toroidal ring with a central channel (15, 19, 58, 143) that encircles single-stranded DNA and unwinds the helix in advance of the replication fork. In order to load onto single-stranded DNA, the DnaB ring must be broken by DnaC-ATP (29 kDa) (130, 251, 252), a structural paralog of DnaA (177). After loading DnaB onto *oriC*, DnaC dissociates from the complex (66). DnaB-C loading also requires DnaA since mutations in the N-terminus region of DnaA disrupt the loading (237), although details regarding DnaA's role in the process are lacking. A recent model (177) suggests a novel mode of interaction among DnaA, B, and C during helicase loading and is discussed below.

DnaC is essential for initiation, and temperature-sensitive, conditional lethal *dnaC* mutants have been used extensively to align initiation of chromosome replication in populations of growing *E. coli* cells (39). Cultures of a *dnaC*(ts) strain that are shifted to 42°C for one hour complete all previously initiated rounds of DNA synthesis, but are unable to start new rounds. During the time at non-permissive temperature, all other components necessary to intiate new rounds are synthesiszed in excess so that upon shifting back to permissive temperature (25°C) all *oriC* copies intiate DNA synthesis simultaneously (91). Initiation potential for two rounds of DNA synthesis accumulates at the nonpermissive temperature and a second burst of initiation follows about 10 min after the first. The time interval between these successive initiations is termed the eclipse period (245) and is considered to be the minimum time during which a cell is capable of successive initiations from the same origin. It is not clear what normally determines the length of the eclipse period, but the topological state of *oriC* and sequestration (see below) may contribute (116).

Analysis of *A. aeolicus* DnaC structure reveals that it shares DnaA's ability to form a right-handed helical filament (177). This structural similarity is sufficent to allow placement of DnaC AAA^+ domains onto the end of a DnaA oligomer in an ATP-dependent fashion. It has been proposed that DnaC-ATP associates with the DnaA bound to the 13-mer region to specifically position DnaB on top and bottom strands within the unwound region of *oriC* (177).

The origin of chromosome replication, *oriC*

E. coli and *S. enterica* each have a single, fixed origin of chromosome replication, termed *oriC*, which in *E. coli* K12, comprises nucleotides 3,923,767–3,924,025 on the *E. coli* K12 genomic sequence (25). In *E. coli* and *S. enterica*, the *oriC* region does not encode any proteins involved in DNA replication (Figure 3). This is not true for all eubacteria, as many harbor *dnaA* within 2–3 kb of *oriC*. In *E. coli* and *S. enterica*, DnaA remains the closest replication gene to *oriC*, but the two loci are separated by 42 kb (186). The genes directly flanking *oriC* are *mioC* (clockwise) and *gidA* (counterclockwise) (Figure 3). *MioC* encodes a flavodoxin implicated in biotin synthesis (21) and *gidA* encodes a flavin adenine dinucleotide-binding protein that modifies tRNA (170, 263). The protein products of both genes are dispensable, but transcriptional activity from both *gidA* and *mioC* promoters is implicated in *oriC* regulation (see below). Immediately beyond *mioC* and *gidA* lie genes encoding asparagine synthetase (*asnC* and *asnA*) and ATP synthetase, respectively.

Cloned versions of *oriC* form minichromosomes—Although the location of *oriC* was precisely mapped on the *E coli* chromosome, the size of the chromosome was prohibitively large for detailed functional analysis. This problem was solved by constructing *oriC* plasmids (termed minichromosomes), selected for autonomous replication in the absence of any other replication origin (250, 262). Minichromosomes share regulatory attributes with the chromosome including bidirectional replication (166), dependence on the same DNA replication factors (250), and synchronous, cell cycle-specific, one round per cell cycle initiation (103, 134, 148). Although minichromosomes replicate only once per cell cycle, they lack an equipartition mechanism and tend to segregate in clumps, causing recipient cells to harbor more than one copy per cell (52). Synchronous cell-cycle replication of up to 30 additional *oriC* copies is permitted, demonstrating that the levels of diffusible factors are not generally limiting (151). Cell cycle-specific minichromosome replication is not dependent on chromosomal *oriC* function, and minichromosomes are retained in cells with randomly replicating chromosomes, in which chromosomal *oriC* is replaced by a plasmid replication origin (62). Combined, these studies demonstrated that the wt *oriC* sequence contains all of the information needed to direct cell cycle-specific timing of initiation.

Sequence features of *oriC*—Functional *oriC* can be dissected into two distinct regions that play different, but inter-related roles in initiating DNA replication. The right/central region (bases 80–270; Figure 3) contains a set of protein recognition sites that are separated by fixed numbers of base pairs (5), forming a platform for ordered assembly of multiple DnaA molecules (discussed in more detail in a later section). The leftmost region of *oriC* (bases 10–66 of Figure 3) contains the **D**NA **U**nwinding **E**lement (DUE) (137); an A-T-rich region with three tandem 13 mer repeats of 5’-GATCTNTTNA/TA/TA/TG/T-3’, where the DNA helix unwinds in response to assembly of a higher order DnaA complex (32). In the single-stranded state, the 13-mers in the DUE are reported to interact directly with DnaA-ATP, at several 6-mer A/TGATCT motifs (referred to in Figure 3 as Speck-Messer, S-M, sites) (231), similar to the ATP-DnaA boxes observed in the *dnaA* promoter (232). Interaction of DnaA with these sites is proposed to stabilize origin unwinding (196, 231). An additional AT-rich sequence is found between bases 10–20 at the left-most end of *oriC*, and

although it is required for proper *oriC* function, its specific role remains to be determined (7).

The DUE is separated from the right/central DnaA loading platform by a precise distance of 13 bp (encompassing bases 67–79); adding or deleting nucleotides in the spacer sequence is not tolerated (109). The right/central region contains at least ten DnaA 9 mer recognition sites whose occupation is detected by DNA footprinting (79, 124, 146, 163). The nucleotide sequence and positions of all DnaA binding sites in *oriC* are highly conserved among all members of the *Enterobacteriacae* (99, 271).

Each DnaA recognition site in *oriC* may be classified based on two criteria: affinity for DnaA and preference for DnaA nucleotide form. There are three high affinity (Kd = 4–20 nM) (215) consensus recognition sites: R1, R2, and R4 (Figure 3). These sites bind DnaA-ATP and DnaA-ADP equally well *in vitro* (79, 215). There are an additional seven low affinity (Kd > 200 nM) (215) sites that have been identified in *oriC*; these deviate from the consensus sequence at one or more bases. Of the lower affinity sites, I sites (I1, I2, and I3) (87) and tau sites (τ1 and τ2) (124) clearly discriminate between nucleotide forms of DnaA, showing a 4-fold preference for DnaA-ATP (124, 165). There are conflicting data regarding binding of DnaA-ADP to R5M and R3; DMS footprinting studies indicated that these two sites have no preference for a particular DnaA nucleotide form (88, 165), while Dnase I footprinting experiments suggested that all low affinity sites, as well as R2, preferred to bind DnaA-ATP (124). The reason for these differing results is not clear, but may reflect a difference in the sensitivity of the methods used to examine binding. DnaA binding to its high and low affinity recognition sites is discussed in greater detail in later sections.

In addition to DnaA recognition sites, *oriC* contains specific binding sites for two DNA bending proteins, Factor for inversion stimulation (Fis) and Integration Host Factor (IHF) (70, 200, 208). The Fis binding site lies in the right half of *oriC* between R2 and R3, and IHF binds between R1 and R5M (Figure 3). Both proteins modulate DnaA interactions, as discussed below.

The entire *oriC* sequence contains an unexpected number of GATC palindromes (Figure 3), with four being located in the 13 mer region and seven found between bases 80–250. No GATC is found within any high affinity DnaA recognition site, but four low affinity recognition sites (τ1, τ2, I2, and I3, Figure 3) contain an internal GATC, and R5M overlaps a GATC. GATC is the recognition sequence for both Deoxyadenosine methyltransferase (*dam* methylase) and the hemimethylated DNA binding protein, SeqA. The DNA replication-dependent conversion of fully methylated GATC to the hemimethylated state plays an important regulatory role in limiting initiations from *oriC* and resetting the origin for the next cell division cycle (described in more detail below).

Several transcriptional promoters were also previously mapped within *oriC* (115, 216), but a specific role during initiation for these transcripts remains unclear.

Mutational analysis of *oriC*—Mutations have been placed at specific locations within *oriC* to evaluate the roles of individual protein binding sites, as well to examine the role of

the intervening nucleotides between DnaA recognition sites (108, 141, 165, 194, 195, 208, 254, 257). Generally, mutations are introduced into *oriC* on plasmids that carry an additional origin of replication, such as *ori* pBR322. This chimeric configuration (149, 255), allows recovery of defective versions of *oriC* and the extent of the defect can be assayed in strains lacking DNA polymerase I (required for initiation from *ori* pBR322, but not *oriC*).

While some single base pair changes in DnaA recognition sites do not affect either DnaA binding or *in vivo* replication (108), mutations that reduce DnaA binding resulted in decreased minichromosomal *oriC* function, as do mutations that alter helical phasing between recognition sites (141, 165). The loss of minichromosome replication *in vivo* indicates that these mutations cripple *oriC* so that it is unable to compete effectively with the wild-type chromosomal copy (108, 151). Combined, the results of these studies support a role for all DnaA recognition sites and spacer regions during normal assembly of higher order complexes. However, with the exception of mutations that eliminate binding to R1, reducing the activity of a single recognition site does not completely inactivate *oriC* when it is functioning as the sole origin on the chromosome, although many mutations alter initiation timing and/or perturb initiation synchrony (20, 203, 254). This resiliency of function suggests that there are likely to be multiple ways for DnaA complexes to assemble, unwind *oriC* and load helicase. However, the failure of mutant origins to function in the presence of wild-type competition suggests that none of the potential alternative assembly mechanisms is as efficient as the normal pathway. A model for the precise assembly of pre-replication complexes based on binding to all high and low affinity recognition sites is discussed in a later section.

