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Mechanisms for Initiating Cellular DNA Replication

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Abstract

The initiation of DNA replication represents a committing step to cell proliferation. Appropriate replication onset depends on multiprotein complexes that help properly distinguish origin regions, generate nascent replication bubbles, and promote replisome formation. This review describes initiation systems employed by bacteria, archaea, and eukaryotes, with a focus on comparing and contrasting molecular mechanisms among organisms. Although commonalities can be found in the functional domains and strategies used to carry out and regulate initiation, many key participants have markedly different activities and appear to have evolved convergently. Despite significant advances in the field, major questions still persist in understanding how initiation programs are executed at the molecular level.

Keywords

DNA replication; AAA+ ATPases; initiators; helicases

INTRODUCTION

The initiation of DNA replication is a central event in the growth and division of all organisms. The assembly of replication machineries, or replisomes, is coordinated by dedicated initiation proteins, which ensure that DNA synthesis begins at the correct chromosomal locus in accordance with cell cycle cues. Faithful execution of initiation is crucial to viability; inappropriate replication onset is linked to changes in gene copy number, DNA damage, and genetic instability (1, 2).

Cellular initiation programs rely on proteinaceous, *trans*-acting factors that process chromosomal start sites (termed origins). The mechanics and control of these proteins are complex. Some factors and their activities are conserved across all three domains of life

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(bacteria, archaea, and eukaryotes); for example, all proteins responsible for recognizing origins (initiators) possess a set of evolutionarily related ATPase domains fused to a helix-turn-helix (HTH)-type DNA binding element (3-7). However, other aspects of initiation—such as DNA-unwinding and strand-synthesis enzymes (e.g., helicases and primases)—are distinct between bacterial and archaeal/eukaryal lineages (3, 8, 9). Hence, although common themes exist in how initiation is executed and controlled, the specific pathways and players that promote this process can vary dramatically.

The goal of this review is to highlight the prevalent mechanisms used to initiate DNA replication in cells. Given that a PubMed search for “DNA replication initiation” turns up >8,000 papers, it is impossible to be comprehensive. We apologize to those colleagues whose work could not be accommodated.

REPLICATION ORIGINS

Bacterial *oriC*

Bacterial chromosomes, which are typically (though not always) circular, generally bear a single origin distinguished by two classes of sequence elements: conserved repeats that are important for initiator recognition and an AT-rich DNA-unwinding element (DUE) where the replication bubble originates (10-12). The ~260 base-pair (bp) origin of *Escherichia coli*, *oriC*, has been a long-standing model system for studying initiation. *E. coli oriC* bears a complex array of conserved sequence motifs. Most of these are recognized by the ATP-dependent initiator, DnaA (13-15), whereas others are recognized by factors that modulate initiator/origin interactions (see Figure 1*a*). One prevalent motif is the DnaA box (sites R1–R5) (15, 16). DnaA boxes are recognized equivalently by ATP-bound and ADP-bound DnaA (ATP-DnaA and ADP-DnaA, respectively), with regions R1, R2, and R4 bound most tightly (17-20). I-sites and τ-sites constitute two other classes of DnaA binding sites that associate more weakly with the initiator and are preferentially recognized by ATP-DnaA (13, 18, 21). The DnaA boxes, I-sites, and τ-sites in *oriC* together form an organizing center that is critical for promoting the higher-order assembly of DnaA and origin activation (discussed below). Interestingly, the spacing between high- and low-affinity sites is important, as even two-base insertions can abrogate origin function (22).

The DUE contains several copies of a fourth type of DnaA binding element termed an ATP-DnaA box. These sites reside within three 13-bp AT-rich repeats historically designated as L, M, and R (10, 12). Similar to I-sites and τ-sites, ATP-DnaA boxes are bound preferentially by ATP-DnaA (14, 23). ATP-DnaA box recognition requires the cooperative assembly of multiple ATP-DnaA protomers and depends on initiator binding to the adjacent R1 element (23). As with the organizing center, the spacing between the DUE and other DnaA binding sites is critical for *oriC* function (24).

The direct, sequence-based readout of specific origin binding sites by DnaA appears preserved across bacteria (25-28). However, the replication origins of bacterial species display a unique number and arrangement of the consensus DnaA box sequence and its variants (Figure 1*a*) (29). DnaA orthologs also appear to differ in their relative affinity for DnaA box sequences (30). For example, *Mycobacterium tuberculosis* DnaA does not stably

associate with a single DnaA box; instead, binding requires that multiple repeats be present in target substrates (31). This variation, coupled with the distinct arrangement of DnaA boxes among different *oriC*s, suggests that DnaA orthologs are fine-tuned to act only on their cognate origin. Consistent with this notion, *Escherichia coli* DnaA cannot initiate replication on *Bacillus subtilis oriC* or vice versa (32). Why origins are so diverse among bacteria is unclear but could reflect a speciation mechanism for preventing the replication of foreign origins acquired through phage infection or DNA uptake.

Eukaryotic Origins

Eukaryotic origins exhibit several differences from their bacterial counterparts. First, eukaryotic chromosomes invariably contain multiple origins. Second, eukaryotic origins frequently appear to be defined more by local DNA structure and chromatin environment than by sequence conservation (33). An exception is budding yeast, whose genome is punctuated by related DNA segments called autonomous replicating sequences (ARSs) (34). ARSs serve as recruitment sites for the origin recognition complex (ORC), a multiprotein assembly that functions as the initiator of replication in eukaryotes (35, 36).

The binding of ORC protects specific regions within certain ARSs known as ARS consensus sequences (ACSs). ACSs typically contain two conserved regions bound by ORC termed A and B1 (Figure 1*b*) (35-37). Interestingly, although ACSs can define a replication start site, they are not always sufficient to do so (38, 39). Initiation in *Schizosaccharomyces pombe* echoes this paradigm: Fission yeast origins frequently contain an AT-rich segment recognized by the Orc4 subunit of ORC (40, 41), but removal of these AT stretches affects origin activation only marginally in vivo (42, 43).

Metazoan replication start sites are more loosely encoded than those of yeast but exhibit some general similarities. For example, metazoan chromosomal regions occupied by ORC often (although not always) correlate with certain skewed patterns of DNA sequence bearing either AT- or GC-rich stretches (44-46). Nonreplicative proteins, such as transcription factors (47), can associate with ORC to help define particular origin loci. However, despite these generalities, a growing body of evidence points to contextual cues—e.g., proximity to promoter elements, DNA accessibility, nucleosome occupancy, and local structure—as major factors in specifying metazoan replication start sites (48-51). Indeed, in *Xenopus* egg extracts, replication can initiate at any nucleotide sequence in an exogenously introduced piece of DNA (52). Overall, the molecular logic that eukaryotic cells use to decide which region serves as a replication start site has yet to be fully defined (see Supplemental Material for more detail; follow the Supplemental Material link from the Annual Reviews home page at http://www.annualreviews.org).

Archaeal Origins

Archaeal origins exhibit a mix of bacterial and eukaryotic features (53). As in eukaryotes, archaeal chromosomes frequently bear more than one origin (54-56). However, archaeal origins, similar to their bacterial counterparts, also contain conserved elements that function as sequence-specific binding sites for initiators (54).

Sulfolobus solfataricus, whose genome bears three loci termed *oriC1*, *oriC2*, and *oriC3*, exemplifies many of the attributes of archaeal origins (Figure 1*c*) (54). *oriC1* contains three conserved sequence motifs: the origin recognition box (ORB) and the C2 and C3 loci. *oriC2* contains similar elements, although its ORB repeats are smaller. Each type of site is bound by one of three distinct initiator paralogs (the Orc1-1, Orc1-2, and Orc1-3 proteins, respectively). Binding sites for archaeal Orc1 paralogs can overlap partially or fully (Figure 1*c*) (54), suggesting that cooperative or competitive interactions may occur among different initiator subfamilies.

As with different bacterial origins, the number, type, and order of Orc1 binding sites are not preserved among the three *Sulfolobus* origins. Nonetheless, the ORB-type sites within *oriC1* and *oriC2* are both arranged as inverted repeats separated by an AT-rich region of varying length that contains a DUE (54, 55). Surprisingly, *oriC3* has no discernable Orc1 binding site and is instead protected by a winged-helix initiator protein (WhiP) that is unrelated to Orc1 and does not appear to bind ATP (55). These features suggest that origin multiplication in *Sulfolobus* likely occurred by the acquisition of extrachromosomal elements rather than by gene duplication (55).

