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Eukaryotic DNA replication control: Lock and load, then fire

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The initiation of chromosomal DNA replication involves initiator proteins that recruit and load hexameric DNA helicases at replication origins. This helicase loading step is tightly regulated in bacteria and eukaryotes. In contrast to the situation in bacteria, the eukaryotic helicase is loaded in an inactive form. This extra 'lock and load' mechanism in eukaryotes allows regulation of a second step, helicase activation. The temporal separation of helicase loading and activation is crucial for the coordination of DNA replication with cell growth and extracellular signals, the prevention of re-replication and the control of origin activity in response to replication stress. Initiator proteins in bacteria and eukaryotes are structurally homologous; yet the replicative helicases they load are unrelated. Understanding how these helicases are loaded and how they act during unwinding may have important implications for understanding how DNA replication is regulated in different domains of life.

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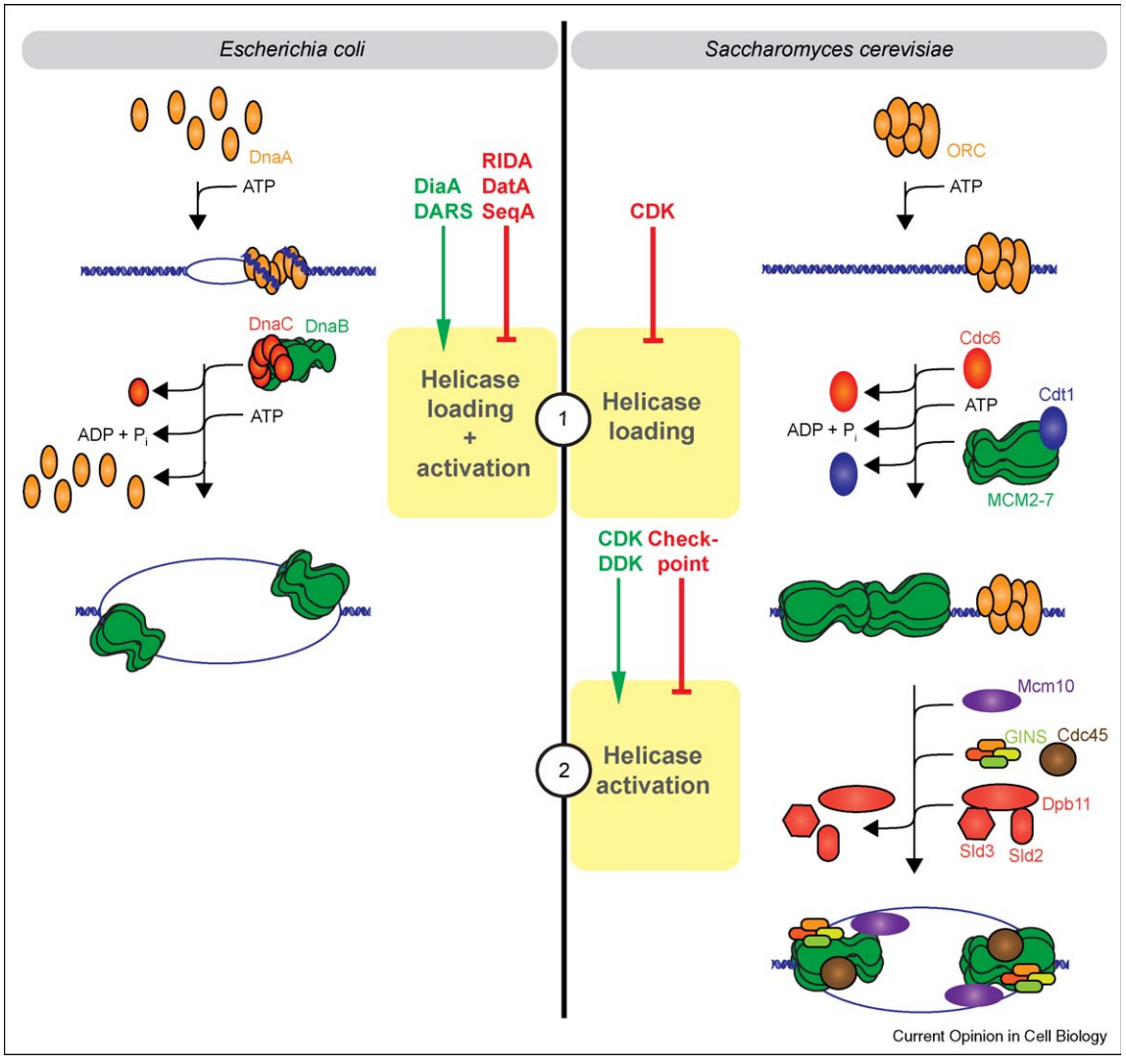
Introduction