Mutations have also been made in the binding sites for Fis and IHF (50, 208), as well as in the GATC sequences in the left part of *oriC* (13). These mutations are discussed below.

Additional regulators of initiation

Although DnaA, DnaB, and DnaC are essential genes, there are other regulators of initiation that can be mutated without loss of viability, but whose inactivation results in abnormal initiation timing during the cell cycle. These regulators generally work by: 1) controlling availability of active initiator DnaA (Figure 4A); 2) enhancing or repressing the ordered assembly of DnaA complexes at *oriC* (Figure 4B); or 3) conditionally blocking initiation in response to unfavorable conditions (Figure 4B). It is sometimes difficult to evaluate the function of these regulators by examining the phenotype of mutant strains with severe growth perturbations, since compensatory mutations that improve cell survival may arise spontaneously in strains with severe initiation defects (205).

Architectural proteins IHF, HU, and Fis: DNA bending proteins that modulate assembly of DnaA complexes at *oriC*—Fis (71), IHF (63), and HU (59) are small, abundant, nucleoid-associated proteins that bend DNA and regulate many aspects of chromosome biology, including specific steps in orisome assembly. IHF places a severe bend (120°) in *oriC* between R1 and R5M, while the Fis-induced bend, between R2 and R3, is less acute, at 55° (85, 200, 201). HU lacks a specific recognition site in *oriC*, but binding of several HU molecules is reported to introduce a curve into DNA (30, 107).

Minichromosomes cannot be stably maintained in cells lacking Fis or HU (70, 85, 189), while IHF mutants are able to maintain minichromosomes unless *polA* is also absent (69). The reason for the *polA* requirement is not clear, but it was proposed that these cells use an alternative mode of DNA replication (69).

Both IHF and HU are enhancers of DnaA-catalyzed unwinding of *oriC* (56, 110), but two different mechanisms are involved (210). IHF binding promotes DNA unwinding by increasing DnaA occupation of low affinity recognition sites (87). IHF mutants initiate DNA replication later in the cell cycle than wild-type cells, and they have asynchronous, rifampicin-resistant initiations (31, 248), a phenotype that is consistent with a requirement for a higher cellular level of DnaA to initiate chromosome replication. Scrambling the IHF binding site on *oriC* eliminates minichromosomal *oriC* function, and causes late and asynchronous initiations on the chromosome (208, 254), providing supporting evidence for IHF's role as a positive regulator of initiation.

The mechanism used by HU to assist *oriC* unwinding is not yet clear. HU does not appreciably alter DnaA binding to *oriC* (211), although it might affect IHF binding under some conditions (30). It is possible that by placing curves in the *oriC* DNA, HU increases torsional stress and stabilizes unwinding. HU was also recently shown to interact directly with DnaA (44), which could be a mechanism to bring HU to specific positions within the orisome. HU mutants, particularly those with defects in the α-subunit of HU, display an asynchronous phenotype (14), indicating that HU and IHF are not interchangeable *in vivo*.

Fis represses *oriC* unwinding (210) and *in vitro* replication of *oriC* plasmids (104, 258) by blocking DnaA binding to low affinity sites (210). Fis also inhibits IHF binding to *oriC* (210). *In vivo*, cells lacking Fis initiate asynchronously (31). Mutant versions of *oriC* with a defective Fis binding site are not functional as minichromosomes (50, 85), and replicate with altered timing (earlier) when operating as the chromosomal origin (203). Fis occupies its primary site in *oriC* throughout the cell cycle in rapidly growing *E. coli*, but DnaA-catalyzed displacement of Fis near the time of initiation (40) promotes binding of IHF (210). Fis is the only bending protein in this group of initiation regulators whose synthesis is growth-rate regulated (18), and its presence at *oriC* is likely to also be growth-rate dependent.

Dam* methylation and SeqA protein—Dam* methylation of GATC (83) plays a role in many different DNA-based activities in *E. coli* including gene transcription, DNA mismatch repair, initiation of chromosome replication and maintaining nucleoid structure (155) (for more details, the reader is referred to EcoSal (4.4.5) DNA Methylation). As mentioned above, *oriC* contains an unusually high number of GATC motifs (Figure 3), and GATC is also found in the promoter region of *dnaA*, allowing methylation to play a regulatory role in both DnaA synthesis and DnaA binding to *oriC* (34, 181).

After the passage of a replication fork, fully methylated GATC on duplex DNA becomes transiently hemimethylated, but in most regions of the genome, re-methylation by *dam* methyltransferase is rapid. However, both *oriC* and the DnaA promoter region remain hemimethylated for about 1/3 of the cell cycle, termed the sequestration period (38). During sequestration, hemimethylated *oriC* is inactive, and for this reason fully methylated

minichromosomes are unable to replicate in Dam⁻ strains (209, 229). Additionally, transcription from both *dnaA*1p and *dnaA*2p is blocked during the sequestration period (38).

Sequestration is mediated by the protein SeqA (21kDa), which preferentially binds hemimethylated GATC sequences (158, 228). *E. coli* mutant strains lacking SeqA activity have asynchronous initiations, and suffer from over-initiation of DNA replication (158). In *oriC*, SeqA binding to hemimethylated GATCs blocks re-binding of DnaA to low affinity sites after initiation (181) (discussed in a later section). Presumably, SeqA also blocks RNA polymerase from binding to the *dnaA* promoter region. The orientation of the eleven GATCs within *oriC* is sufficient to allow prolonged binding of SeqA and slow remethylation, since this pattern of sites also shows delayed remethylation if inserted at non-*oriC* regions of the chromosome (12). Further, mutation of GATC sequences in the left region of *oriC* resulted in loss of sequestration and over-initiation (13). Hemimethylated *oriC* is also associated with *E. coli*'s membrane fraction (51, 191) and this interaction is reported to require SeqA (228).

SeqA forms homo-tetramers that bind to two hemimethylated GATCs that are separated by no more than 31 base pairs (35). Based on structural analysis, SeqA forms higher order oligomeric filaments (89) similar to DnaA (64). With an intracellular concentration of about 1000 molecules (228), there is also sufficient SeqA to perform other duties in the cell including formation of aggregates that travel with the replication forks (106, 261) and a role in chromosome organization (243). While these attributes of SeqA are beyond the scope of this chapter, interested readers are referred to an excellent recent review (253).

DiaA: a DnaA binding protein that enhances DnaA oligomerization—DiaA is a recently discovered positive regulator of initiation, identified in a screen for mutations that suppress the over-initiation phenotype of *dnaAcos* (113). DiaA-deficient strains are viable, but show perturbed initiation timing, with delayed, asynchronous initiations in rich media (113). DiaA stimulates initiation by enhancing oligomeric DnaA binding to *oriC*, so that less DnaA is required for unwinding of the 13 mer region (77, 128) (Figure 4B). The active form of DiaA (50–60 kDa) is a homo-tetramer that interacts specifically with the N-terminus of DnaA (128). A single homo-tetramer can bind to multiple DnaA molecules simultaneously and enhance assembly of both DnaA-ATP and DnaA-ADP complexes at *oriC* (128). About 3 DiaA tetramers are assciated with a complete DnaA complex at *oriC* (113, 128). DiaA also contains a putative phosphosugar binding domain that may regulate DiaA activity.

Dps and IciA: Conditional repressors of initiation—Dps (178) and IciA (242) are negative regulators that act in response to stress or starvation conditions to decrease initiation from *oriC* (Figure 4B). Dps is a DNA binding protein that is abundant in stationary phase cells and in starved cells (2), where it appears to play a role in protecting the bacterial genome from environmental stress . Dps also is responsible for increased chromosome condensation in stationary phase (193). Recently, Dps was shown to interact directly with the N-terminus of DnaA and to impede *in vitro* replication by interfering with *oriC* unwinding (45). This repression of initiation may allow stress-induced DNA damage to be repaired in the absence of new replication forks.

IciA is a 33 kDa protein that blocks *oriC* unwinding by interacting directly with *oriC* within the A-T rich 13 mer region (111, 112). IciA is a member of the LysR family of transcription regulators, and was shown to be identical to ArgP, a regulator of arginine transport (42). IciA/ArgP levels increase in response to phosphate starvation (90), and presumably block initiations under conditions where successful elongation is unlikely. IciA also binds to the *dnaA* promoter region, simultaneously enhancing transcription and blocking DnaA autoregulation (145). Thus, during phosphate starvation, while *oriC* is inactivated, levels of DnaA-ATP would be expected to increase and remain high enough to trigger initiation once phosphate was no longer limiting. When conditions are appropriate for initiation, IciA levels are reduced by specific proteolysis (264).

Hda: post-initiation inactivator of DnaA—As discussed above (section on DnaA), DnaA-ATP is rapidly converted into the inactive ADP form during RIDA (120) (Figure 4A). Initially, the factors involved in RIDA were termed IdaA and IdaB (119, 121, 139); IdaA was subsequently found to be the sliding clamp of the DNA polymerase holoenzyme (121), and IdaB was isolated and renamed Hda (123), since the protein is **h**omologous to **D**na**A**. Hda forms a stable complex with the sliding clamp, where its primary function is to stimulate the intrinsic ATPase activity of DnaA (121, 236). The mechanism used by Hda to do this is not fully characterized, but it appears that active Hda must be bound to a DNA-loaded clamp (126). The interaction of Hda with DnaA also requires amino acid residues in DnaA's N-terminal region (236). Hda is a member of the AAA^+ family of ATPases (123), but there is no evidence that it binds ATP (235). Rather, a conserved arginine (arg-168) in the box VII motif (arginine finger) is required for the stimulation of hydrolysis, and a recent structural analysis of Hda suggests that the box VII region interacts directly with the ATPase region of DnaA (260). ADP binding to Hda may also be important for i*n vivo* activity (235).

