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Cell Cycle Regulation of DNA Replication

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Abstract

Eukaryotic DNA replication is regulated to ensure all chromosomes replicate once and only once per cell cycle. Replication begins at many origins scattered along each chromosome. Except for budding yeast, origins are not defined DNA sequences and probably are inherited by epigenetic mechanisms. Initiation at origins occurs throughout the S phase according to a temporal program that is important in regulating gene expression during development. Most replication proteins are conserved in evolution in eukaryotes and archaea, but not in bacteria. However, the mechanism of initiation is conserved and consists of origin recognition, assembly of pre-replication (pre-RC) initiative complexes, helicase activation, and replisome loading. Cell cycle regulation by protein phosphorylation ensures that pre-RC assembly can only occur in G1 phase, whereas helicase activation and loading can only occur in S phase. Checkpoint regulation maintains high fidelity by stabilizing replication forks and preventing cell cycle progression during replication stress or damage.

Keywords

origins; kinase; helicase; checkpoint; replisome; replicons

INTRODUCTION

> *Le rêve d'une bactérie doit devenir deux bactéries* (*The dream of a bacterium is to become two bacteria*)
>
> François Jacob, 1965

As François Jacob said poetically over 40 years ago, it is a cell's "dream" to become two cells with identical copies of the genome. Thus the replication of the genome must be an exact process. Errors that result in underreplication or overeplication of the genome in any cell cycle have disastrous consequences and can produce a large array of human genetic diseases, including cancer, birth defects, and many developmental abnormalities (58). Molecular regulatory mechanisms have evolved to ensure that the genome is replicated once and only once and then segregated equally to the resultant daughter cells. This review summarizes recent developments in the field and focuses mainly on cell cycle regulation of DNA replication in eukaryotic cells.

In all cells studied, DNA replication is regulated by recruiting the replication machinery or "replisome" to sites called origins on the chromosome (Figure 1). The replisome is a molecular machine that replicates the DNA bidirectionally from origins in a semiconservative fashion. The recruitment process is called initiation, whereas subsequent replication of the DNA by the replisome is called elongation. It is initiation and hence the recruitment process that is the site

DISCLOSURE STATEMENT
The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

of regulation. When a change in replication rate is needed, adjustments are made to initiation. For example, the rate of DNA replication is almost two orders of magnitude faster in embryos than in somatic cells because more origins are used, resulting in more initiations. This review focuses on the important molecules used in the initiation process in the context of the cell cycle. Readers are advised to refer to other excellent reviews (12,13,273) for additional information.

The basic mechanism of regulation is to identify the origin by having an initiator protein bind to it (Figure 1). Protein complexes that load or recruit the replisome to the origin then recognize the initiator protein bound to origin chromatin. The replisome opens the DNA helix, stabilizes the ssDNA that is formed, and allows enzymes (polymerases) to copy the DNA. Coliphage λ replication is an excellent model for the initiation process due to its simplicity (244,261) because only two viral proteins are required for initiation (Figure 1*a*). The λO-protein recognizes and binds the single origin on the phage chromosome. λP protein recruits dnaB helicase to the λO-origin complex but inhibits it (7). Replication begins only after heat shock proteins remove λP (6) and the replisome is loaded.

EVOLUTION OF CHROMOSOMAL REPLICATION

One would think that the basic mechanism of regulation has been conserved during evolution as it has adapted to the basic problem of copying a double-stranded antiparallel helix. However, comparative genomic studies of the evolution of the three domains of life (306) have shown that the cellular replication machinery diverged when eubacteria separated from archaea and eukaryotes billions of years ago (83,158) (Table 1). Many of the replication proteins of eubacteria have no clear orthologues in eukaryotes, yet proteins used for transcription and translation are conserved among the domains (83,158). Notably, dnaB helicase is used by bacteria whereas archaea and eukaryotes use the MCM (mini-chromosome maintenance) helicase for replication. Similarities in structure and function between the two helicases suggest that they probably resulted from convergent evolution. One explanation is the “replicon takeover” hypothesis, which states that eukaryotic and archaeal replication proteins evolved by nonorthologous gene displacement by viral or plasmid invaders in a common ancestor at the time of the separation (83,158). Consistent with this hypothesis, a prophage found in the eubacterial *Bacillus cereus* was found to contain a MCM-homologue (181). The implication is that the MCM helicase evolved from viral invaders and replaced a dnaB-like helicase used by the ancestor of eukaryotes and Archaea. Another possibility is that some replisomal proteins evolved twice in an independent way from the last common ancestor that had a hybrid genome of RNA and DNA (158).