Additional aspects of *Sulfolobus* replication origins are echoed in other archaeal species. One is origin multiplicity (55, 57). Others are GC sequence skew (58) and the existence of DUEs flanked by inverted ORB repeats (for review, see Reference 59). Whether contextual cues contribute to archaeal origin discrimination is still unclear.

INITIATORS AND ORIGIN PROCESSING/PRIMING/REMODELING

Bacterial DnaA

The DnaA initiator not only recognizes bacterial replication origins but also melts DNA to promote replication onset (12, 15). DnaA is modular, composed of four distinct domains (Figure 2*a*) (60, 61). The first (domain I) is a small, globular K-homology (KH)-type fold (62-64) that both homodimerizes and associates with many diverse regulatory proteins (for review, see References 65, 66). The second (domain II) is a variable and likely flexible linker element of unclear function (65). Domain III consists of an AAA+ (ATPases associated with various cellular activities) fold (3, 5, 6, 67, 68) that can form large homo-oligomeric arrays (61, 68-71), bind single-stranded (ss)DNA (Figure 2*b*) (72, 73), and promote *oriC* melting (71, 73, 74). The fourth region (domain IV) comprises a C-terminal HTH element (5, 75) that recognizes specific sites within the *oriC* organizing center (26, 28, 76). Domain III is the only functional fold conserved with eukaryotic and archaeal initiators (3, 5), indicating that this region was extant in an ancient initiator protein before bacteria and archaea split into different lineages.

Prior to replication onset, strong DnaA boxes in *oriC* are bound by domain IV of ATP-DnaA or ADP-DnaA (77, 78). As initiation commences, additional copies of ATP-DnaA localize to *oriC* (78), filling weaker DnaA binding sites and coassociating into a large nucleoprotein complex (13, 22, 69, 79, 80). Domain III is the primary mediator of oligomerization (7, 21, 71, 72), although domains I and IV also participate in assembly (71, 74, 81). DnaA forms a helical oligomer that reconstitutes a hydrolysis-competent ATPase

site through the joint action of several signature amino acid motifs (7, 70), including the Walker A, Walker B, Sensor I, and Sensor II elements from one DnaA subunit and a *trans*-acting arginine finger from a partner protomer (reviewed in Reference 65). Upon assembly, the DnaA helix appears to wrap duplex DNA around itself, stabilizing one or more positive DNA supercoils (Figure 2*c*) (7, 82).

The complex formed between DnaA and the organizing center of *oriC* serves as an essential prerequisite to DUE melting (10, 12, 23). Negative supercoiling is required for melting (12), suggesting that DNA opening might be aided by torsional strain (7). However, ATP-DnaA also associates directly with the DUE (23, 73) and can actively melt short DNA duplexes in an ATP-dependent manner by binding and stretching ssDNA along the helical axis of the DnaA oligomer (Figure 2*b*) (72). Although the precise mechanism is not fully understood, the available evidence indicates that either concomitant with or following DnaA self-assembly, the ATPase domains of the initiator deform and open the DUE directly (72). At least two models account for how DnaA organizes both ssDNA and double-stranded (ds)DNA to promote melting (Figure 2*c*,*d*), although the precise architecture of the nucleoprotein complex is not firmly established.

Eukaryotic Origin Recognition Complex

The eukaryotic initiator, ORC, was first discovered from biochemical studies using budding yeast (35, 36). Unlike the single-subunit DnaA protein, ORC comprises six protomers that transiently associate with a seventh factor known as Cdc6 (Cdc18 in fission yeast) (36, 83-85). Together, ORC and Cdc6 identify and mark chromosomal origins (35, 36) and promote the loading of the replicative eukaryotic helicase onto DNA (86-88).

As with DnaA, ORC is composed of many functional elements (Figure 3*a*). Orc1, Orc4, and Orc5, along with Cdc6, contain AAA+ ATPase domains closely related to that found in the bacterial initiator (3). Orc2 and Orc3 also appear predicated on an AAA+ scaffold, albeit a highly diverged one (84, 89), whereas Orc6 (at least in metazoans) is structurally related to the transcription factor TFIIB (90-92). Many of the AAA+ domains within ORC and Cdc6 are linked to other modules, in particular a predicted variant of an HTH fold termed a winged-helix domain (WHD), which may be involved in DNA binding (4, 84, 93). The N terminus of *S. pombe* Orc4 is further decorated with several AT-hook repeats that promote interactions between the initiator complex and origin DNA (40, 41, 94), whereas the N terminus of Orc1 universally bears a conserved bromo-adjacent homology (BAH) domain implicated in binding multiple factors (50, 95, 96), including transcriptional silencing factors such as Sir1 (in yeast) and HP1 (in human and *Drosophila*) (97-99). The Orc1 BAH domain binds to nucleosomes, specifically recognizing dimethylated histone H4 tails (100). BAH-dependent association of ORC with nucleosomes assists with sequence-independent recognition of replication origins (50, 96) and may have a role in the demonstrated ability of ORC to reposition nucleosomes (44, 51, 101). Contacts between the Orc1 BAH domain and histones are important physiologically, as their ablation has been linked to primordial dwarfism (100, 102).

As expected from the high degree of interspecies sequence conservation among Orc proteins, 3D electron microscopy (EM) reconstructions have revealed similar (although not

identical) architectures for different Orc homologs. For example, *Drosophila* and *Saccharomyces cerevisiae* ORC form crescent-shaped particles (Figure 3*b*, *c*) (84, 89), both of which can accommodate five AAA+ initiator protomers (89, 103). The first five protomers of ORC appear to form the core of the crescent, associating in the order Orc1→Orc4→Orc5→Orc2→Orc3 (103-106). The location of Orc6 is less certain but likely maps near Orc2 or Orc3 (103, 105-107). Cdc6 binds directly to Orc1 and, in the yeast ORC model, contacts the open end of the crescent, converting ORC into a closed-ring-like particle (Figure 3*c*) (84).

Akin to DnaA, ORC relies on ATP to control several aspects of its activity. Some ATP-dependent responses observed in different ORC orthologs are not universal; for example, ATP promotes the ordered assembly of human ORC subunits (104, 105), whereas ORC from budding yeast and *Drosophila* can be purified as hexameric complexes even when nucleotide is not added (36, 108). Nonetheless, ATP frequently appears to control how ORC interacts with specific types of DNA substrates and certain classes of binding sites (109, 110). In *S. cerevisiae*, ATP also promotes the association of ORC with Cdc6 (111, 112), extending the footprint of the initiator on origins such as ARS1 (84, 103, 113). The ability of ATP to elicit specific structural and functional responses likely involves the pairwise coordination of AAA+ ATPase domains from adjoining subunits. Consistent with this idea, Orc4 of budding yeast bears a conserved arginine that maps to a secondary structure position typically occupied by AAA+ arginine fingers and that is critical for stimulating the ATPase activity of Orc1 in *trans* (111, 114, 115).

The action of ORC on DNA is not fully established. Although ORC binds readily to DNA (36) and is capable of altering local DNA structure and writhe (59), research has yet to show that the eukaryotic initiator can actively melt duplexes. Why ORC might have lost an ancestral ability to unwind replication origins (or why DnaA gained such a function) is unclear. The AAA+ domains of cellular initiators form a distinct subgroup (3) and possess a distinguishing α-helical appendage termed an initiator specific motif (ISM) (116). However, the specific architecture of the DnaA ISM differs from that of archaeal and eukaryotic initiators, as it forms a wedge-shaped structure that pushes adjoining subunits out of plane and into a helical oligomer (7). Differences in ISM configuration may cause adjoining subunits in ORC to adopt a less extreme helical organization, which in DnaA is necessary to support DNA stretching and melting (72, 74). Additional studies are needed to clarify how evolutionary divergence of the AAA+ scaffolds used by ORC and DnaA has led to distinct functions on DNA.