The transmission of genetic information from one generation to the next requires that the parental genome is replicated exactly once in each generation. The copying of the parental DNA duplex is carried out by a multiprotein machine, the replisome, which is assembled in a controlled manner at chromosomal sites, termed replication origins. The entire circular genome of bacteria like *Escherichia coli* is replicated bi-directionally by a pair of replisomes assembled at a single origin (*oriC*). One consequence of this replication strategy is that the time it takes to replicate the genome is directly proportional to genome length: doubling the genome size will double the time required to replicate it. By contrast, eukaryotic genomes are replicated from multiple replication origins distributed along multiple chromosomes. This strategy has an important advantage: the time required to replicate the entire genome is no longer proportional to genome size, but, to a first approximation, is proportional to the inter-origin distance. Thus, large genomes can be replicated in short periods of time. However, in order to achieve precise genome duplication, it is crucial that the activity of these multiple origins is carefully coordinated.

A key event in replisome assembly is the loading of the replicative helicase onto the DNA (Figure 1). This loading is mediated by initiator proteins, and precedes the recruitment of the DNA polymerases. The *E. coli* initiator protein, DnaA, binds to specific sequences within the origin (*oriC*) and oligomerises [1]. ATP binding by DnaA leads to the formation of a region of unwound DNA in tandem 13mer sequences adjacent to the bound DnaA. The hexameric helicase, DnaB, is then loaded around each single strand in the unwound region with the aid of the DnaC protein. In yeast, the six subunit initiator Origin Recognition Complex (ORC) specifies origin location and acts together with the Cdc6 and Cdt1 proteins to load the hexameric Mcm2-7 complex, which serves as the replicative helicase during S phase [2].

The helicase loading step is tightly regulated in both bacteria and eukaryotes (Figure 1). DnaA is negatively regulated by several mechanisms: the hydrolysis of its bound ATP is stimulated (ADP·DnaA does not promote oriC unwinding), its access to *oriC* is prevented, and its effective free concentration is reduced. DnaA is positively regulated by promoting exchange of bound nucleotide and by DnaA multimerisation [1,3,4]. Helicase loading is also negatively regulated in eukaryotes (Figure 1): the proteins of pre-replicative complexes (pre-RCs), which form at origin sites to load the Mcm2-7 helicase, are inhibited by cyclin-dependent kinase (CDK)-dependent and CDK-independent pathways during cell cycle stages that are non-permissive for Mcm2-7 loading. Mechanisms involved in the regulation of this step in eukaryotes have been thoroughly discussed elsewhere [5]. Mcm2-7 activation and concomitant origin firing is under further regulation requiring the activities of CDK and Cdc7-Dbf4 kinase (DDK) during S phase, whilst the DNA damage checkpoint counteracts this activation step [6,7]. In this review we compare the mechanism and regulation of helicase loading and activation in the bacterium *E. coli* and the unicellular eukaryotic yeast, *Saccharomyces cerevisiae*.

Figure 1



Stepwise loading and activation of the replicative helicase in *Escherichia coli* and *Saccharomyces cerevisiae*. Events at the *E. coli* origin *oriC* are depicted in the cartoon flow-chart on the left, those in *S. cerevisiae* on the right. **(1)** DnaB loading and activation in *E. coli*; Mcm2-7 loading in eukaryotes (pre-RC formation). In *S. cerevisiae*, Mcm2-7 loading is prevented by CDK-dependent inhibition of pre-RC proteins [5]. In *E. coli*, pathways that regulate helicase loading control the nucleotide-bound state of DnaA (RIDA [1]; DARS [3]), reduce its free concentration (DatA [1]), prevent access to newly replicated *oriC* (SeqA [1]) or promote DnaA multimerisation (DiaA [4]). **(2)** Activation of Mcm2-7 during origin firing. Activation of loaded Mcm2-7 is inhibited by the DNA damage checkpoint [6], but induced by CDK and DDK phosphorylation.