Lessons from Archaea

Many archaeal genomes are small and only about 1.5–2.0 Mbp. Investigators have exploited the fact that archaeal replication is similar yet simpler than eukaryotic replication with regard to the number of proteins required (Table 1) [for excellent reviews of archaeal replication, see (12,133)]. Therefore, Archaea represent a simpler and less complicated model system to analyze the function of the human replication apparatus. For example, the eukaryotic MCM-helicase (see below) is composed of six, paralogous proteins and is inactive either when purified from cells or made using recombinant DNA technology (81), making it difficult to analyze on a molecular level. However, a recombinant form of an archaeal homologue was shown to be an active DNA helicase and both atomic crystal and cryo EM reconstruction structures have been solved (Figure 2). Furthermore, eukaryotic ORC (origin recognition complex) is also composed of six proteins (Orc1–6), and Cdc6 protein is needed to load the MCM helicase at the origin. Cdc6 and Orc1 are homologues and in some Archaea, a single protein Orc1/Cdc6 can act as both ORC and Cdc6.

Several groups have utilized the biochemical and structural information gleaned from archaeal proteins to analyze the function of the eukaryotic homologue in vivo using genetic and molecular techniques. For example, the structure of the archaeal Orc1/Cdc6 was used to test the importance of the winged helix domain in binding in eukaryotic cells of *Schizosaccharomyces pombe* (164). Conversely, the significance of mutations in yeast Mcm5 protein (76) and in mouse Mcm4 (252) was determined by using structural information from the archaeal Mcm protein (36,76,77). Thus, the power of each system, Archaea for biochemistry and structure and yeast and mouse for genetics, can be used to determine function both in vivo and in vitro.

ORIGINS AND REPLICONS

Origins of DNA replication are sites in the genome at which replication begins. The DNA replicated from a single origin is called a replicon. Usually, replication begins at an origin and proceeds bidirectionally to complete a single replicon (Figure 3). Eventually, replicons fuse resulting in complete genomic duplication. The chromosome of *Escherichia coli* has one origin and its entire genome of about 4 Mbp is a single replicon. The replicon hypothesis postulated that origins would be specific DNA sequences (*in cis*) that would be recognized by DNA-binding proteins (*in trans*) much like the *lacO* operator is recognized by the *lacI* repressor in transcription (119). To a first approximation, the replicon hypothesis is correct with regard to many single-celled prokaryotic organisms (bacteria and Archaea) and to many viruses. In eukaryotes, the situation is more complex in that there are many origins present on a single chromosomal molecule of DNA (Figure 3). To further complicate matters, in many eukaryotes, the replicon hypothesis may not be completely correct in that there seems to be very little sequence-specificity of origins except in the budding yeast *Saccharomyces cerevisiae* (203, 227). In this review, recent studies about origins in both unicellular and multicellular eukaryotes are compared and contrasted to reveal the important similarities and differences.

In budding yeast *S. cerevisiae*, origins were isolated using extrachromosomal plasmid-based ARS (autonomously replicating sequence) assays. Simply, the ARS allowed circular bacterial plasmids to replicate as mini-chromosomes and “shuttle” between yeast and bacteria (13). Using 2D-gel analysis, the ARS were shown to be *bona fide* origins of replication (28). The ARS is about 100 bp and consists of a simple 17-bp consensus A-domain region with an 11-bp ACS (ARS consensus sequence A/TTTTAT/CA/GTTTA/T) that is A-T rich and flanked by poorly conserved B domains (196). The A and B1 domains are binding sites for the ORC, while the other B domains (B2, B3, etc.) act as enhancers of origin efficiency. In some ARS regions, there are degenerate repeats of the ACS instead of the B regions. Thus, the context of the chromatin surrounding the ACS is important for function (13).

Using a combination of classical Meselson-Stahl and high-resolution DNA-DNA microarray technologies, it was shown that budding yeast has about 332 origins among its 16 chromosomes (224,311), which are spaced apart according to a normal distribution indicating a nearly uniform arrangement (215). More recently, ssDNA genomic mapping was used to identify and confirm these 332 active origins (73). Information about yeast origins can be found in a user-friendly computerized database called OriDB (199) that is based on a phylogenic analysis of *sensu stricto Saccharomyces* species (200). In this latter study, the ACS in ARS was conserved but not the flanking B2 regions.