Because Hda activity is dependent on association with DNA-loaded sliding clamp, RIDA is coordinated with movement of replication forks. It was first proposed that, as the newly formed replication forks proceed from *oriC*, the associated clamp/Hda interacts with DnaA-ATP, stimulating ATP hydrolysis (236). In this scenario, DnaA would have to be present at the fork. It is also possible that some clamps are left behind on the DNA after lagging strand synthesis, and these "leftover" clamp/Hda complexes might also stimulate DnaA-ATP hydrolysis. In either case, DnaA-ATP inactivation would not be expected during cell cycle periods devoid of ongoing DNA synthesis, which occur in slowly growing cells (see Figure 1).

There is controversy in the literature regarding the phenotype of *hda* mutants. Loss of Hda activity is reported to produce effects of varying severity, ranging from lethality (123) to significant over-initiation (36) or only modest over-initiation and asynchrony (37, 205). However, several compensatory mutations were identified in Hda-defective strains (205), and much of the variation in phenotype could be explained by differences in the compensatory mutations generated in the strains used in the studies.

***datA* and other chromosomal DnaA titration sites—**There are approximately 1000 molecules of DnaA per *E. coli* cell (93, 221). Despite the need to activate multiple *oriC* copies in rapidly growing cells, this level of DnaA is unexpectedly high, and it is clear that

there must be negative regulators of DnaA activity to ensure that excess free DnaA-ATP is not available to bind *oriC* and promote unscheduled initiation events. There are several ways this can be accomplished, two of which (RIDA and sequestration) were discussed above. An additional mechanism is to titrate DnaA away from *oriC* by providing competing initiator recognition sites on the chromosome. As originally formalized in the initiator titration model (94), ongoing DNA replication causes the duplication of DnaA recognition sites dispersed along the chromosome that bind DnaA and lower its availability. Orisome assembly is then prevented until the number of newly synthesized DnaA molecules is greater than the number of titration sites. In order for initiator titration to be effective, the relative affinities of DnaA recognition sites within *oriC* must be lower than the titration sites dispersed on the chromosome. This requirement raises interesting issues for mechanisms that precisely order orisome assembly (see below).

Although some DnaA recognition sites on the *E. coli* chromosome are in gene promoter regions, where DnaA acts as a transcriptional regulator (168), there are other chromosomal DnaA recognition sites that appear to serve no role other than to titrate available DnaA away from *oriC*. The primary DnaA titration activity on the *E. coli* chromosome is localized within a 950 bp region, termed *datA*, that carries 5 DnaA recognition sites site (190). *DatA* is reported be able to bind large amounts of DnaA (up to 300 molecules) (129). Such high titration capacity from only a few recognition sites must require cooperative protein-protein interactions and a higher order structure among assembled DnaA molecules. Although loss of *datA* is not lethal, initiations from *oriC* become asynchronous and an over-initiation phenotype was observed by flow cytometry (129). More recently, *datA* mutants were shown to continue to initiate in the presence of rifampicin (175), resulting in the apparent asynchronous phenotype. Rifampicin-resistant initiations do not occur in normal cells, and generally indicate that there is an increased availability of DnaA in the cells. Excess *datA* delays initiations from *oriC* (173, 174), as would be expected if it were titrating DnaA away from *oriC*.

Membrane phospholipids and DARS: two DnaA-ADP recharging mechanisms—DnaA-ADP produced by RIDA does not spontaneously reactivate by exchanging bound ADP for ATP (218). Rather, two different recycling pathways exist to regenerate DnaA-ATP, and these pathways may be required, at least under some growth conditions, to supplement the amount of DnaA-ATP generated by new synthesis (76, 147, 259). The first pathway is dependent on acidic phospholipids (26). DnaA interacts directly with phospholipids via a short stretch of amino acids between Domains III and IV (26, 65) (Figure 2), and *in vitro*, acidic phospholipids can catalyze exchange of DNA-bound DnaA-ADP to DnaA-ATP (48, 220). In cells, DnaA associates with phospholipid membranes (27, 179) and about one half of the intracellular DnaA appears to be retained in the insoluble lipid fraction after cell lysis (221). *E. coli* cells that are unable to make the acidic phospholipids phosphotidylglycerol and cardiolipin are growth arrested at the initiation stage (49, 259), and this growth arrest can be suppressed by mutations in the DnaA membrane binding domain (e.g. DnaAL366K) (270), although the mechanism for the suppression is not yet clear. It is also suggested that a high local DnaA concentration in the membrane may be necessary to regulate ADP to ATP exchange (3).

A second recharging mechanism requires specialized chromosomal DNA sequences termed DARS (**D**na**A R**eactivation **S**equences) (75, 76). Although originally identified in the ColE1 replication origin, DARS1 and DARS2 are found on the *E. coli* chromosome near *bioD* and *mutH*, respectively (76). While the exact recharging mechanism remains to be elucidated, DARS share two closely spaced DnaA recognition sites that are oriented in back-to back positions, and this configuration is required to stimulate the conversion of bound DnaA-ADP into the apo-DnaA (no-nucleotide) form. Once the nucleotide is removed, DnaA quickly recharges back to DnaA-ATP spontaneously, due to the higher cellular ATP concentration. Loss of DARS delays initiation during the cell cycle (76), suggesting these sequences normally contribute to the threshold level of DnaA-ATP required for initiation. A yet to be indentified soluble factor has also been implicated in regulating the activity of DARS2 (76).

Topoisomerases and RNA Polymerase—Initiation of *E. coli* chromosomal DNA replication requires supercoiled *oriC* DNA (32), and any changes in the level of DNA supercoiling or changes in transcriptional activity from promoters adjacent to *oriC* have the potential to alter origin function. For this reason, both DNA topoisomerases and RNA polymerases must be considered regulators of *oriC* activity.

DNA supercoiling is altered in strains carrying mutations in topoisomerase genes (84). Increased supercoiling, as is seen in strains with mutations in *topA* encoding Topoisomerase I, suppresses the temperature sensitivity of the *dnaA46 (ts)* allele, which normally under-initiates (157). Decreased supercoiling also causes asynchronous initiations from *oriC* (246, 247), as well as adversely affecting minichromosome maintenance (150). These findings suggest a direct relationship between DNA supercoiling and initiation efficiency, most likely by modulation of the amount of DnaA required to unwind *oriC*.

Transcription near *oriC* generally promotes initiations under conditions where DnaA levels are deficient (17). *In vivo*, the *gidA* promoter, located to the left (counterclockwise) of *oriC*, is the most likely candidate for a transcriptional activator of initiation, since the direction of *gidA* transcription should generate negative supercoiling within *oriC* (see Figure 3). In support of this idea, *gidA* transcription is reported to be required for normal origin function (7), and the *gidA* promoter is shut down during *oriC* sequestration (28), as would be expected for a positive modulator of initiation. The *mioC* gene on the right (clockwise) side of *oriC* was also originally thought to play a role in initiation timing (*mioC* stands for **m**odulator of **i**nitiation at ***oriC***). *MioC* transcripts enter and pass through *oriC* (28, 29, 115, 156, 216). Normally, *mioC* transcription is shut down by DnaA binding to the *mioC* promoter, shortly before initiation (29, 187, 234). Continuous *mioC* transcription is reported to be deleterious to *oriC* function (183, 234), but does not completely repress new rounds of DNA synthesis (29). However, despite its intriguing cell cycle-specific regulation, *mioC* is not necessary for proper timing of initiation, since its deletion does not affect replication timing of either minichromosomes (148) or the chromosome (152).

Direct interaction of DnaA and RNA polymerase was recently detected by immunoprecipitation (72), and this observation is consistent with previous findings that several mutations in *rpoB* (encoding RNA polymerase beta subunit) are able to suppress

temperature sensitive *dnaA* initiation mutants (8). Further examination of this interaction may reveal new roles for both proteins at specific stages of initiation.

OVERVIEW OF NUCLEOPROTEIN COMPLEXES FORMED AT *ORIC*

In vitro replication systems: defining stages leading to replisome assembly

The development of *in vitro* systems to study *E. coli* DNA replication revolutionized our understanding of the steps used to assemble the replication machinery at *oriC* (135, 219). By characterizing the complexes generated by combining purified replication proteins and supercoiled *oriC* plasmids (16, 79, 80, 219), the Kornberg laboratory defined a series of stages required to initiate DNA synthesis *in vitro* from *oriC* (described below).

Initial complex—The initial complex was originally defined as the association of DnaA with R boxes on *oriC* templates (219). Neither supercoiled *oriC* nor ATP are required to assemble this complex. When examined by electron microscopy, the initial complex was classified further based on correlation of a specific structure with *in vitro* replication activity (50). The active initial complex was a compact ellipsoid containing approximately 200 bp of *oriC* wrapped around approximately 20 molecules of DnaA-ATP, with the DNA strands crossing as they emerge from the ellipse (50, 80). At lower DnaA concentrations, smaller complexes were observed, suggesting that initial complexes are built from distinct sub-complexes (50). The observation that small complexes transitioned into a reproducible and uniquly shaped active initial complex was one of the first indications that DnaA interaction with *oriC* is ordered (discussed in greater detail below). A complex that is similar in appearance to the compact ellipse can be formed with DnaA-ADP, but the DnaA-ADP complex is inactive in the *in vitro* replication assay (50).