Archaeal Orc1

Archaea lack a DnaA ortholog, instead containing proteins more closely related to eukaryotic Orc1 and Cdc6. Archaeal Orc1 is the most streamlined of the cellular initiators, consisting only of an N-terminal AAA+ ATPase domain and a C-terminal WHD (4, 5, 116-118). Archaeal Orc1 proteins bind to origins (54, 119, 120), and the WHD contributes to both sequence-specific origin recognition and DNA affinity (121-123). Interestingly, the AAA+ domains of archaeal Orc1 proteins also bind to DNA, using their ISM to contact the phospho-diester backbone of target duplexes through either the major or the minor groove

(116, 118). Although origin recognition relies on very few base-specific contacts (only one in *Sulfolobus* Orc1-1), the joint action of the Orc1 WHD and AAA+ domain creates an extended footprint that bends and underwinds the target DNA substrates (28, 116, 118). Biochemical data suggest that, as with eukaryotic replication start sites, local DNA structure is an important determinant for origin discrimination by the archaeal initiator (122).

Although many archaeal Orc1 proteins both possess hallmark AAA+ ATPase motifs and hydrolyze ATP (3, 4, 117, 124), the role of nucleotide in controlling initiator function is poorly understood. As with the HTH domain of DnaA (5, 7), the Orc1 WHD can adopt different orientations with respect to its adjoining AAA+ core in a manner influenced by both nucleotide and substrate DNA (Figure 3*d*) (4, 116-118). However, ATP has not been observed to foster the formation of higher-order Orc1 assemblies seen with DnaA. Moreover, although the binding of archaeal Orc1 to origin regions can alter the superhelical state of the DNA and promote melting in vitro, this effect is not nucleotide dependent and does not always result in duplex opening within the DUE (59, 120, 125). How Orc1 initiators respond to ATP binding/hydrolysis remains to be determined.

HELICASE LOADING AND ACTIVATION

Helicase Loading in Bacteria

The marking and processing of origins by initiator proteins facilitate the next phase of initiation, namely, the loading of replicative helicases onto DNA (reviewed in Reference 126). In bacteria, a single helicase—termed DnaB in *E. coli*—serves as the front end of the newly emerging replication fork. DnaB forms a twin-tiered, ring-shaped hexamer (Figure 4*a*) (127-129), in which a conserved N-terminal domain (NTD) structurally homologous to the helicase interaction domain of the DnaG primase forms one tier and the C-terminal ATPase domain (CTD) forms the other (128, 130-132). Adjoining NTDs in the DnaB hexamer self-assemble into a trimer-of-dimers configuration (127-129, 133), creating a collar that binds ssDNA (127). The CTD tier also interacts with nucleic acid substrates but serves as the site for DNA translocation (134-137). During replication, DnaB encircles the lagging template DNA strand (138), moving 5′→3′ with the ATPase domains oriented toward the duplex side of the fork (138-141). DNA unwinding likely occurs by a steric mechanism that excludes the leading template strand from the helicase interior during translocation (139, 142).

E. coli DnaB is loaded onto the single-stranded DUE regions of *oriC* by the concerted action of both DnaA and a dedicated loading factor, DnaC (64, 143-146). DnaC is an AAA+ ATPase and paralog of DnaA (67, 147), but it lacks the C-terminal, duplex-DNA binding domain of the initiator. DnaC, like DnaA, bears a DnaB binding domain at its N terminus (148, 149); however, the folds of these regions are unrelated between the two protein families (62, 150, 151). DnaC also binds ssDNA in a cooperative and ATP-dependent manner (147, 152) by using its AAA+ domains to form a right-handed helical oligomer (Figure 4*b*) that exhibits a bipartite ATPase site similar to that of DnaA (147). When bound to ATP or poorly hydrolyzable ATP analogs, DnaC represses DnaB activity (146, 153, 154). Although ATP binding is not required for DnaC to load DnaB onto a ssDNA circle, productive association with nucleotide is necessary for DnaC to support the DnaA-

dependent initiation of DNA replication on an *oriC* substrate in vivo and in vitro (155). Overall, the role of nucleotide in DnaC function remains enigmatic.

How DnaB is placed onto a melted origin is a central question in the field. DnaC forms a 6:6 complex with DnaB (156, 157) and has been proposed to crack open the helicase ring to allow ssDNA to engage the motor interior (126, 158, 159). Recruitment of DnaB to *oriC* is mediated, at least in part, by an interaction between domain I of DnaA and the helicase NTDs (63, 143). Coprecipitation studies using *Aquifex aeolicus* DnaC have further shown that the loader, whose N terminus binds to the C-terminal face of DnaB (149, 157), can use its ATPase domain to bind the AAA+ domain of DnaA in a nucleotide-dependent manner (147). Given that biochemical studies have shown that DnaA preferentially loads DnaB onto the bottom strand of the DUE in vitro (160) and that two DnaB hexamers are loaded onto opposite strands and in opposing orientations from one another at *oriC* (145), DnaA and DnaC may collaborate in orienting DnaB hexamers on the complementary strands of a melted DUE (Figure 4*c*). Recent studies have shown that the DnaG primase can bind to the DnaB · DnaC complex, stimulating ATP hydrolysis by the loader and causing DnaC to release the helicase (155).

Replicative helicase loading in other bacterial species exhibits both similarities to and differences from that of *E. coli* (126). For example, *B. subtilis* bears a DnaB helicase (named DnaC) as well as an AAA+-family loading protein (DnaI) that is an ortholog of *E. coli* DnaC. *B. subtilis* also utilizes two other proteins, DnaB (unrelated to *E. coli* DnaB) and DnaD, to aid helicase recruitment and deposition (161). During initiation, *B. subtilis* DnaB and DnaD, together with their cognate initiator DnaA, associate with *oriC* in the order DnaA→D→B (162, 163). DnaB and DnaD then interact with DNA, the DnaC helicase, and the DnaI · DnaC complex to facilitate helicase loading (161). As in *E. coli*, the DnaI loader can both bind ssDNA in an ATP-dependent manner and form a stable 6:6 complex with a replicative helicase target (148); however, data have also suggested that the *B. subtilis* helicase is assembled on the origin rather than loaded as a preformed hexamer (161).

The apparent lack of DnaC/DnaI loader orthologs in many species [e.g., *Helicobacter pylori* and *M. tuberculosis* (164, 165)] has confounded efforts to establish a general framework for helicase loading in bacteria. This absence raises the question of whether certain bacteria rely on as yet unrecognized loading factors, or whether their helicases are self-loading. In this respect, *H. pylori* DnaB has been observed to form a distinctive double hexamer, which appears predicated on a sixfold symmetric configuration of the N-terminal domains that differs from the trimer-of-dimers state seen in other DnaB orthologs (166). Moreover, expression of *H. pylori* DnaB in *E. coli* can complement a *dnaC* temperature-sensitive mutation (167). These data suggest that dedicated helicase-loading proteins may not be required in all bacterial species; DnaA may serve as the sole factor responsible for mediating the recruitment of the hexameric motor to replication origins in these instances (65). Overall, much remains to be discovered concerning the mechanisms by which initiation factors collaborate to facilitate helicase loading and the extent to which these strategies differ between species.

Helicase Loading and Activation in Eukaryotes

The replicative helicase of eukaryotes comprises an assembly of six distinct but evolutionarily related minichromosome maintenance (MCM) subunits termed Mcm2–7 (168). Mcm2–7 is superficially similar to DnaB, as it forms a hexameric ring composed of two tiers (169, 170)—one corresponding to an N-terminal oligomerization collar and the other to a set of C-terminal motor domains (6, 171)—that encircles and translocates along DNA (Figure 5*a*) (172-175). However, Mcm2–7 fundamentally differs from its bacterial counterpart in several ways. First, the NTDs of Mcm2–7 and DnaB are structurally unrelated (171, 176, 177). Second, the C-terminal motor domain of MCMs is an AAA+ protein (6, 178) and hence is evolutionarily and organizationally distinct from RecA-type motors such as DnaB (179-181). Third, Mcm2–7 translocates 3′→5′ along the leading strand template of the replication fork (182, 183), opposite to DnaB (140). Finally, whereas DnaB-type helicases exhibit closed-ring architectures in the absence of DNA and appear to switch into a lock washer configuration during DNA unwinding (127-129, 184-187), Mcm2–7 appears capable of spontaneously forming open-ring states when free of substrate and converts into a closed-ring form when bound to ATP and specific activating factors (Figure 5*b*) (170, 188). These differences support the idea that certain replication fork components evolved independently at least twice (189).