Although the efficiency of these origins varied greatly, many were very efficient, used in every cell cycle, and account for most of the replication of the genome. Most origins were found in intergenic regions that were later shown to lack nucleosomes in the region 3′ to the ACS (200,319). These studies also showed that origins are fired continuously throughout S phase. Most of these origins were also found in combined chromatin immunoprecipitation-microarray

(ChIP-chip) studies that determined genome-wide ORC and MCM binding sites (309,310). Using tiling microarrays and ChIP, 529 ORC-MCM binding sites were found with the ORC-MCM coincidence having the highest predictive value, suggesting that only a subset of origins is used, that is, 332/529 origins (310).

Therefore, budding yeast has specific sequences at origins (ARS), which are recognized and bound by initiator proteins (ORC) according to the replicon hypothesis. The origins are efficient and evenly positioned allowing for complete genome replication in a reasonable amount of time. *S. cerevisiae* arose from *Kluyveromyces waltii* by genome duplication, gene selection, and divergence (131). During this evolution, sequences produced at random that contained evenly spaced origins might have been selected to allow optimal chromosomal replication. It is possible that *S. cerevisiae* uses simple sequences as replication origins because it is "streamlined" from domestication by humans in industrial fermentation. In support, centromeres of *S. cerevisiae* are also simple sequences of about 100 bp that bind one microtubule but in other eukaryotes they are much larger and bind many microtubules (40).

S. cerevisiae is clearly an exception in that other eukaryotes have many inefficient origins spread randomly throughout their genomes and in many cases, the origins are without any identifiable specific sequences (203,227). In the distantly related fission yeast *S. pombe*, combing studies using DNA fibers labeled by DNA analogue precursors and FISH (fluorescence in situ hybridization) showed that origins spacing is distributed in an exponential fashion, indicating that origins fire stochastically and are not inherited in that different origins are used in each generation (215). A low average origin efficiency (<30%) and stochastic origin selection was also seen in other studies using different methods (49). Although the ORC binds a specific sequence in budding yeast and is conserved (see below), there is little sequence conservation among fission yeast origins, which are about 1 kb, A-T rich (about 70%), and are also in non-nucleosomal intergenic regions (49,246).

Thus, in contrast to budding yeast, fission yeast cells have many inefficient origins that fire randomly and are scattered haphazardly throughout the genome. As pointed out, this could result in large regions being missed by chance, resulting in a "gap" that needs to be replicated passively from very distant origins (227). A similar gap problem also exists in metazoan embryos such as *Xenopus* and *Drosophila* (109,113). One possibility is that origin efficiency may increase as S phase progresses, thereby increasing the chance that an origin will fire in the problematic area (113,227).

In metazoan cells, the situation is less clear (93,170,203). As seen in budding yeast, there are clearly active origins that act as defined, sequence-specific sites and can also function at ectopic sites such as the 1.2-kb human lamin B2 origin (213). In the HBB (human β-globin) locus, there are two 2—3 kb adjacent regions that can act as origins even at ectopic sites (296). Other origins are not sites but broad zones of initiation such as in DHFR (dihydrofolate reductase) with more than 40 sites of initiation in a 55-kb intergenic region (61).

Temporal Program of Initiation

Origins are activated at different times during the S phase according to a temporal program. In budding yeast, origins are activated continually during the S phase with the majority of events in mid-S phase (224). Timing in yeast is regulated after the previous mitosis in early G1 phase (223). Similarly in mammalian cells, the decision to replicate the DHFR locus before β-globin was also made in early G1 phase at the "timing decision point" (162).

However, the function of the temporal program remains unknown. Originally, because transcriptionally active euchromatic regions replicated early and inactive heterochromatic regions late, it was thought that early replication is a prelude to transcription (95). In this

hypothesis, transcription "opens up" chromatin to allow easy access to replication factors. Again an analogy to coliphage λ can be made, in which the λcI transcriptional repressor blocks the initiation of DNA replication even if replisomal proteins are supplied *in trans* (281). Later studies showed transcription is important for the binding of initiator proteins λO and λP (88).

Early evidence in mammals had come from studies in which the active X chromosome replicated early, while the homologous inactive X chromosome in females replicated late (30). Similarly, the heterochromatic inactive B chromosomes of maize also replicate late, while the active A chromosomes replicate early (222). More recent studies using microarray analyses have supported the idea in *Drosophila* (169) and in human cells (169). The choice of the DHFR for initiation is influenced by transcription (236). In contrast, there is no correlation with transcription and origin usage in the budding yeast (224), and in fission yeast origins fire randomly in each generation (215). However, there is a correlation between origin and transcriptional terminators (200)

The relationship between transcription and replication in regulating the temporal program is unclear in that it has never been established whether transcription of these regions causes replication or vice versa (203). Furthermore, there are a number of exceptions in which transcriptional activation during development correlates with decreased origin usage as in the mouse HoxB locus (100,203). In fact, the timing of origin activation correlates with a developmental program rather than with transcription per se in chicken cells (54,204). Large chromatin effects in highly transcribed regions are responsible for increased origin efficiency suggesting a change in global chromatin structure rather than local repressive effects at 5′ ends of specific genes (169,170).