Open complex—The second distinctive stage, the open complex, is characterized by DnaA-mediated unwinding of supercoiled *oriC* templates in the absence of DNA helicase (219). The location of the single-stranded bubble has been mapped by its sensitivity to P1 endonuclease, and is found within a 26 bp region between the middle and rightward 13 mer repeat in the DUE (32, 85). Patterns of cutting are different for the top and bottom strands (32) and may result from one strand interacting with DnaA more extensively than the other strand (196, 231). DnaA-ATP is sufficient to unwind *oriC in vitro* at 37° C, but HU or IHF must be included for any unwinding to take place at lower temperatures (110). Presence of HU or IHF also reduces the amount of DnaA that is required for unwinding at 37°C (56, 87, 110). Additionally, while it is clear that some of the DnaA used to make the open complex must be in the ATP-bound form, a combination of DnaA-ADP and DnaA-ATP can support *in vitro* replication of *oriC* plasmids (265). The DnaA-ATP requirement for initiation is discussed in more detail below.

Conversion of the initial complex, made with either all DnaA-ATP or a combination of DnaA-ATP and DnaA-ADP, to the open complex requires the additional presence of high ATP concentrations (5 mM) (265), even though the K_D for ATP binding to DnaA is much lower (30 nM) (218). The reason for this requirement remains unclear, since there is no evidence suggesting that there is more than one ATP binding site in DnaA.

Pre-priming complexes I and II are stages of helicase loading that follow open complex formation. Pre-priming complex I is defined by two DnaB-C complexes (251, 252) loaded sequentially at unwound *oriC* (top and bottom single strands). In pre-priming complex II, the loaded helicases are translocated within *oriC*, expanding the unwound region sufficiently to assemble the replisome (16, 80).

Pre-priming complex I requires addition of DnaA-ATP, DnaB, and DnaC to the supercoiled *oriC* template, with HU, if present, stimulating the reaction (80, 218). DnaA-ATP is necessary to produce both the open complex and for stable retention of DnaB in the pre-priming complex (237). The latter function is mediated through direct interaction of the N-terminal domain of DnaA with DnaB (237).

DnaC loads replicative helicase by interacting with a 1:1 stoichiometry on one face of the DnaB hexamer, creating a gap in the ring through which single-stranded DNA can pass (54). DnaC binds both ATP and ADP, and the dual ATP/ADP switch activity is necessary to form the pre-priming complexes. DnaC-ATP is proposed to first interact with single-stranded DNA, expanding the unwound bubble at *oriC* as it delivers DnaB. DnaC-ATP also inhibits helicase activity, preventing the DnaB oligomer from translocating until it is properly loaded (53). DnaC is also modeled to interact directly with DnaA-ATP oligomeric filaments, directing helicase loading to the correct location on the top strand of the unwound 13 mer region (177). In the presence of DnaB and single- stranded DNA, DnaC-ATP is hydrolyzed to DnaC-ADP, activating helicase and allowing its translocation along the DNA (53), forming Pre-priming Complex II. The association of DnaC with *oriC* is transient, and DnaC cannot be detected in the pre-priming complex after it is assembled (80).

Helicase hexamers are loaded face to face (one on each strand) and pass one another as they move along the DNA (66). By passing one another in this way, the unwound region remains melted and can be coated with single-strand binding protein prior to priming and replisome assembly.

Priming complex and replisome assembly stages do not require DnaA and for details the reader is referred to EcoSal (4.4.2) DNA Replication Machinery. Limited helicase unwinding (65 bp or more) is required prior to the interaction of DnaG primase. Primase interacts with the moving helicase and for this reason RNA/DNA junction positions are not fixed (66). Predominant priming sites fall within *oriC* (between the rightmost 13 mer and R1) for counter-clockwise synthesis, but outside *oriC* (about 60–70 bp left of the left boundary) for clockwise synthesis (131). The primases also appear to pass one another during bidirectional replication. Following the synthesis of two 10–12 bp primer RNAs per *oriC*, two β-clamps assemble on the DNA followed by two DNA polymerase III complexes (66).

Cell cycle assembly of *in vivo* complexes: Defining the bacterial ORC and pre-RC

Although characterization of *in vitro* complexes provided invaluable information regarding the biochemical processes needed to begin DNA replication, these studies did not address how the orisome was assembled in growing cells at the correct time during the cell cycle. In order to answer this critical question, high resolution *in vivo* footprinting methods were

developed, making it possible to follow the occupation of DnaA recognition sites in *oriC* as a function of cell cycle timing (40, 181, 211, 212). These studies revealed that the three highest affinity sites, R1, R2, and R4 are the only sites that become occupied immediately after initiation during the sequestration period (181). Further, these three sites remain the only *oriC* sites bound by DnaA throughout the majority of the cell cycle (40, 181, 211, 212). This persistent DnaA complex is temporally similar to the nucleoprotein structure observed at replication origins in budding yeast, in which the Origin Recognition Complex (ORC) proteins remain associated with replication origins throughout the yeast cell cycle (233). For this reason, the DnaA complex bound to R1, R2, and R4 is defined as the bacterial ORC (181). Interestingly, X-ray crystallographic analysis of DnaA from *Aquifex aeolicus* support this definition, by revealing that DnaA has remarkable structural similarity to components of the eukaryotic ORC as well as to archeal initiators (60, 65). In addition to regulation by ATP, bacterial and eukaryotic initiator proteins appear to share the ability to form right-handed helical filaments (46, 64, 185), and the structural similarites are sufficiently strong that it is possible to dock a helical pentamer of DnaA into the core of Drosophila ORC (46). Thus, it seems that all domains of life may utilize similar mechanisms to regulate initiation of DNA synthesis.

In *E. coli*, at the time of initiation of DNA replication, *in vivo* footprinting studies revealed that additional DnaA is bound to *oriC*, where it occupies the lower affinity sites R5M, R3, I1, I2, I3, and τ2 (40, 181, 211) and mediates localized strand separation in the DUE (87, 165). This distinctive fully assembled DnaA/*oriC* complex with an unwound DUE is reasonably defined as the bacterial pre-replication complex, pre-RC (181). In yeast, prior to entering S phase, a similar increase in protein binding to replication origins is observed, where additional proteins (Cdc6, Cdt1 and MCM2–7) associate with ORC to form yeast pre-RC prior to loading DNA polymerase (55). The similarities between eukaryotic and *E. coli* initiation can be further extended to include mechanisms used to load replicative helicase, with DnaA, DnaB and DnaC being the functional equivalents of ORC/Cdc6, replicative helicase MCM 2–7, and Cdt1, respectively. However, the distinct DNA strand melting observed in the *E. coli* pre-RC prior to helicase loading has not been observed at eukaryotic origins.

BUILDING AND UNBUILDING THE ORISOME

Ordered assembly of *E. coli* pre-RC

In vitro, the differing affinities of the DnaA recognition sites in *oriC* causes DnaA loading to take place in the order: R1=R4>R2>R5M=I2=I3>I1=τ2=R3 (87, 160, 165). *In vivo*, only two stages of DnaA occupation of *oriC* are easily observed: filling of R1, R2, and R4 (forming the bacterial ORC), and the filling of the remaining, lower affinity sites to form the fully occupied pre-RC (181, 211). Conversion of the *E. coli* ORC to the pre-RC must proceed identically during every cell division cycle to ensure correct initiation timing. Since DnaA binding to high affinity sites persists throughout the majority of the cell cycle, their occupation cannot be part of the timing mechanism, nor is the formation of bacterial ORC likely to be the target of mechanisms that regulate initiation timing. Rather, it is the filling of the lower affinity sites by DnaA that is the rate-limiting and regulated process. Although *in*

vivo, low affinity sites appear to fill abruptly at the time of initiation, it seems likely that the ORC to pre-RC transition takes place in separate and successive stages, since this type of assembly would provide more opportunities for regulation.

Evidence for the existence of separate stages of pre-RC assembly comes from analysis of the binding patterns of accessory bending proteins Fis and IHF to *oriC* during the cell cycle, and a model for staged pre-RC assembly is shown in Figure 5A. During rapid growth, Fis is associated with the bacterial ORC, occupying its primary recognition site immediately rightward of R2 throughout most of the cell cycle (40). *In vitro* analysis of the effect of Fis on DnaA binding to *oriC* revealed that the occupation of low affinity recognition sites R5M, I2 and I3 requires higher DnaA levels than are needed in the absence of Fis (210).

The next observable stage of pre-RC assembly is the displacement of Fis. As DnaA levels are raised *in vitro*, and at the time of initiation *in vivo*, Fis is lost from its primary site in *oriC* (40, 210). Although the mechanism responsible for the displacement of Fis is unknown, one attractive possibility is that Fis is displaced by DnaA oligomers that extend between R2 and R4.

Immediately after Fis displacement, IHF is detected at its primary recognition site between R1 and R2 (40, 210). *In vitro* footprinting analysis demonstrated that Fis represses the ability of IHF to bind to *oriC* (210), although the mechanism for this repression is unclear. Once bound, IHF promotes DnaA occupation at low affinity sites R5M, I1, I2, and I3, and it is this IHF-mediated stimulation that led to the naming of I sites (for IHF-stimulated sites) (87). The tau sites, located to the left and right of R5M, are also reported to become occupied coincidently with the other lower affinity sites *in vitro* and *in vivo* (124, 165, 181). It is possible that the bending of *oriC* DNA by IHF promotes interaction of the DnaA molecules bound to R1 and R5M, providing a stable anchor site from which to extend an oligomeric filament toward R2. This would help explain the rapid filling of the lower affinity sites in this region, but again, such structures bound to *oriC* have yet to be observed.

The transition from Fis-bound ORC to IHF-bound pre-RC appears to be a switch-like mechanism for rapid conversion of ORC to pre-RC in a DnaA-dependent fashion. Because of the dynamic interplay among DnaA, Fis, and IHF (210), cell cycle specific DNA bending by Fis and IHF would be an efficient mechanism to rapidly change positional relationships of DnaA binding sites during the cell cycle to either promote or inhibit the occupation of lower affinity DnaA recognition sites.