Mcm2–7 subunits assemble in the order Mcm5→3→7→4→6→2(→5) (170, 190-192). Interactions between Mcm2 and Mcm5 are weak (190, 191) and appear to constitute a natural break point in the ring (170, 183). This discontinuity likely disrupts the processive coordination of ATPase activity among subunits and may explain why DNA unwinding by Mcm2–7 occurs in a narrow window of experimental conditions (183, 193). The Mcm2/5 gap may be a gate that topologically breaches the MCM motor domains (194). In *S. cerevisiae*, ATP binding appears capable of opening or closing the Mcm2/5 gate (174, 195); however, ATP does not exert such an effect with MCMs from *Drosophila melanogaster* (170) or the microsporidian parasite *Encephalitozoon cuniculi* (188). The functional implications of these differences on Mcm2–7 mechanism are unknown.

The loading of Mcm2–7 onto DNA [an event that marks the licensing of replication origins (196)] is accomplished by the joint action of ORC and Cdc6, together with a third protein called Cdt1 (reviewed in Reference 197). These factors form a distinct initiation intermediate known as the prereplicative complex (pre-RC) (198-200). As in bacteria, eukaryotes use an AAA+-type initiator to catalyze helicase deposition; however, the mechanics of Mcm2–7 loading differ from those used for DnaB. In budding yeast, Mcm2–7 is bound first by Cdt1, in part through an interaction with a WHD at the C terminus of Mcm6 (201-203). ORC and Cdc6 then collaborate with Cdt1 to load Mcm2–7 complexes onto DNA (204-206). ATP is required for loading, with ATP hydrolysis by Cdc6 assisting Mcm2–7 recruitment and nucleotide turnover by Orc1 promoting the stable association of the helicase with DNA (114, 207). Mcm2–7 loading appears highly cooperative, with two helicase rings deposited onto duplex DNA in a head-to-head (collar-to-collar) double hexamer for every round of action by ORC · Cdc6 (Figure 5*c*) (175, 208). How the Mcm2–7 double hexamer forms is not known, but the ability of Orc6 to bind two copies of Cdt1 may help ORC and Cdc6 count the number of MCMs to be used in each loading event (205,

206). Once loaded, Mcm2–7 complexes can passively slide along DNA in vitro but cannot unwind the duplex, suggesting that the double hexamer configuration corresponds to an inactive state (175).

The available structural and biochemical data on Mcm2–7 have several implications for how the helicase loads onto DNA. The break in the Mcm2/5 interface provides a way for DNA to enter the helicase interior (170, 183). Spontaneous adoption of a cracked-ring state suggests that the eukaryotic loading machinery may not use the energy of ATP to actively open the Mcm2–7 ring per se; instead, ATP may be involved in a switching mechanism that coordinates helicase recruitment with ring splitting and DNA entry (170). Because an open Mcm2–7 complex could in principle bind to any accessible DNA region in a chromosome, the initiation machinery, particularly Cdt1, may actually help prevent aberrant loading events as much as aid origin deposition. Curiously, multiple Mcm2–7 complexes can be loaded in vitro and during initiation in cells, even though only two are destined to become part of an active replication fork (209, 210). This so-called MCM paradox (211) differs from bacterial initiation, in which only two molecules of DnaB are deposited per round of origin firing (145, 212) and may have a role in helping to overcome replication fork stalling, for example, by the activation of dormant origins (212).

Although Mcm2–7 serves as the primary motor for unwinding DNA during replication, it lacks robust activity until two additional factors—Cdc45 and the heterotetrameric GINS assembly (comprising the Psf1, Psf2, Psf3, and Sld5 subunits)—associate with the helicase (193, 213-215). Both Cdc45 and GINS travel with Mcm2–7, forming a stable complex known as the CMG (216-219). The CMG exhibits dramatically higher ATPase and 3′→5′ DNA-unwinding activity than Mcm2–7 alone (193, 213). Activation of the CMG appears to occur through a conformational change in the helicase (193) promoted by GINS · Cdc45 binding to one edge of the Mcm2–7 ring and the concomitant closure of the Mcm2/5 interface (Figure 5*e*) (170). Several lines of evidence indicate that the CMG acts as a discrete, single particle during DNA unwinding and DNA replication (193, 210, 213, 214), implying that the Mcm2–7 double hexamers formed during loading separate once replication commences. How double hexamer separation occurs is not understood.

The observation that Mcm2–7 is initially loaded onto dsDNA fits with the apparent inability of ORC to melt DNA directly (see Eukaryotic Origin Replication Complex, above). However, this finding raises questions such as how origin DNA is melted and how Mcm2–7 associates with DNA in the context of the CMG to promote duplex unwinding. Although multiple models have been advanced for DNA unwinding by MCM proteins (reviewed in Reference 220), recent data appear to support a steric exclusion model akin to that of DnaB. For example, the CMG unwinds forked DNA substrates but not blunt-ended duplexes (193, 214). EM reconstructions of the CMG indicate that the GINS tetramer binds near the edge of the Mcm2–7 hexamer, precluding it from separating a DNA duplex that might emerge from the pore of the helicase ring (170). Perhaps most compellingly, functional replisomes formed in *Xenopus* egg extracts can bypass roadblocks on the lagging strand but not the leading strand, indicating that only one DNA strand enters and moves through the CMG during translocation (221).

How Mcm2–7 might transition from a ds-DNA to a ssDNA binding mode is unclear. One possibility is that as eukaryotic MCMs are activated upon entry into S phase (discussed below), they undergo a conformational rearrangement—possibly linked to a second ring-opening event—that couples the separation of the Mcm2–7 double hexamer with the concomitant melting of duplex DNA (222, 223). The disposition of DNA strands within the helicase once the CMG is formed is similarly unknown. Similar to the CMG, the GINS tetramer can bind to DNA substrates containing single-stranded regions but not to blunt-ended duplexes (193, 224). Cdc45, which appears to be a catalytically inactive homolog of the bacterial/archaeal 5′→3′ RecJ exonuclease (225), also associates with ssDNA but not ds-DNA (226). Within the CMG, two topologically segregated conduits exist, one formed by the Mcm2–7 ring and the other between the external face of Mcm2 · 5 · 3 and one side of GINS · Cdc45 (Figure 5*e*) (170). This configuration suggests that GINS and Cdc45 may help capture the lagging DNA strand as it is ejected from the Mcm2–7 complex during origin melting (170, 193, 223, 227). Alternatively, GINS and/or Cdc45 might prevent the leading strand from escaping the CMG during replication fork stalling or collapse; consistent with this idea, work in *Xenopus* egg extracts has shown that DNA damage causes part of the replisome (including GINS) to disassemble, whereas Cdc45 stays bound to Mcm2–7 at the fork (228). Overall, much remains to be discovered regarding how eukaryotic initiation machineries collaborate to open origin DNA and expand the resulting nascent replication bubble.

Helicase Loading in Archaea

Archaea do not utilize a bacterial DnaB-type helicase but rather retain one or more homologs of the eukaryotic MCM proteins (229-232). With the exception of certain MCM paralogs that appear catalytically defunct (233), archaeal MCMs typically form homohexameric rings that exhibit 3′ → 5′ helicase activity (230, 231, 234). Archaeal MCM protomers are composed of three elements: an N-terminal region that bears both a zinc binding domain and OB fold, a central AAA+ ATPase core, and a predicted C-terminal WHD of unknown function (reviewed in Reference 220). The NTD forms a singly or doubly hexameric collar with cyclic, sixfold symmetry that can bind ssDNA and dsDNA (171, 234, 235). The AAA+ motor elements assemble into a second ring coaxial with the N-terminal collar and may constitute the advancing end of the replisome during translocation (169, 234). A set of β-hairpin loops, present in both the NTD and AAA+ regions, forms a complex network that responds allosterically to ATP binding and hydrolysis to promote translocation and DNA unwinding (178, 234, 236, 237). A detailed understanding of the MCM-unwinding mechanism has yet to emerge.

As with eukaryotic Mcm2–7, full-length archaeal MCMs can assemble into both individual hexamers and NTD-to-NTD dodecamers (Figure 5*d*) (169, 235). Although the roles of the two oligomeric forms are not known, they may (as with eukaryotic Mcm2–7) reflect different functional states, with the double hexamer corresponding to a loaded configuration and the single hexamer corresponding to an active unwindase. Unlike eukaryotic Mcm2–7, open hexameric rings have not been observed in archaeal MCM structures; nonetheless, archaeal MCMs are both polymorphic and dynamic. For example, *S. solfataricus* MCMs undergo rapid subunit exchange (236), whereas the *Methanothermobacter*

thermautotrophicus helicase forms heptameric assemblies that revert to hexamers upon binding duplex DNA (235). An open-end helical configuration, somewhat reminiscent of oligomerized DnaA, has even been observed by EM (238, 239). This latter observation suggests that archaeal MCMs might have a propensity to enter into a spiral state, possibly during helicase loading. Whether such a state engages ssDNA or aids in DNA melting is unresolved.