As described below, origin activation results from the recruitment of the replisome that requires the loading of Cdc45 protein, which is dependent on the action of two protein kinases, CDK (cyclin-dependent kinase) and DDK (Dbf4-dependent kinase). However, because both kinases are active at the beginning of S phase, downstream effects must be responsible for timing (193,243). One possibility is that the chromatin structure at late origins makes it more difficult for CDK and DDK to act. This is supported by the fact that Cdc45 loading correlates with origin timing as first demonstrated in budding yeast (8,9,327). In budding yeast, changing the chromatin context influences timing (85,260,320). Posttranslational modification of histones also clearly plays a part in temporal program as deletion of the budding yeast Rpd3 histone deacetylase or targeting the histone acetylase Gcn5 to a late origin, which both increase more acetylated and "open chromatin" at origins, advances replication timing (291).

Another connection between chromatin and replication timing involves the DNA damage and replication checkpoints (see below) (206). Rad53 kinase (known as Chk2 in vertebrates), which is needed for checkpoint control, regulates free histone levels (101) and binds to DNA replication origins (63,129). Loss of Rad53 protein kinase or its upstream kinase Mec1 (known as ATM/ATR in vertebrates) advances timing (235,253). When DNA replication is inhibited, Mec1 and Rad53 kinases are activated and prevent replication at late origins in both budding yeast and frog extracts (44,248). Even during normal replication, the checkpoint pathway is important in regulation of timing in human and frog cells (248,268).

MODEL IN VITRO SYSTEMS: YEASTS (BUDDING AND FISSION), FROGS, AND MAMMALS

Great progress has been made in dissecting the mechanism of initiation using fully reconstituted in vitro systems with purified proteins and defined DNA substrates in *E. coli* and in animal viruses such as SV40 (132,143). However, a eukaryotic in vitro system to measure initiation with all purified components is not yet available. Nonetheless, a number of crude extract in

vitro systems have been successful in measuring protein function and assessing biochemical mechanism.

Although budding and fission yeast molecular genetic systems are being used to dissect the mechanism of initiation (79), no yeast in vitro system completely reconstitutes DNA replication. However, partial reconstitution of the pre-RC (prereplication complex) on origin DNA in vitro has been shown (247). In this system, extracts from G1-arrested cells will support assembly of pre-RC containing the ORC (origin recognition complex) and Mcm2–7 DNA helicase onto exogenously added and immobilized ARS DNA. In a more recent version, ORC is depleted from the extracts by ion-exchange chromatography and replaced by recombinant ORC1–6 proteins made in insect cells (26).

In frogs (*Xenopus laevis*), several embryonic systems are available. The original system consists of injection of DNA into an egg (104). Later a cell-free egg extract was used in which a DNA template is added (21). In both cases, any DNA that is added will replicate once and only once per cell cycle and is packaged into chromatin in a nucleus. Therefore, this system does not require a defined origin sequence, and origin selection is random. A newer, more soluble version does not require the presence of the nuclear membrane (293). This latter system uses two extracts: a concentrated NPE (nucleoplasmic extract) and an egg cytosolic extract. Both of these cell-free systems rely on depletion of the target protein, usually with antibodies (immunodepletion), and then reconstitution with recombinant wild-type or mutant proteins to determine function. As described below, the combination of using budding and fission yeast molecular genetics to identify the protein important for initiation of DNA replication in vivo and the frog extract system to investigate biochemical function in vitro has been very powerful. This is also true of studies of the entire cell cycle (188). Because many of the proteins are conserved in evolution, the conclusions can also be applied and tested using mammalian model systems.

Several mammalian in vitro systems are being used successfully [reviewed in (145)]. Most of the systems use template nuclei isolated from cells in G1 phase and a soluble cytosolic extract made from cells in the S phase. A major caveat is that the system is inefficient and only a small fraction of the nuclei replicate. Furthermore, only early origins are used. As in frog (293), an intact nuclear membrane is not required (144). DNA replication is dependent on the same important proteins (Table 1) (45,46,171) found in vivo and in the other systems. These systems have also been used to identify novel regulatory factors such as MCM3AP, a Mcm3 protein acetylase (275). Another use of this type of system is to substitute a frog egg extract to replicate the mammalian nuclei (162), which has been used successfully to analyze the temporal program (236).