The binding of IHF and the IHF-stimulated filling of low affinity sites leads to localized unwinding of the DNA in the DUE (32, 87, 110, 210). After the 13 mer DNA becomes single stranded, there is evidence that DnaA-ATP associates with, and stabilizes the unwound region (196, 231, 266). Although this interaction has been proposed to be directed to single-stranded ATP-DnaA boxes (AGatct; labeled S-M sites in Figure 3) found in each 13 mer repeat (231, 232), it seems likely that DnaA-ATP does not use Domain IV for binding of single-stranded DNA. Instead, both structural and mutational analyses suggest that the central region of a DnaA-ATP filament associates with the single-stranded DUE (64, 196).

The mechanism by which the DnaA that is bound to high and low affinity sites in *oriC* mediates strand separation in *oriC* and directs helicase loading is among the least understood aspects of initiation in *E. coli*. Two models for unwinding, which are not mutually exclusive, have been proposed. In one, *oriC* DNA wraps around a right-handed oligomeric filament of DnaA-ATP. This wrapping would generate positively supercoiled DNA, but could simultaneously produce localized negative supercoiling in the adjacent DUE (64). In another model, the DnaA complex bound to *oriC* would prevent the DNA helix outside of the DUE from unwinding in the presence of topological change directed by transcriptional activity, presumably from *gidA* (6, 188). This inhibition of helical twist would thereby direct local unwinding to AT-rich regions within the DUE. Models for DNA helicase loading currently focus on the putative DnaA-ATP filament that extends into, but not through, the unwound 13 mer region. The availability of an oligomeric DnaA structure fixed in location is an attractive target for the interaction of DnaB-DnaC and the delivery of helicase to a specific location on both strands (177).

The role of DnaA-ATP in forming the pre-RC—Although it is clear that DnaA-ATP is the active form of the initiator, the reason for this requirement is not immediately obvious, since R-boxes bind DnaA-ATP and DnaA-ADP with equal affinities (165, 215, 218). Two compatible scenarios seem likely; first, that *oriC* contains binding sites with a preference for DnaA-ATP, and second, that DnaA-ATP is uniquely capable of forming a complex with the capability of unwinding the 13 mer region. Current data provide supporting evidence for both cases. Sites that preferentially bind DnaA-ATP have been identified, and they fall into two major classes. The DnaA-ATP-specific S-M sites are in the 13 mer region and preferentially bind DnaA as single stranded DNA (231). I sites and tau sites are located in the body of *oriC* between high affinity sites (87, 124), and must be filled prior to strand separation (87, 165). I sites and tau sites have a 3–4 fold preference for DnaA-ATP (124, 165). The placement of these sites may be critical for pre-RC formation, to ensure that DnaA-ATP is correctly positioned to form a specific structure such as the previously mentioned right handed helical filament (64).

Questions remain about the role of DnaA-ADP in the assembly of pre-RC. *In vitro*, only a fraction of the DnaA needed to support DNA replication needs to be DnaA-ATP (265), and our unpublished results indicate that open complexes can be made when only 30% of the DnaA in the reaction is in the ATP form. There is also *in vivo* evidence that DnaA-ADP is used during pre-RC formation, since increasing the requirement for DnaA-ATP by converting R5M to I2 makes the mutated *oriC* less efficient (88). Since structural studies indicate that only DnaA-ATP can form a helical filament (64), it remains to be determined how DnaA-ADP is accommodated in the pre-RC. It also remains uncertain to what degree oligomer formation *in vivo* requires assistance from other cellular factors. For example, DiaA stimulates formation of both DnaA-ATP and DnaA-ADP oligomers and may play a role in promoting specific interactions between DnaA molecules in growing complexes.

Synchronous initiation from multiple copies of *oriC*: the initiation cascade model—It is clear that every *oriC* copy must build a complete pre-RC in order to unwind the DUE. However, potential problems arise in rapidly growing cells or cells containing

minichromosomes. These cells, containing multiple copies of *oriC*, (see Figure 1) (101), initiate chromosome replication at the same mass per chromosomal origin (57), and all origins initiate synchronously during the cell cycle (148, 225). To account for the fact that the cell does not need to make more DnaA in order to initiate more copies of *oriC*, the "initiation cascade" model was proposed, in which newly triggered origins are able to release active DnaA that becomes available for rapid pre-RC assembly at remaining "uninitiated" *oriC* copies (94, 153). For an initiation cascade to function properly, there must be mechanisms to prevent pre-RC from being reassembled at origins that have recently initiated. These mechanisms are discussed in the following sections.

Disassembly, inactivation, and resetting of the orisome: ensuring one properly timed initiation every cell cycle

In order to trigger DNA synthesis only once from each *oriC* copy per cell cycle, DnaA interacting with each newly initiated *oriC* must be removed and prevented from reassembling pre-RC for one cell generation. Once all *oriC* copies initiate, any remaining free DnaA-ATP must become unavailable and ultimately inactivated to ensure that new synthesis of active initiator is required for the next round, so that initiation frequency remains coupled to growth rate. Additionally, the mechanisms that disassemble and inactivate the orisome must also allow each *oriC* copy to be reset by reforming the bacterial ORC, so that the next round of pre-RC assembly can proceed in the correct order. All of these activities are accomplished by complementary and cell cycle-specific mechanisms, described below and shown in Figure 5B.

Removal of *oriC*-bound DnaA—*In vivo* DMS footprinting studies have demonstrated that the pre-RC is a transient complex, with *oriC* being fully occupied by DnaA-ATP for only a small fraction of the cell cycle (181). However, the mechanism by which DnaA is removed from *oriC* is not known. It is likely that either translocation of DNA helicase through *oriC*, or the assembly and movement of the replisome would generate enough force to displace DnaA. If displaced by DNA helicase, then DnaA-ATP may leave *oriC* while remaining in the active form for use at other unfired origins in the initiation cascade. If the replisome were to remove the DnaA, then it is possible that the Hda bound to the sliding clamp could stimulate hydrolysis of DnaA-ATP, which might preclude its reuse at another origin. However, the rapid and multiple re-initiation events that take place in SeqA mutants (158) suggest that if the replisome is the factor that removes DnaA from *oriC*, RIDA activity may not be sufficient to inactivate enough DnaA-ATP to prevent re-formation of the pre-RC.

***OriC* sequestration and resetting of ORC—**Replication of *oriC* DNA results in hemimethylation of the 11 GATC residues located in the origin, followed by binding of SeqA to the hemimethylated sites (181, 227, 228). These SeqA-*oriC* interactions mark the start of the sequestration period, where *oriC* remains refractory to re-initiation and blocked from remethylation by Dam methyltransferase for approximately 1/3 of the cell cycle (38, 158). During sequestration, bound SeqA prevents reassembly of pre-RC at newly replicated origins by blocking DnaA binding sites that contain or overlap a GATC (181, 240). These sites include the low affinity sites in the body of *oriC* (R5M, τ2, I2, and I3), as well as the

ATP-DnaA/S-M sites in the 13 mer region (see Figure 3). In mutants lacking SeqA, loss of this blocking activity allows rapid rebinding of DnaA to the lower affinity sites and reformation of the pre-RC, (181), leading to inappropriate re-initiation and over-replication (158).

The high affinity sites R1, R2, and R4 do not contain or overlap a GATC sequence, and SeqA does not block DnaA from binding to these sites (181). Therefore, early in the sequestration period, DnaA rebinds the high affinity sites in *oriC*, resetting the bacterial ORC so that the origin is ready to begin the process of pre-RC assembly so that chromosome replication will start at the correct time in the next cell cycle.

Does the bacterial ORC need to be recharged to the ATP form?—There is no known mechanism that would ensure that the DnaA that rebinds to high affinity sites during sequestration is in the ATP form, since R1, R2, and R4 have equal affinity for both DnaA-ATP and DnaA-ADP, and both forms are likely to be available immediately after initiation. However, if all origins are to build orisomes identically each and every cell cycle, it seems logical to propose that they should all start from the same ground state, although there is little evidence to suggest or refute that this is, indeed, the case. If the transition from ORC to pre-RC requires that the ORC contain only DnaA-ATP, then special mechanisms would be needed to exchange any DnaA-ADP or replace bound DnaA-ADP with DnaA-ATP. Since hemimethylated *oriC* DNA interacts with the membrane fraction (191), there is an opportunity during the sequestration period for any *oriC*-bound DnaA-ADP to interact with acidic phospholipids that stimulate the nucleotide exchange to recharge DnaA-ATP (41, 49). Alternatively, DnaA is detected near the membrane surface (27, 179), and it is possible that the membrane provides a localized reservoir of DnaA-ATP that could replace any DnaA-ADP bound to newly replicated *oriC*.

Decreasing the cellular levels of available DnaA-ATP—SeqA blocks reformation of the pre-RC for approximately 1/3 of the cell cycle (38). During this time, several other mechanisms are active, working to reduce the cellular levels of available DnaA-ATP, so that at the end of sequestration, the pre-RC does not reform prematurely. During sequestration SeqA binds and sequesters the *dnaA* gene promoter, shutting down transcription (38, 241), and causing a rapid decrease of cellular *dnaA* mRNA levels, and a halt to new DnaA-ATP synthesis for the duration of the sequestration period. Additionally, as each new round of DNA synthesis begins, RIDA becomes active and Hda associated with the replication forks stimulates the intrinsic ATPase activity of bound DnaA-ATP (123). Thus, RIDA, coupled with the lack of new DnaA-ATP synthesis, results in a rapid decrease in cellular DnaA-ATP levels after initiation (140). Mutational studies have demonstrated that both timely *dnaA* promoter sequestration and Hda/RIDA are necessary to prevent reinitiation (123, 204). Moving the *dnaA* gene to a location more distant from *oriC*, thus delaying its shut down by sequestration, results in asynchronous initiations (204). While this latter study indicates that turning off *dnaA* expression at the wrong time in the cell cycle perturbs initiation regulation, the result of keeping the *dnaA* gene on throughout the entire cell cycle is less clear. In studies where DnaA, driven by the inducible *plac*, was constitutively expressed from a plasmid, changes in *dnaA* expression levels altered the mass at initiation, but did not change

once per cycle regulation, unless the DnaA levels were raised to such an extent as to cause over-initiation (10, 154). There is no clear explanation for these conflicting results, but they may indicate that there is a very strict balance between DnaA levels, RIDA, and sequestration in preventing re-initiation events.