Although the establishment of an origin-dependent, archaeal MCM loading reaction has not been reported, the candidate helicase loader for archaea is Orc1. Interestingly, many archaeal origins contain inverted Orc1 binding sites (ORBs) separated by a distance sufficiently large to accommodate an MCM double hexamer (54, 118). The polar binding of Orc1 to these inverted ORBs is expected to orient the initiator's C-terminal WHD toward this intervening region (116, 118), which often contains a DUE (59). In *M. thermautotrophicus*, the Orc1-1 C-terminal WHD associates with the MCM-NTD, whereas Orc1-2 destabilizes the helicase ring (240, 241). Together, these observations have led to a symmetrized model for the bidirectional loading of the archaeal replicative helicase, in which two Orc1 proteins chaperone an MCM double hexamer onto origin DNA (Figure 5*f*) (59). Future efforts are needed to establish whether helicase-loading factors exist in addition to Orc1.

The DNA-unwinding activity of the archaeal MCM is intrinsically more robust than that of the isolated eukaryotic Mcm2–7 (172, 183, 230, 234). Nevertheless, homologs of the eukaryotic helicase activators GINS and Cdc45 are present in archaea. Archaeal GINS is simpler than its eukaryotic counterpart, as it comprises a homotetramer of only one GINS subunit (GINS15) (242) or a heterotetramer of two subunits (GINS15 and GINS23) (243). Archaeal GINS interact with their respective MCM counterparts both in vitro and in vivo (243, 244), but a consistent effect of this interaction on helicase function has yet to emerge. GINS also forms a stable complex with archaeal RecJ-type proteins (243), although we do not know whether these prokaryotic Cdc45 homologs support MCM function.

REGULATION OF INITIATION FACTORS

Cellular organisms use an array of mechanisms for regulating initiation that are generally distinct among bacteria, archaea, and eukaryotes. Nonetheless, there exist common strategies that appear to have evolved convergently. One is the control of origin accessibility by DNA architecture factors [e.g., histones and nucleotide-associated proteins, which are discussed in the Supplemental Material and several recent reviews (18, 47, 245)]. Another strategy is the regulation of initiation proteins by posttranslational modifications and/or protein-protein interactions. In this section we compare and contrast a few of the many approaches used by cells to govern when and where initiation can occur.

Control of DnaA Function

A prevalent regulatory theme in bacteria is the control of DnaA's nucleotide status and availability (Figure 6*a*). Although replication initiation depends on the binding of ATP to DnaA (see Bacterial *oriC*, above), once hydrolyzed, the initiator remains tightly bound to ADP, entering an inactive state that disfavors DnaA-*oriC* assembly (246). In *E. coli*, the

intracellular concentrations of ATP-bound DnaA vary with the bacterial cell cycle, peaking at initiation (247). A portion of this active material derives from newly synthesized DnaA, which rapidly binds ATP (19). However, ADP-DnaA can be reactivated, both by acidic phospholipids [e.g., cardiolipin (248)] and by specific DNA loci [DnaA-reactivating sequences (DARSs)] (249). *E. coli* DnaA pools can also be adjusted transcriptionally (250) and by titrating free ATP-DnaA molecules to chromosomal loci such as *datA* (251), which contain multiple DnaA binding sites. In organisms such as *E. coli* and *Caulobacter crescentus*, Hda, a DnaA paralog, can stimulate initiator ATPase activity (246, 252). Hda functions by associating with polymerase sliding clamps that are loaded onto DNA following initiation and providing a catalytic arginine finger that activates the nucleotide binding site of DnaA in *trans* (253, 254). *B. subtilis* and other gram-positive bacteria appear to lack Hda but possess a functional analog termed YabA. As with Hda, YabA binds to both DnaA and the sliding clamp to block further DnaA binding to *oriC*, thereby preventing inappropriate reinitiation (255-257).

Additional aspects of DnaA function are also subject to direct control. For example, the DnaA-binding protein DiaA is a positive regulator of initiation that, in *E. coli*, enhances DnaA-*oriC* assembly and aids DUE unwinding (258). DiaA and its homologs appear to function by forming homotetramers that bind to the N terminus of DnaA (259, 260), facilitating cooperative interactions between initiator protomers to maintain replication synchrony (260). Other proteins also bind to the N-terminal domain of *E. coli* DnaA; however, some of these interactions (such as the L2 ribosomal subunit) interfere with DnaA assembly to negatively regulate initiator activity (261). For its part, *B. subtilis* utilizes a different set of factors that binds to and controls DnaA, in particular SirA and Soj, which govern how the initiator associates with both itself and *oriC* (70, 262-264). Overall, a diverse array of mechanisms for regulating DnaA appears to exist among bacterial species, indicating that many new factors and pathways await discovery.

Regulation of Eukaryotic Initiation Proteins

Eukaryotes regulate initiation through a complex strategy that predominantly targets the assembly and activation of Mcm2–7 helicases through posttranslational modifications (197, 265). The cyclin-dependent kinases (CDKs) are a key set of players whose activities fluctuate in accordance with the phase of the cell cycle (1). In G1, CDKs are inactive, allowing ORC to recruit Mcm2–7 to replication origins and form the pre-RC. Upon entry into S phase, CDK activity increases, blocking pre-RC assembly but allowing for the recruitment of replisome components that activate Mcm2–7 and promote origin firing (Figure 6*b*) (266).

Mcm2–7 is activated by the action of two distinct cyclin-dependent kinases: CDK and a CDK variant termed the Dbf4-dependent kinase (DDK). DDK phosphorylates serine/threonine-rich sites—found predominantly in the unstructured N-terminal tails of Mcm2, 4, and 6 (reviewed in Reference 267)—that have been prephosphorylated by other kinases (268). Interestingly, two alterations in the Mcm2–7 NTDs overcome the requirement for DDK phosphorylation and either prematurely drive cells into S phase or bypass S phase checkpoints (269, 270). Although it is not known how DDK action and N-terminal

mutations activate Mcm2–7, the available data suggest that these events may reshape the interface formed between newly loaded double hexamers (175), promoting the separation of the two helicase rings upon formation of the CMG.

Studies in budding yeast reveal that CDK also regulates Mcm2–7 function by controlling how two non-MCM factors, Sld2 and Sld3, chaperone CMG assembly. Modification of Sld2 and Sld3 by CDK promotes their association with Dpb11, a scaffolding protein that binds phosphorylated targets using BRCT repeats (Figure 6*b*). Formation of the CMG is completed by the arrival of Cdc45 via a (CDK-independent) interaction with Sld3 and of GINS through an interaction with phospho-Sld2 (265, 271, 272). Following entry into S phase and the initiation of DNA replication, GINS and Cdc45 continue to travel with Mcm2–7 as part of the CMG (273), whereas Sld2, Sld3, and Dpb11 are lost (218). The mechanism by which specific proteins are linked to or removed from Mcm2–7 is unclear but requires the transient association of another origin activator, Mcm10 (274, 275). At least part, if not all, of the yeast Sld2 · Dbp11 · Sld3 system appears present in metazoans (276-278). In vertebrates, this system is further embellished by additional regulatory factors [e.g., GemC1 (279)].

Metazoans employ several additional layers of control to ensure that helicase loading is tightly coordinated with cell cycle cues. For example, proteolysis can degrade Cdt1 following entry into S phase (280). In a strategy reminiscent of RIDA in bacteria (see Control of DnaA Function, above) (253, 254), proteolysis occurs only after Cdt1 binds to the PCNA sliding clamp and is ubiquitinated (280, 281). Mcm2–7 recruitment can also be prevented through Geminin, a Cdt1-sequestration factor that becomes active upon reaching a critical concentration threshold in the nucleus (282, 283). Structural and biochemical studies show that Geminin exerts this concentration-dependent effect by initially binding Cdt1 in a 2:1 complex (284), which subsequently dimerizes as protein levels increase to mask Cdt1 regions required for replication licensing (285).