Distinct helicase mechanisms may require distinct helicase loading mechanisms in bacteria and eukaryotes

Surprisingly, whilst DnaA and ORC (subunits 1–5) belong to the same clade of AAA+ ATPases [8], the respective replicative helicases DnaB and Mcm2-7 are not orthologous [9]. Thus, although certain aspects of initiator function may be conserved between bacteria and eukaryotes, structural differences in the helicases suggest that detailed mechanisms of helicase loading may be different. Moreover, DnaB and Mcm2-7 differ in their mode of DNA unwinding and may thus need to be loaded onto DNA templates differently. DnaB forms hexameric rings around the single-stranded lagging strand template of a replication fork and translocates in the 5′–3′ direction, sterically displacing the leading strand. The mechanism for DNA unwinding by Mcm2-7 during replication is unknown, but, unlike DnaB, Mcm2-7 tracks along DNA in a 3′–5′ direction [10,11••]. Alternative modes of unwinding by Mcm2-7 have been suggested by analogy to related enzymes. Mcm2-7 belongs to a superclade of AAA+ ATPases that contains the simian virus 40 large T-antigen and papilloma virus E1 helicases, as well as the bacterial recombination motor RuvB [8]. T-antigen and

E1 both form hexameric or double hexameric rings around DNA and track on DNA in the 3′–5′ direction. Models have been proposed in which these helicases encircle either single-stranded or double-stranded DNA [12]. Recent structural work has shown that E1 can encircle single-stranded DNA during unwinding [13]. RuvB, however, is thought to bind to and pump double-stranded DNA through the central channel of its hexameric ring during Holliday junction migration [14]. If Mcm2-7 act analogously to RuvB, Mcm2-7 may be loaded around dsDNA rather than ssDNA. As discussed below, this may be important to understand the cell cycle regulation of unwinding. RuvB requires an additional, non-AAA+ protein, RuvA, to coordinate DNA strands during Holliday Junction branch migration. Mcm2-7 also requires additional, non-AAA+ factors, Cdc45 and GINS, for helicase activity, and it will be important to understand the roles of these proteins in DNA unwinding.

Loading onto single-stranded origin DNA is sufficient to activate *E. coli* DnaB

Origin specification in *E. coli* begins with the multimerisation of ATP-bound DnaA at *oriC*, which requires DnaA binding to multiple short sequence motifs in *oriC* (Figure 1) [1]. DnaA can form helical filaments in which monomers are arranged head-to-tail to form composite ATP-binding sites at subunit interfaces. Wrapping of origin DNA around this DnaA filament may cause the melting that occurs at specific 13mer DNA sequences within the unwinding element (DUE) adjacent to the DnaA binding site [15]. Following origin melting, DnaA recruits DnaB, which is initially in complex with DnaC, to the unwound origin. DnaC is a close structural paralog of DnaA and, similarly, can form helical filaments, which may provide a structural scaffold for transient opening of the DnaB ring during loading [16••]. DnaC restrains DnaB from unwinding DNA until bound ATP is hydrolysed by DnaC, which induces the release of DnaB. DNA replication thereupon commences bi-directionally from *oriC* without physical linkage of the sister replication forks as soon as DnaB has generated sufficient single-stranded DNA for the assembly of DNA polymerase III holoenzyme [17,18••]. How DnaA can load the DnaC·DnaB complex twice at *oriC* in opposite orientations and on opposite strands is unclear, but bi-modal interaction of DnaA with DnaC·DnaB may be involved [16••]. Important for this review, ATP hydrolysis by DnaC and release of DnaB occur without further delay after DnaB loading [19]. Thus, DnaB is active as soon as it is loaded.

Mcm2-7 is inactive after loading in M/G1 phase and requires activation in subsequent S phase

The chromosome replication cycle in eukaryotes is strictly divided into a period when replication origins acquire replication competence (licensing) but are inactive, and a subsequent period during which origins can be activated but cannot be re-licensed [2,7]. Replication competence is conferred by Mcm2-7 loading, which occurs during pre-RC formation at the end of mitosis and in G1 phase. During this period, Mcm2-7 remain stationary at replication origins and unwound DNA is not yet detected [20]. Similarly, processive DNA unwinding does not occur upon Mcm2-7 loading in *Xenopus* extracts before initiation [21]. The lack of ssDNA detected during G1 phase may suggest that Mcm2-7 are bound to double-stranded DNA.