As replication forks proceed, DnaA recognition sites on the chromosome are duplicated and become available to titrate any freely available DnaA in the cell (94), including DnaA displaced from *oriC* that is precluded from rebinding by sequestration. The primary DnaA titration locus, *datA* is located 450,000 bp rightward of *oriC*, and, based on the fixed rate of replication fork movement, becomes duplicated approximately 8 min after initiation, producing a cell cycle-specific titration activity during the sequestration period. However, it is not clear if this location is critical, since moving *datA* further away from *oriC* has little effect until relocation places it near the terminus region (129)

Regulating the availability of DnaA-ATP during the cell cycle: a proper balance of synthesis, inactivation, and recharging—Although newly synthesized as DnaA-ATP (218), in exponentially growing *E. coli*, DnaA-ADP predominates, making up approximately 70% of total DnaA (140). In synchronized cells, the relative levels of DnaA-ATP and DnaA-ADP fluctuate. Near the time of initiation of DNA synthesis, DnaA-ATP comprises 80% of total DnaA. After initiation, there is a rapid decrease in DnaA-ATP levels (to approximately 30% of total DnaA), and then, after sequestration ends and approximately half of the chromosome is replicated, the DnaA-ATP levels begin to rise again as cells approach the time to start the next round of DNA replication (140).

Regulating this dramatic cell cycle-specific fluctuation in DnaA-ATP levels over a wide variety of growth rates demands careful orchestration among different regulatory pathways. As described above, decreased DnaA activity immediately following initiation can be explained by the combination of RIDA and sequestration of the *dnaA* promoter. After sequestration is completed, new DnaA-ATP synthesis will conflict with RIDA in establishing DnaA-ATP levels, and this conflict will continue throughout the cell cycle, since there are always active replication forks in rapidly growing cells (Figure 1). However, for a cell to begin the next new round of DNA synthesis, at some cell cycle time the production of DnaA-ATP must outpace inactivation by Hda so that DnaA-ATP levels can reach the threshold needed to trigger the initiation event. Resumption of DnaA-ATP synthesis alone, at least initially, does not appear to be able to raise DnaA-ATP levels in the face of ongoing RIDA, since there is a gap in time between the end of sequestration and the rise in DnaA-ATP levels (140). It has been proposed that re-activation of DnaA-ADP is required to supplement the DnaA-ATP levels during the “gap” (76, 259) using the two independent DnaA recharging mechanisms described in a previous section. It remains to be determined whether or not the recharging mechanisms operate over specific fractions of time during the cell cycle. For example, the phospholipid-based exchange mechanism could be restricted to a particular time since phospholipid synthesis and assembly of membrane domains is cell cycle-specific (171, 198). Sequestration may also provide a specific time period in which DnaA-ADP bound to strong sites in hemimethylated *oriC* associates with the membrane (191) and helps mediate phospholipid-stimulated nucleotide exchange.

The two DARS loci are mapped approximately equidistant from *oriC*, located on opposite sides of the replicore (76). Both are replicated approximately three quarters of the way through the C period, and since their duplication is later than the time that DnaA-ATP levels begin to rise, it seems improbable that the increase in DARS copy number plays a significant timing role. DARS could, however, have a role in cell cycle-specific recharging if the sites were supplied with DnaA-ADP substrate at a specific time during the cell cycle. *DatA* is an obvious "sink" for DnaA-ATP, with the ability to produce a cell cycle-specific increase in DnaA-ADP available to DARS as replication forks move through the titration locus and Hda hydrolyses the bound DnaA-ATP. It should be noted, however, that RIDA appears to be functional in *datA* mutant strains (120), suggesting that other chromosomal recognition sites also contribute to DnaA-ATP inactivation and supply of DnaA-ADP for DARS-mediated recharging.

It also remains possible that DnaA-ADP is selectively removed from the pool of available free DnaA causing an increase in DnaA-ATP relative to DnaA-ADP. The membrane is the most likely destination for DnaA-ADP, since much of the DnaA in the cells has been shown to be localized near the surface (27, 179), and in cell lysates, approximately half of the cellular DnaA is found in an insoluble fraction associated with phospholipids (221). However, it is not yet known if any particular nucleotide form of DnaA predominates in the membrane-bound fraction.

OTHER BACTERIAL ORIGINS

The conservation of DnaA suggests that efforts to dissect the mechanisms that control initiation of replication in *E. coli* will assist our understanding of the process in other eubacteria. However, there are surprisingly large differences in the nucleotide sequences that constitute eubacterial replication origins, with a wide range of numbers and placements of DnaA recognition sites (81). These differences suggest that eubacterial ORC and pre-RC are likely to be assembled and regulated by mechanisms that are most appropriate for the life-style of the bacteria. For example, large numbers of DnaA recognition sites within *oriC* may be an advantage for slow growing bacteria, for examples see (269), that initiate exclusively from one copy of *oriC* and have extended periods of the cell cycle devoid of ongoing DNA replication. Adding *oriC* recognition sites to titrate DnaA may enhance the sensitivity of the initiation timing mechanism during slow growth, particularly if extremely low rates of DnaA synthesis are difficult to achieve. In the case of enteric bacteria like *E. coli* and Salmonella, relatively low numbers of DnaA recognition sites within *oriC* may allow for additional modulators to extend the dynamic range of initiator complex assembly to accommodate both fast (multi-fork replication) as well as slow growth.

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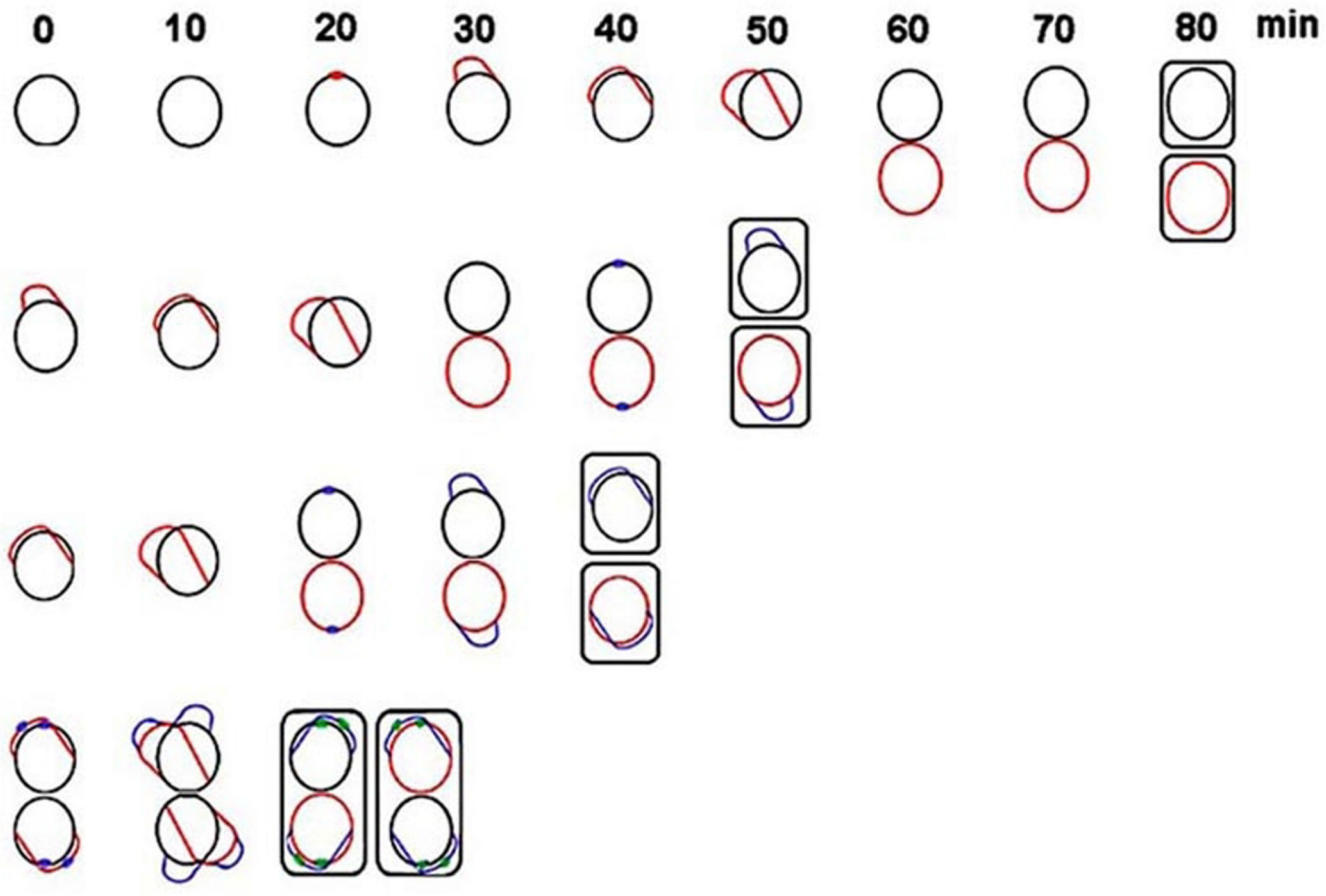

Figure 1. *E. coli* chromosome configurations and initiation time at different growth rates

Chromosome configurations for four different growth rates (20, 40, 50 and 80 minutes) are shown. Cell cycle time is indicated by the time line at the top of the figure. For all growth rates, the C period is 40 minutes, and the D period is 20 minutes. In cells growing with doubling times less than 40 minutes, a new round of chromosome replication is initiated prior to completion of the previous round, resulting in multi-fork replication (see 30 and 20 minute doubling times; bottom 2 panels) . Faster growth rates produce larger cells with increased numbers of origins and increased DNA content per cell. Cells growing slower than C + D minutes (e.g. 60 minutes) have gaps in DNA synthesis (see 80 minute doubling time, top panel). The time of initiation is independent of cell cycle time, and cells initiate chromosome replication when they accumulate sufficient mass per origin. Initiating origins are marked by colored ovals. For all panels, the oldest (parental) chromosome is drawn in black, the next oldest is drawn in red, the next oldest in blue, and the youngest is green.