Despite a focus on Mcm2–7, the helicase is not the sole nexus for regulating initiation in eukaryotes. For example, many organisms target Orc1 for ubiquitination and/or degradation to prevent re-replication (reviewed in Reference 1). CDK activity can stimulate the expression of Cdc6 and Mcm2–7 prior to origin firing (286, 287), as well as target pre-RC components to avoid aberrant secondary initiation events; interestingly, this latter activity can lead initiation factors to different destinies both within and between organisms. For instance, in budding yeast, phosphorylated Mcm2–7 and Cdt1 are routed out of the nucleus (288-290), whereas phosphorylated Cdc6 can be both sequestered and ubiquitinated for targeted proteolysis (291). Although the effect of CDK-dependent phosphorylation on yeast ORC is unclear, Cdk1 activity impairs the ability of ORC to bind DNA in *Xenopus* and mammalian systems (292, 293), precluding origin relicensing (294). Given the diverse actions seen in the regulation of eukaryotic initiator function, new investigations will likely reveal additional complexities.

Control of Initiation in Archaea

Compared with their bacterial and eukaryotic counterparts, the pathways and mechanisms by which archaea regulate initiation are far less well defined. Nonetheless, a growing body

of evidence suggests that archaea likely possess cell cycle–dependent initiation control systems. For example, a strong consensus Orc1-1 binding site immediately precedes the *orc1-1* open reading frame, which itself lies just downstream of *S. solfataricus oriC1* (54), suggesting that archaeal initiators (like DnaA) may autoregulate their own expression. The *whiP* gene, which is adjacent to *S. solfataricus oriC3*, similarly can achieve extensive self-protection by the cooperative binding of WhiP protomers, at least in vitro (55). Genome-wide mapping of *Sulfolobus acidocaldarius* transcripts even shows a cell cycle–specific induction pattern for certain serine-threonine protein kinases belonging to the same family of kinases as eukaryotic CDKs (295). Overall, uncovering and defining the approaches used by archaea for regulating initiation is still very much a frontier area.

CONCLUDING REMARKS

All replicating systems must perform two fundamental tasks: (*a*) selecting the right time and place for initiating replication and (*b*) orchestrating DNA opening and replisome assembly. Cellular organisms have developed multiple strategies to overcome these challenges, often employing sets of proteins that appear to have evolved independently. The only common strategy universally adopted by cells is the use of an AAA+ ATPase to mark replication origins and to promote helicase recruitment; however, even these factors greatly differ in their specific molecular mechanisms. Future investigations will undoubtedly continue to reveal many unexpected surprises in this area.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

Origin	chromosomal site for initiating DNA replication
Initiator	an ATP-dependent protein or protein complex responsible for recognizing origins and recruiting replicative helicases to sites of replication
Helix-turn-helix (HTH)	a type of DNA binding domain that includes specialized folds such as winged-helix domains
Helicase	an enzyme that unwinds DNA; cellular replicative helicases form hexameric rings
DUE	DNA-unwinding element
ORC	origin recognition complex
AAA+	ATPases associated with various cellular activities
ssDNA	single-stranded DNA
dsDNA	double-stranded DNA
WHD	winged-helix domain
Loader	a protein or set of proteins that assists with reorganizing replicative helicases to promote their association with DNA
MCM	minichromosome maintenance
pre-RC	prereplicative complex
CMG	Cdc45·Mcm2–7·GINS
RIDA	regulatory inactivation of DnaA

SUMMARY POINTS

1. The initiation of DNA replication in cells involves the timely recognition and melting of chromosomal origins and the appropriate loading of replicative helicases.
2. Initiation in cellular organisms relies on evolutionarily related AAA+ ATPase initiators and ring-shaped, hexameric helicases.
3. Despite certain commonalities, the participants, mechanisms, and regulation of initiation differ greatly among bacterial and archaeal/eukaryal lineages.

FUTURE ISSUES

1. How do replication initiators bind and remodel origin DNA?
2. How are replicative helicases loaded onto origins?
3. How do different regulatory systems ensure that initiation of protein function accords with cell cycle cues?

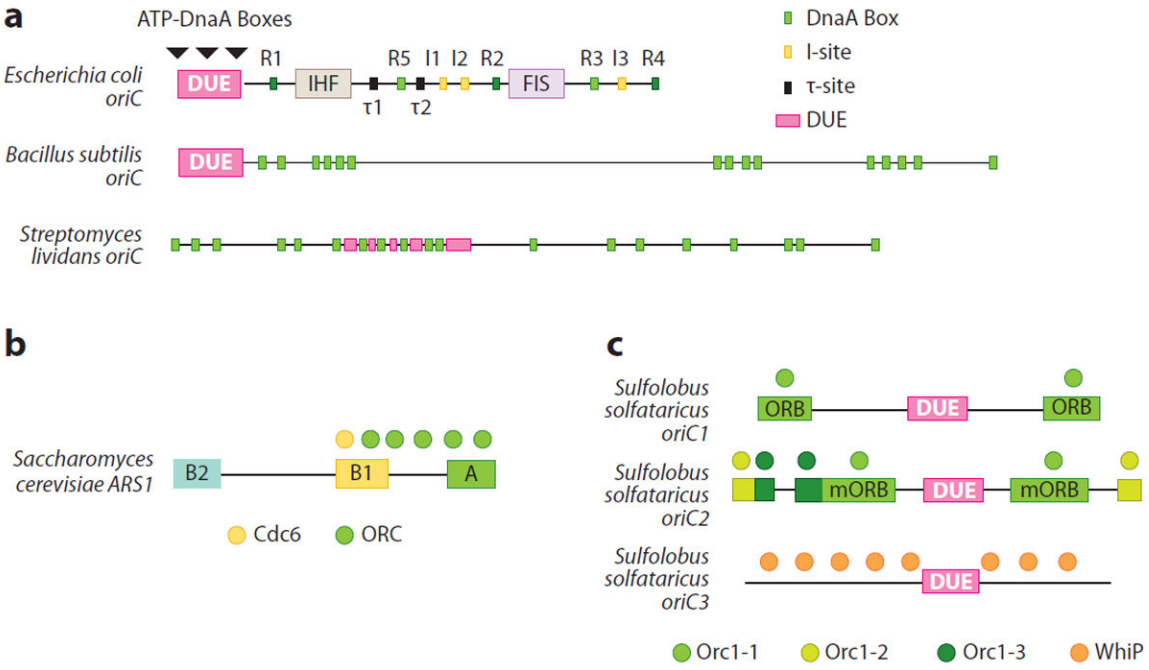

Figure 1.

Overview of origin organization. (*a*) Bacterial *oriC*s from three representative species (*Escherichia coli*, *Bacillus subtilis*, *Streptomyces lividans*). Sites bound by DnaA and other factors, as well as the DNA-unwinding element (DUE), are labeled. (*b*) *Saccharomyces cerevisiae* ARS1 (autonomous replicating sequence 1). ARS consensus sequence elements (A, B1, B2) and ORC · Cdc6 binding sites are shown. (*c*) The three origins of *Sulfolobus solfataricus*. DUEs and binding sites for replication initiators are labeled. Abbreviations: ORB, origin recognition box; ORC, origin recognition complex; WhiP, winged-helix initiator protein.