Origin unwinding in eukaryotes is first detected upon entry into S phase, which requires CDK and DDK activity [2,21,22,23••,24,25••]. These kinases promote the binding of Cdc45, GINS, Sld3, Sld2 and Dpb11 to replication origins, all of which are essential for origin firing (Figure 1). DDK and CDK act locally at origins throughout S phase, with some origins being activated early and others late in S phase. Replication timing variations thus reflect the selective activation of subsets of Mcm2-7 complexes. Timing of origin activation correlates with the timing of association of Cdc45, Sld3 and GINS [2,22]. It is not clear why activators do not bind to all origins synchronously, but limiting protein concentrations, stochastic selection of origin sites, and local chromatin structure might all be determining factors [26,27,28•,29•,30•]. Furthermore, checkpoint pathways, when activated by DNA damage or replication fork stalling, actively inhibit any further origin firing [6]. Time also appears to be a key factor. For example, allowing more time for pre-RC assembly in *S. pombe* increases the efficiency of inefficient origins [30•], whilst preventing passive replication allows sufficient time for otherwise dormant origins to fire [6,31••,32]. The latter case provides an important mechanism to allow completion of DNA replication in the event of replication fork failure under replication stress, at a cell cycle stage when new loading of Mcm2-7 is not allowed [33].

Specific origin DNA sequences and origin melting do not appear to be required for helicase loading in eukaryotes

Unlike homo-hexameric DnaB, the eukaryotic Mcm2-7 helicase comprises six related and conserved AAA+ subunits. Both hetero-hexameric complexes of Mcm2-7 and subcomplexes containing only two to three different subunits can form ring-shaped helicase assemblies *in vitro* [34•,35•]. However, the hetero-hexamer is probably the active helicase *in vivo*, because firstly, all six Mcm2-7 subunits are essential for replication [10]; secondly, all six Mcm2-7 subunits are present in purified replication fork progression complexes (RPCs) in budding yeast and in active Mcm2-7 helicase complexes purified from *Drosophila* embryos [11••,36••]; and thirdly, at least five subunits are required for replication fork progression in budding yeast *in vivo* [10].

Origin loading of Mcm2-7 in all eukaryotes depends on ORC, Cdc6 and Cdt1, which associate sequentially at origins to form pre-RCs with Mcm2-7 before initiation (Figure 1) [2]. Higher eukaryotes may employ additional proteins for Mcm2-7 loading. For example Mcm9, one of two metazoan-specific homologues of Mcm2-7 proteins, and the histone acetyl transferase Hbo1 may assist Cdt1 in Mcm2-7 loading in *Xenopus* and humans [37,38••,39,40]. Mcm2-7 loading with purified *Xenopus* proteins, however, requires neither Hbo1 nor Mcm9 [41], suggesting that these proteins are not principal loading factors, but may instead facilitate loading on chromatin.

In *E. coli*, specific short DNA sequences are crucial both for DnaA binding and melting of *oriC*. Conserved eukaryotic origin DNA sequences have not been identified, and, although specific origins exist in many eukaryotes, origin specificity is often degenerate. In fact, any DNA sequence can exhibit replicator function in both *Xenopus* and human cells and heterologous targeting of loading factors is sufficient to create an origin in mammalian cells [42]. This suggests that specific DNA sequences are not strictly required for Mcm2-7 loading and origin function in eukaryotes. Specific DNA sequences can however preferentially localise pre-RC assembly. ORC was identified on the basis of its sequence-specific DNA binding activity to the *S. cerevisiae* ARS1 origin and ORC's DNA binding specificity here might be sufficient to localise pre-RCs [2]. *Schizosaccharomyces pombe* ORC (SpORC) subunit Orc4 is fused to AT-hook domains that target SpORC to AT-rich sequences. No specific DNA binding activity has been detected for ORC from other eukaryotes. Instead, ORC may be targeted to origin sequences by transient interaction with factors including TRF2 and rRNA [43–46]. Thus the intrinsic non-specific DNA binding activity of ORC may be functional for Mcm2-7 loading, whilst accessory DNA binding factors can target this loading to specific chromosomal sites.