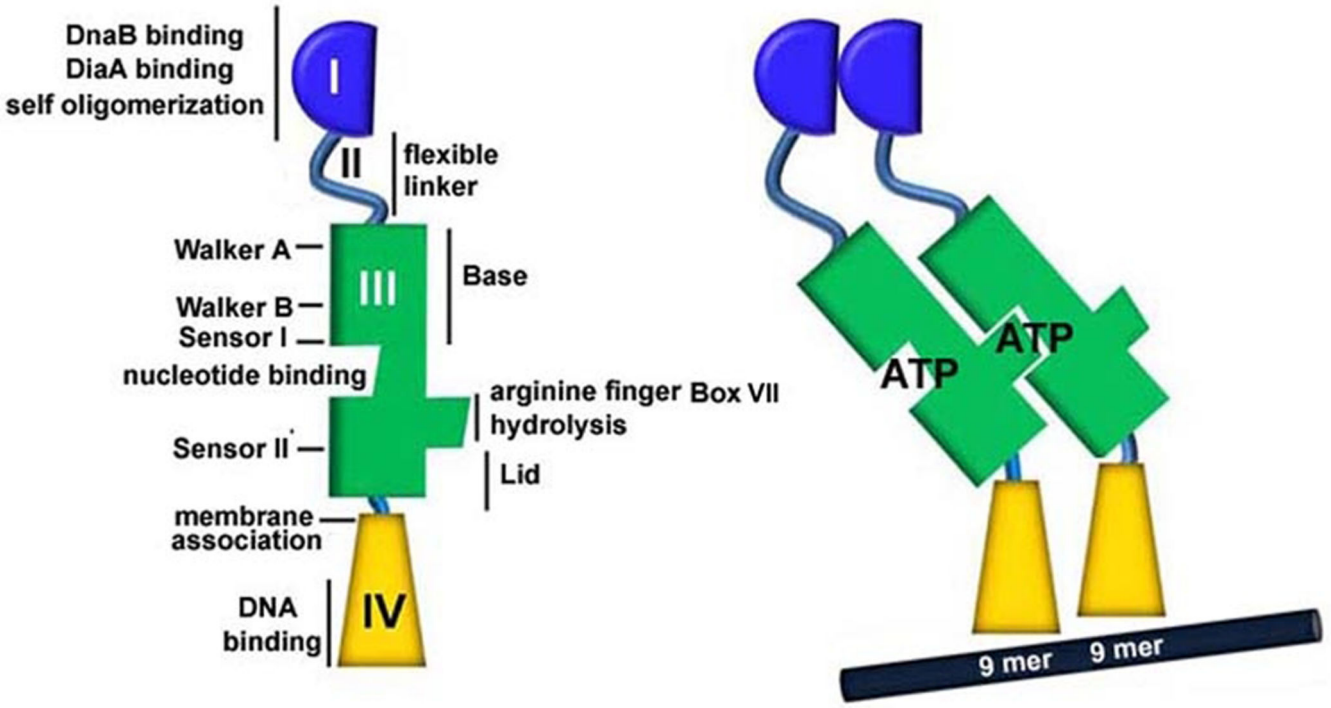

Figure 2. Structural features of the initiator protein DnaA

Left panel) A cartoon of DnaA is shown. Domain I is bright blue, Domain II is light blue, Domain III is green, and Domain IV is yellow. Locations of key structural features, described in the text, are labeled. **Right panel)** Model of the binding of a DnaA dimer to two adjacent 9 mer recognition sites in DNA (shown by black line). The binding of ATP to DnaA causes a structural change in Domain III, such that the arginine finger of Box VII interacts with the ATP bound to the adjacent DnaA molecule. Interaction between the two N-terminal domains is also shown.

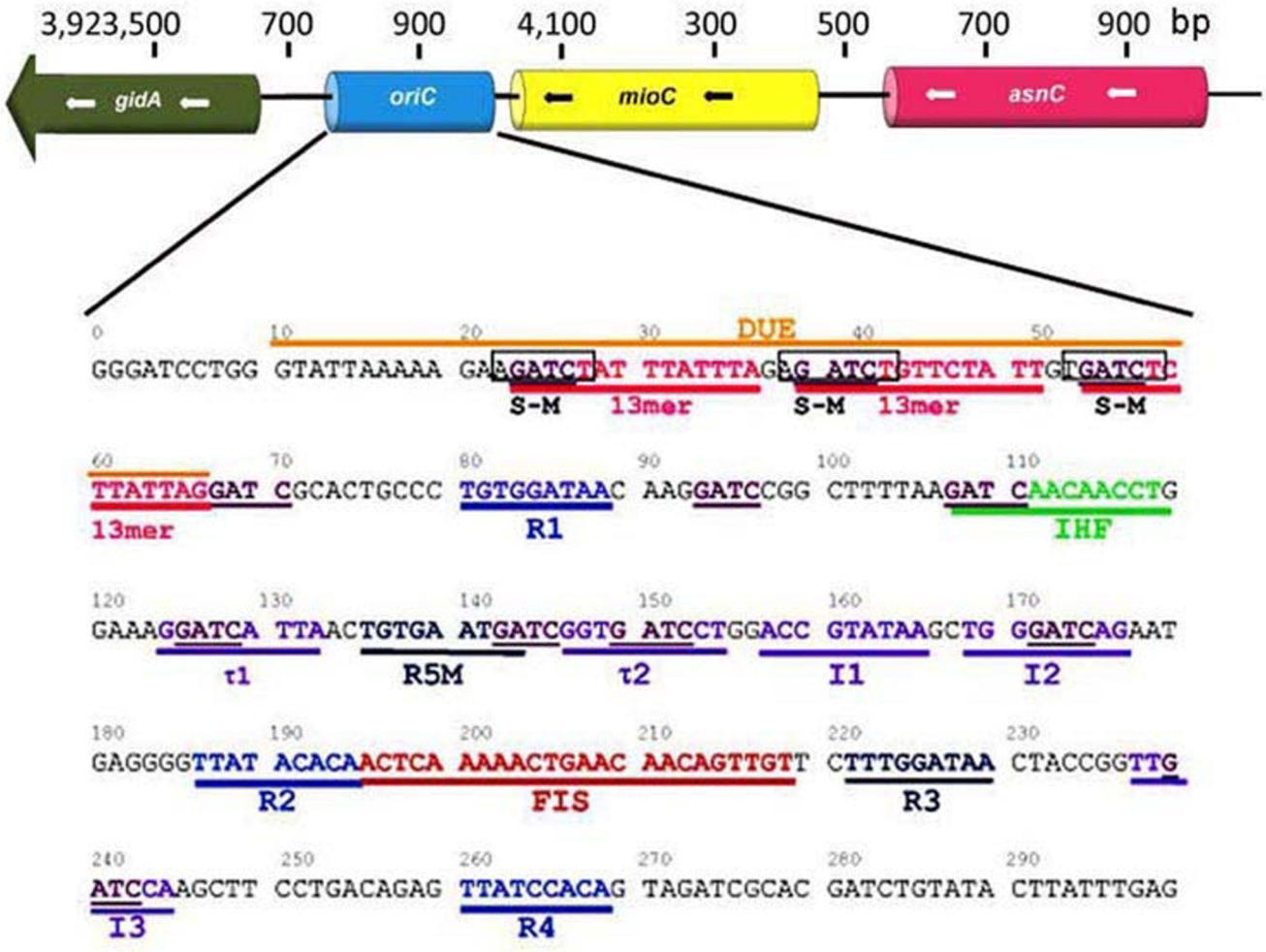

Figure 3. *OriC* geography and nucleotide sequence

Top panel) The unique origin of replication, *oriC*, is located between the genes for *gidA* and *mioC* on the *E. coli* chromosome. The top numbers indicate the nucleotide position in the genomic sequence. The arrows indicate the direction of transcription. **Bottom panel)** At the left side of *oriC*, a gold overline indicates the location of the Duplex Unwinding Element (DUE), the AT-rich 13 mer repeats are highlighted by pink, and the ATP-DnaA Speck-Messer (S-M) sites are outlined by black boxes. The 11 GATC sequences highlighted in dark purple are the sites for SeqA binding and DAM methylation. High affinity DnaA binding sites (R1, R2, and R4) are indicated by bright blue, while low affinity non-discriminatory R boxes are drawn in dark blue. The tau sites and I sites, highlighted in violet, preferentially bind DnaA-ATP. The binding sites for the DNA bending proteins IHF and Fis are highlighted by green and blue, respectively.