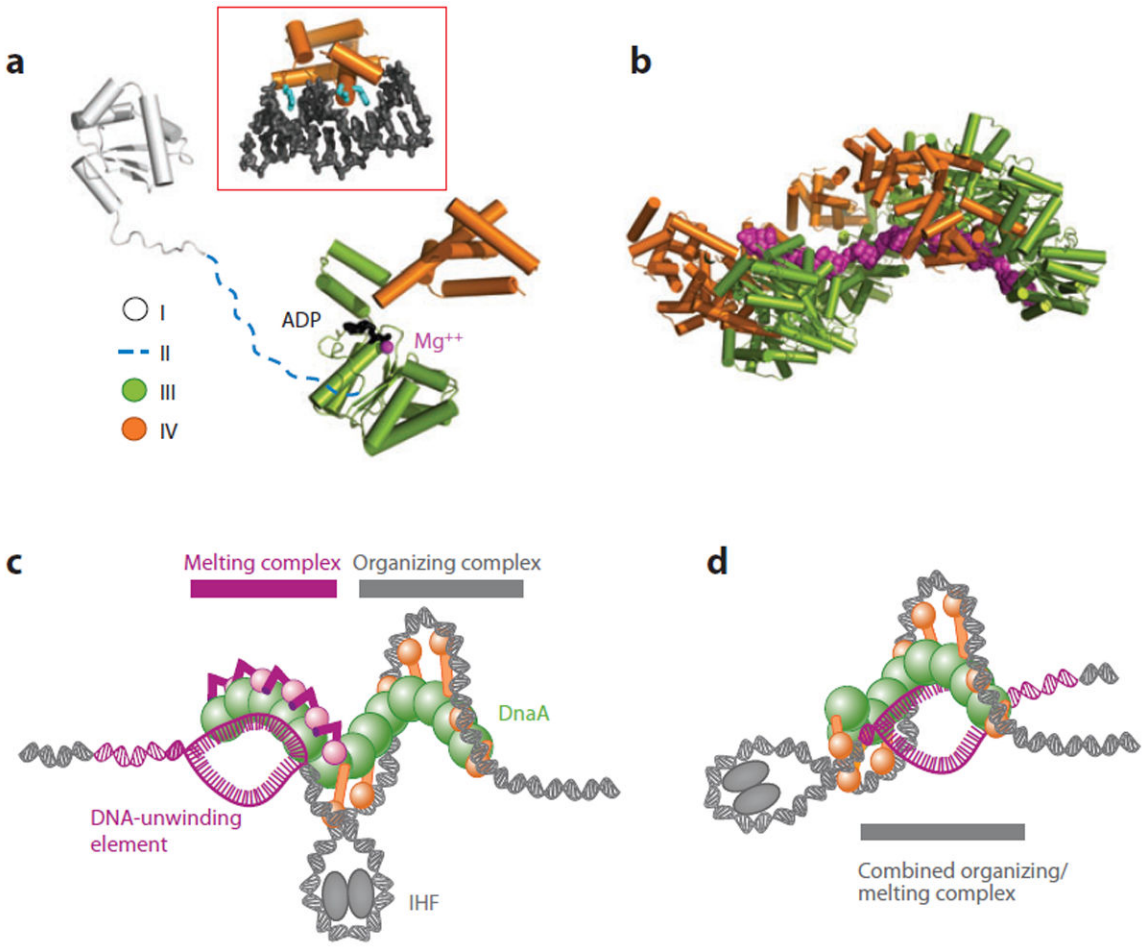

Figure 2.

Structure and mechanism of DnaA. (*a*) Domain architecture of DnaA. Protein Data Bank models for domain I and an ADP state of domains III/IV of DnaA are shown (7, 62, 151). Inset: structure of domain IV bound to duplex DNA (28). (*b*) Structure of an AMPPCP-assembled DnaA helix (domains III and IV) bound to an extended single-stranded DNA substrate (*magenta*) (72). (*c*) Two-state model for IHF-assisted DnaA melting of *oriC* (7). This mechanism accounts for the existence of two different DnaA oligomers (12, 74), one of which wraps the organizing center of *oriC* and another that melts the DNA-unwinding element (DUE). (*d*) Loopback model for IHF-assisted melting, in which a single DnaA complex bound at the organizing center of *oriC* opens the DUE (296).

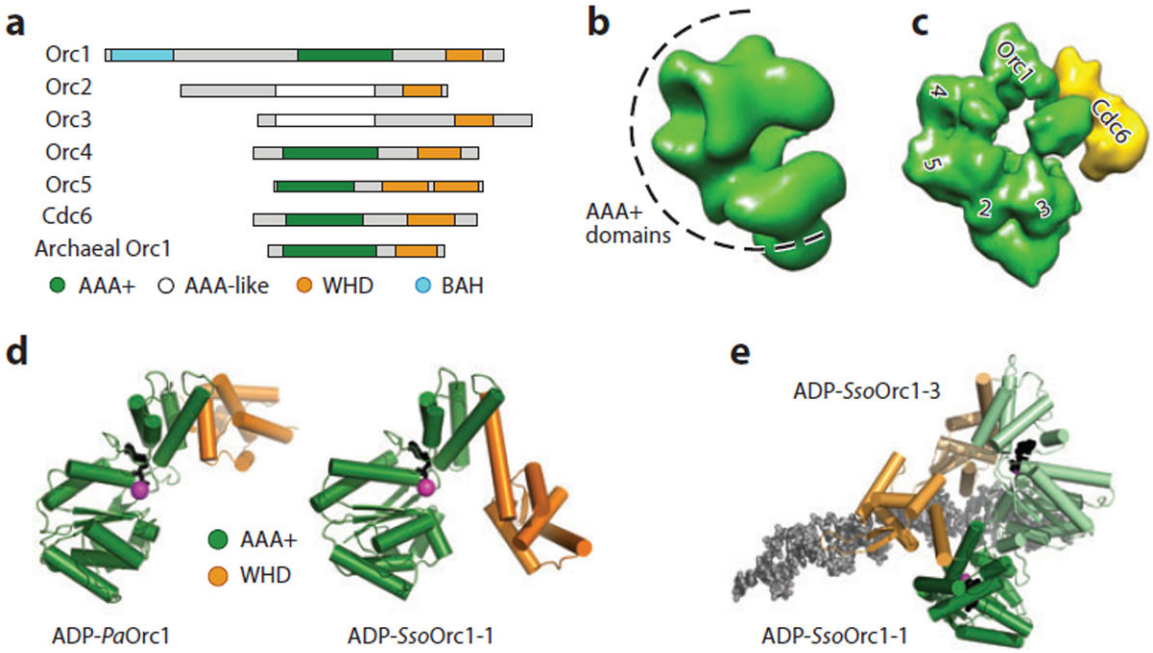

Figure 3.

Structure of origin recognition complex (ORC)-type initiators. (*a*) Schematic of domains in Orc proteins. (*b*) Three-dimensional electron microscopy (EM) model of the *Drosophila* ORC indicating where the AAA+ (ATPases associated with various cellular activities) domains of the complex reside (89). (*c*) Three-dimensional EM model of the budding yeast ORC · Cdc6 complex. The positions of AAA+ subunits are labeled (103). (*d*) Structures of archaeal Orc1 proteins show variability in AAA+/WHD (winged-helix domain) position [*Pyrobaculum aerophilum* (4); *Sulfolobus solfataricus* (116)]. (*e*) Structure of *S. solfataricus* Orc1-1 and Orc1-3 paralogs complexed with overlapping sites in *Sso-oriC2* (116). Abbreviation: BAH, bromo-adjacent homology.