ORC and Cdc6 utilise ATP during Mcm2-7 loading and ORC subunits 1–5 and Cdc6 are structurally homologous to DnaA [8,47], suggesting that Mcm2-7 loading involves molecular remodelling. However, DNA melting does not occur upon eukaryotic ORC and Cdc6 binding to DNA or at budding yeast replication origins in G1 phase when Mcm2-7 are loaded [2]. Structural studies of archaeal ORC homologues bound to origin DNA suggest that ORC DNA binding can induce DNA untwisting; however, this untwisting is not accompanied by the disruption of base pairs [48]. Thus generation of ssDNA may not be a prerequisite for Mcm2-7 loading. The only two non-AAA+ proteins in the pre-RC, Orc6 and Cdt1, make essential contacts during Mcm2-7 recruitment to the origin [49••]. Once Mcm2-7 is loaded, ORC, Cdc6 and Cdt1 are not required for subsequent steps leading to helicase activation [2]. Thus, the loaded and inactive Mcm2-7 complex is likely to be a key target for pathways regulating origin activation.

Mechanism of CDK-dependent and DDK-dependent activation of the Mcm2-7 helicase

The essential positive role for CDK in promoting budding yeast DNA replication is to induce the phosphorylation-dependent binding of Sld2 and Sld3 to Dpb11 [23••,25••]. Sld3 and Dpb11 are required for initiation only, but their molecular function in initiation is unknown [50]. Cdc45 and the hetero-tetrameric GINS complex, in contrast, have essential functions both during initiation and elongation and, as described above, might play some direct role with Mcm2-7 in DNA unwinding. Consistent with this notion, both proteins form an active helicase complex with Mcm2-7 in *Drosophila* and associate with active Mcm2-7 complexes in *Xenopus* extracts [11••,51••]. The structure of GINS does not reveal an obvious molecular function, but GINS does have a prominent role in maintaining the integrity of replication fork complexes [22,52–55].

DDK preferentially phosphorylates Mcm2-7 complexes that have been loaded onto DNA [56••]. Phosphorylation of the N-terminus of Mcm4 in these complexes is required for subsequent binding of Cdc45 [57••]. Because a heterologous serine-rich protein sequence can functionally substitute for the Mcm4 N-terminus, it is unlikely that DDK phosphorylation generates a specific phosphorylation-dependent docking site for Cdc45. Moreover, a point mutation in a different Mcm subunit, Mcm5, can bypass the requirement for DDK in DNA replication [10]. These results suggest that phosphorylation of Mcm2-7 might induce a structural change in Mcm2-7 that promotes its association with activating factors like Cdc45. As in *E. coli*, DNA replication forks proceed bi-directionally from eukaryotic replication origins, but how this bi-directionality is established is not understood. However, similar to T-antigen and E1 helicases, archaeal Mcm proteins can form head-to-head double hexamers, that are held together at their N-termini [58]. Such an arrangement during initiation could provide a mechanism for establishing bi-directionality. Although live-cell imaging suggests that sister replication forks remain in close proximity to one another during DNA replication in budding yeast [59••], sister forks can progress independently from each other when one of the forks is specifically blocked [31••], and purified RPCs from budding yeast and the purified *Drosophila* Cdc45/Mcm2-7/GINS (CMG) complex contain only a single Mcm2-7 hexamer [11••,36••]. Thus phosphorylation of Mcm2-7 N-termini might promote the dissociation of a double hexamer into two single hexamers that may be permissive for Cdc45 and GINS binding.

Conclusions

The cell cycle regulation and differential timing of origin activation throughout S phase, as well as the presence of

excess Mcm2-7 on chromatin at dormant origin sites, illustrate that helicase loading and activation are distinct events in eukaryotes. Because helicase loading occurs in G1 phase in the absence of DNA replication, and several hours might pass until the activation of origins, eukaryotes may load the helicase on double-stranded DNA to avoid the generation of fragile single-stranded DNA. The rapid cell division cycles of bacteria, where re-initiation can occur before cell division has been completed, may not require a similar delay between helicase loading and activation. Delineating the steps involved in Mcm2-7 loading, identifying the mode of DNA unwinding by Mcm2-7 in the context of the replisome, and characterising the molecular events involved in Mcm2-7 activation will yield important insight into the molecular mechanisms by which cell cycle progression is controlled.

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