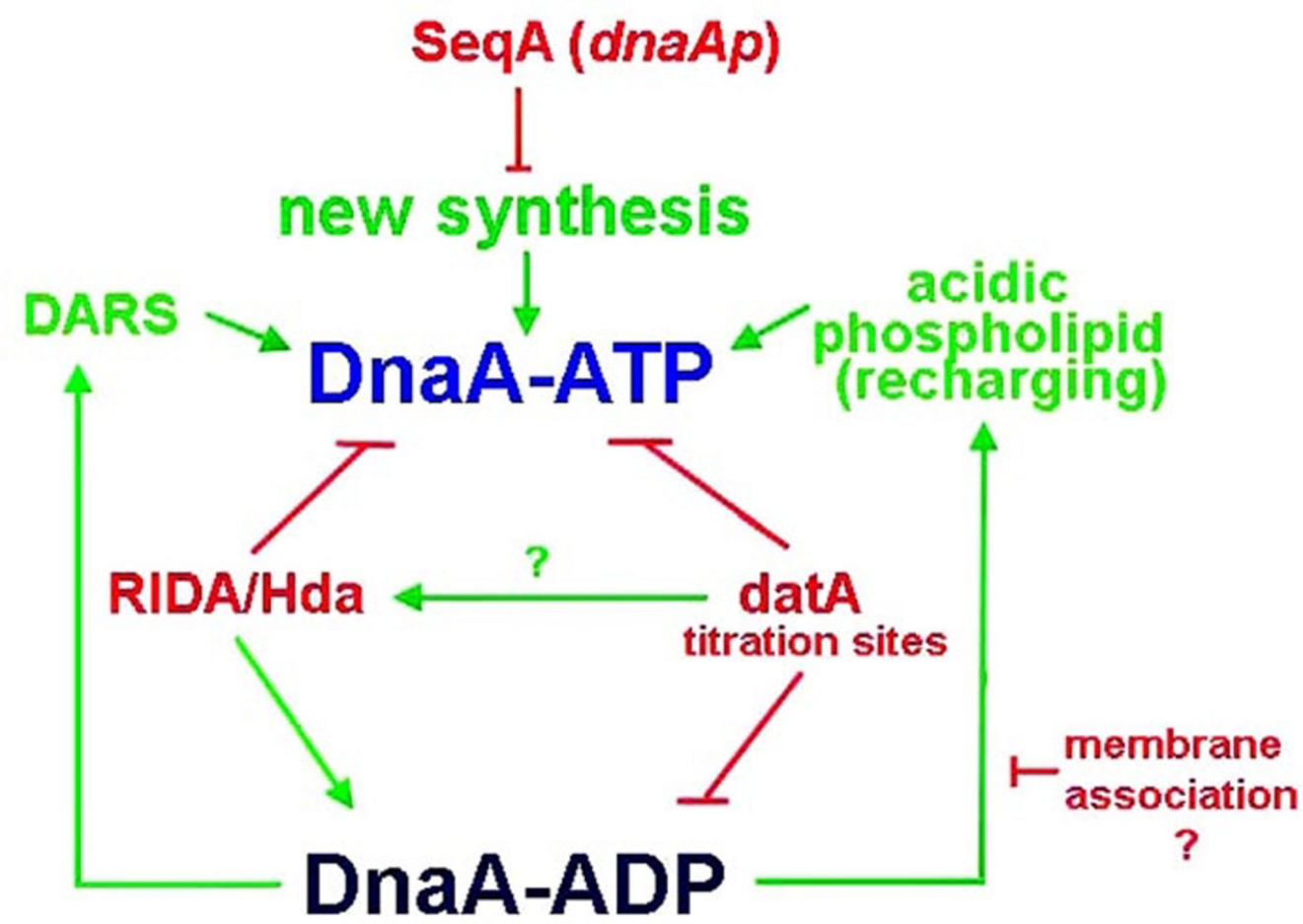

Figure 4. Regulators of orisome assembly

A) Factors or processes that affect DnaA-ATP availability include those that increase DnaA-ATP levels (shown in green): new transcription, the DARS regions, and acidic phospholipids. Factors or processes that decrease availability of DnaA-ATP (shown in red) include sequestration of the *dnaA* promoter region to prevent new synthesis, RIDA/Hda mediated hydrolysis of DnaA-ATP to DnaA-ADP, titration of DnaA to the *datA* locus and other chromosomal titration sites, and long-term association with the membrane, removing DnaA from the pool of protein in the cell. **B)** Regulators of complex assembly that act by inhibiting DnaA from binding to low affinity sites (Fis and SeqA), or by directly preventing unwinding by interacting with DnaA (Dps) or the 13 mer region (IciA) are indicated by labeled octagons and shades of red. Regulators that stimulated orisome formation by facilitating DnaA binding to low affinity sites (IHF, DiaA) or stimulating unwinding (HU, transcription from the *gidA* promoter) are indicated by labeled circles and shades of green. High affinity DnaA boxes are indicated by bright blue boxes, low affinity non-discriminatory R boxes are drawn as dark blue boxes, and tau and I sites, which have a preference for DnaA-ATP are drawn as purple boxes.

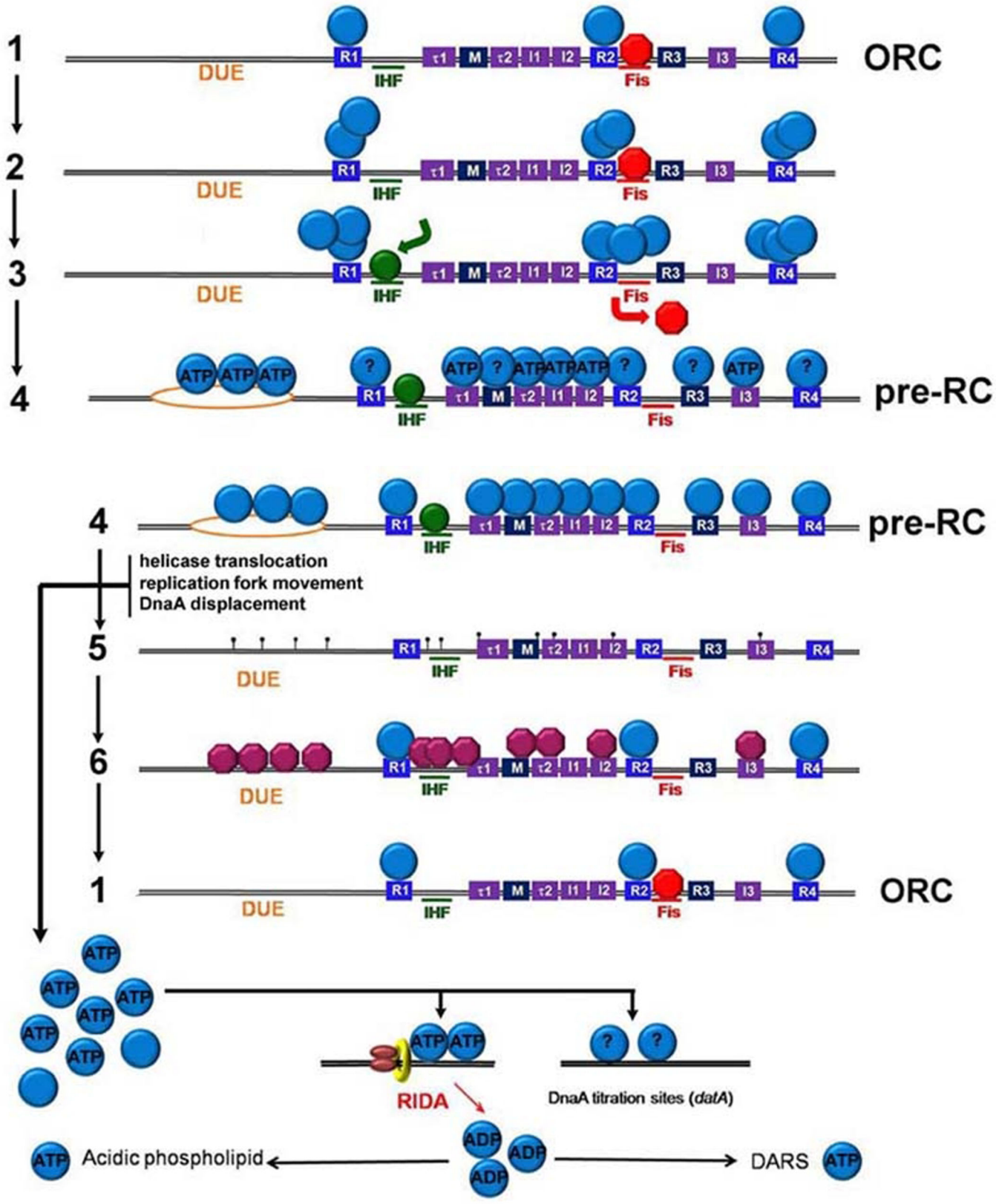

Figure 5. Model of staged orisome assembly and disassembly

A) The orisome is assembled in stages. In stage 1, DnaA (blue circles) is bound to R1, R2, and R4, and Fis (red octagon) is bound to its cognate site, forming the bacterial ORC. DnaA accumulates at the higher affinity sites (Stage 2), until enough DnaA is present to displace Fis and allow binding of IHF (green circle) to its site between R1 and tau1 (Stage 3). Binding of IHF stimulates DnaA binding to all the lower affinity sites, with the tau sites and I sites binding DnaA-ATP. It is not known what form of DnaA binds to the other sites, and this ambiguity is indicated by a question mark. Full occupation of *oriC* by DnaA results in DNA strand separation in the DUE (gold) and binding of DnaA-ATP to the single stranded region, forming the bacterial pre-RC (Stage 4). Attributes of the DnaA binding sites are indicated by colors, as described in the legend to Figure 4B. **B)** Disassembly of the orisome, and resetting of *oriC* to the ORC is also staged. DnaA is removed from the pre-RC (stage 4) by helicase translocation and replication fork movement. Replication of oriC causes hemimethylation of the 11 GATC residues (small black circles) (Stage 5), creating binding sites for SeqA (purple octagon) (Stage 6). The displaced DnaA rebinds the high affinity sites (Stage 6), but is blocked from rebinding to the weaker sites. The end of sequestration results in resetting of the ORC (Stage 1). The excess DnaA displaced from the pre-RC binds other chromosomal sites, including the *datA* locus, and is inactivated by RIDA/Hda. Hda is shown by dark red ovals and the β-clamp is shown by a yellow circle. DnaA-ADP can be recharged to DnaA-ATP by DARS or by acidic phospholipids.