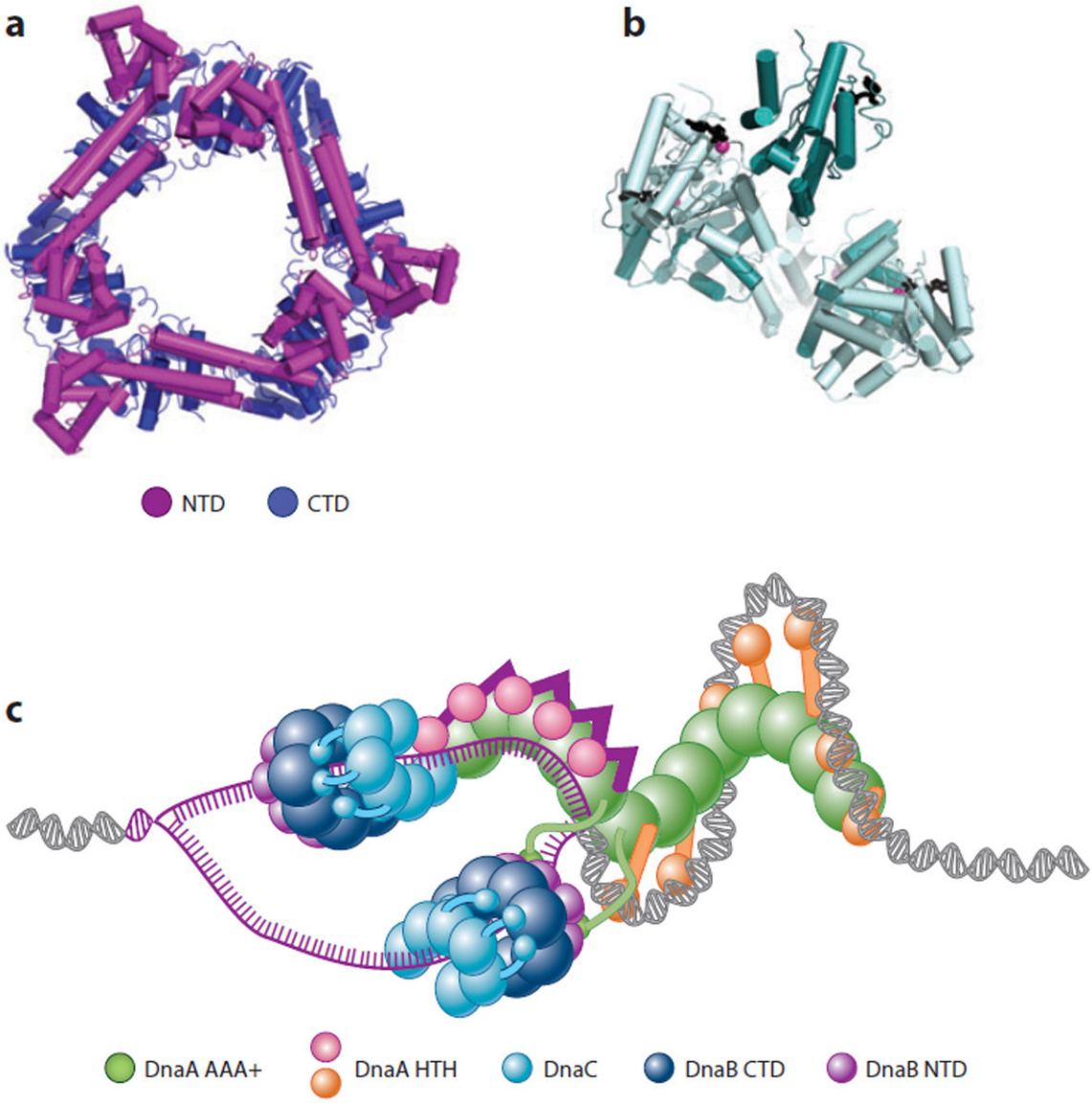

Figure 4.

DnaB structure and loading. (*a*) Closed-ring DnaB hexamer from *Bacillus stearothermophilus* (128). (*b*) Structure of a spiral DnaC oligomer [AAA+ (ATPases associated with various cellular activities) domains] bound to nucleotide (147). (*c*) Model for loading of DnaB onto *oriC* following the two-state mechanism shown in Figure 1*c* (147). Abbreviations: CTD, C-terminal domain; HTH, helix-turn-helix; NTD, N-terminal domain.

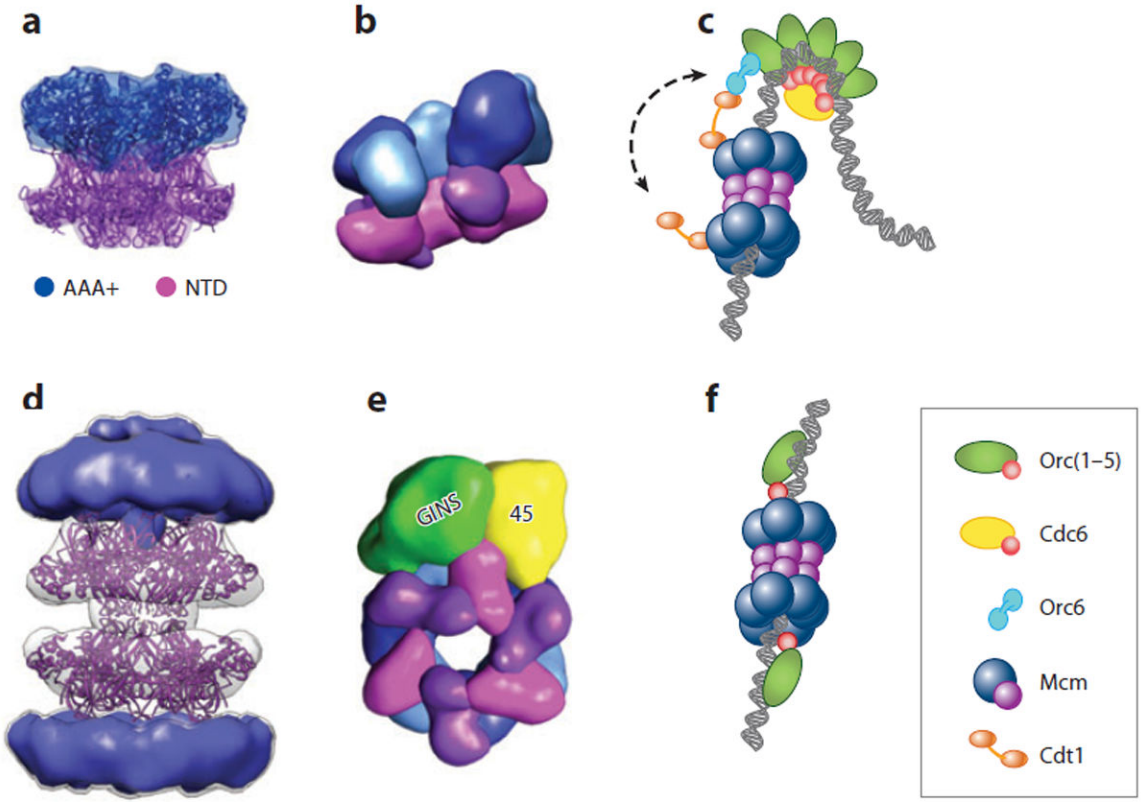

Figure 5.

Minichromosome maintenance (MCM) structure and loading. (*a*) A closed-ring MCM homohexamer from *Sulfolobus solfataricus* (178). (*b*) Three-dimensional electron microscopy (EM) model for an open Mcm2–7 heterohexamer from *Drosophila melanogaster* (170). (*c*) Model for the loading of an Mcm2–7 double hexamer onto DNA by ORC · Cdc6 and Cdt1 (84, 175, 206). (*d*) Three-dimensional EM model for the *Methanothermobacter thermautotrophicus* MCM (235) showing a crystallographic model for an N-terminal domain (NTD) dodecamer (171) docked into the central region. (*e*) Three-dimensional EM model for the *D. melanogaster* CMG showing how GINS · Cdc45 latch onto and close an Mcm2–7 ring (170). (*f*) Model for the loading of archaeal MCMs onto DNA by Orc1 (54, 59).

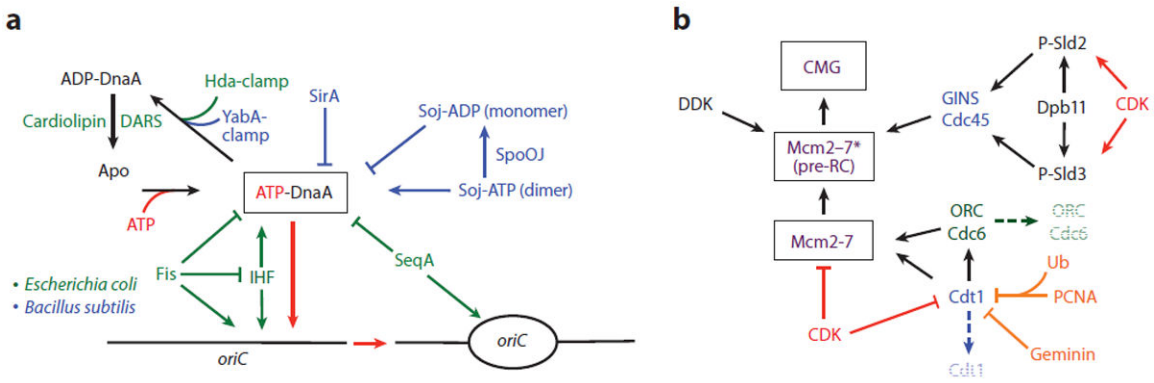

Figure 6.

Overview of initiation factor regulation in cells. (*a*) Regulation of DnaA in bacteria, comparing and contrasting *Escherichia coli* and *Bacillus subtilis* systems. (*b*) Regulation of initiation proteins in eukaryotes. Arrows, activating interactions/events; inhibition lines, repressive interactions/events; hatched text, degradation. Abbreviations: CDK, cyclin-dependent kinase; DARSs, DnaA-reactivating sequences; DDK, Dbf4-dependent kinase; ORC, origin recognition complex; RC, replication complex.