CELL CYCLE REGULATION OF THE INITIATION OF DNA REPLICATION

As seen in coliphage λ (Figure 1*a*), the basic mechanism of initiation occurs in several steps and results in bidirectional replication from the origin:

1. Recognition: label the origin with the ORC
2. Initiative assembly or licensing: load the DNA helicase to form the pre-RC
3. Unwinding: activate the DNA helicase
4. Elongative assembly: load the replisome including DNA polymerase (POL) holoenyzmes and SSB (single-stranded DNA binding protein).

In the model of eukaryotic replication (Figure 1*b*), ORC marks the origin and provides a “landing-pad” for other proteins. A simple metaphor for the process is “if you build it, they will come” from the movie “Field of Dreams,” where the building of a baseball field brings

about the coming of dead baseball players. In this case, the building of a complex on the origin brings about the coming of important replication proteins and the replisome. Again keeping with the similarity to the centromere, this metaphor also has been used to describe the building of the kinetochore on the centromere (56).

DNA replication is regulated in the cell cycle in the following manner (Figure 1*b*): ORC recognizes and binds to the origin. In G1 phase, Cdc6 and Cdt1 proteins load the MCM helicase onto the ORC to form the pre-RC. To initiate DNA replication and enter S phase, two protein kinases, CDK (cyclin-dependent kinase) and DDK (Dbf4-dependent kinase), activate the helicase and load the replisome.

"Replication licensing" is a useful term that is used to describe the process in which origins are "licensed" when the MCM helicase is loaded onto them in G1 of the cell cycle (20). Thus, pre-RC formation equates with "licensing." To avoid confusion, it should be pointed out that the original "licensing" hypothesis is incorrect in that it proposed that the nuclear membrane prevented the "licensing factor' from entering the nucleus (21), which is not the case [see below; (156)].

Re-replication is prevented by blocking MCM loading during S, G2 and M phases. In other words, pre-RCs can be assembled only in G1 phase, but are activated only during S phase. There never exists a cell cycle phase in which pre-RC formation (licensing) and activation can occur (193). In binary or Boolean terms, if off = 0 and on = 1, then there is no phase in which replication licensing (L) and activation (A) both = 1. In G1 phase, L = 1 and A = 0, while L = 0 and A = 1 in S phase (Figure 1*b*).

Recognition: ORC Labels the Origin

The ORC is a six-protein complex containing Orc1–6 proteins in equal stoichiometry that was discovered by identification of proteins that bound to the ACS of budding yeast and via genetic screens involving mating-type silencing (15,262). Although the Orc1–6 proteins are conserved in evolution, the sequence dependence is lost in most other eukaryotes as expected because their origins have no consensus primary DNA sequence. The most striking example of this phenomenon is when recombinant Orc1–6 protein from human replaced the frog Orc1–6 protein in vitro to initiate DNA replication in a sequence-independent manner (94,290). Human, frog, and *S. pombe* ORCs clearly prefer an A-T-rich sequence, but there is no consensus (39,141,290). The AT content could be important for the "helical instability" of origins that is important for facile unwinding (195,200).

From these data, it is not clear what DNA or chromatin structure the ORC from most organisms recognizes. In budding yeast, the ACS is necessary but not sufficient for ORC binding in the genome and most ACS do not act as origins (310). Of an estimated 12000 ACS in the *S. cerevisiae* genome, only about 300 are active, which are conserved among four *sensu stricto Saccharomyces* species (200). In some cases, two ACS sites may act together in directing ORC binding at origins such as at ARS603 (22). The Orc4 protein in *S. pombe* has nine AT hook domains that are responsible for binding A-T-rich DNA (89), which is consistent with the A-T-rich origin preference (49,94). Other Orc4 homologues also prefer A-T-rich sequences but do not have an obvious AT hook domain. Binding of ORC to DNA does not require the Orc6 subunit in budding yeast, whereas all six subunits are needed in *Drosophila* (13).

ORC may recognize a unique chromatin structure dictated by epigenetic determinants and not primary DNA sequence. In budding yeast, a simple 100-bp sequence is needed (see above), which is not conserved, but the ORC is conserved. Again an analogy can be made to that of the centromere in which a self-replicating chromatin structure assures kinetochore inheritance (40), i.e., the origin is the origin because it has always been the origin. In support, histones at

the origins in *Drosophila* follicle cells are hyperacetylated and changes in the acetylation level affect ORC binding (2). In frog, acetylated histones are preferentially found at active origins (50). As in budding yeast (291), the Rpd3 deacteylase is an important regulator in fly, suggesting that even with the acquisition of specific sequence determinants in evolution, budding yeast still retains this important epigenetic regulation.

What are the roles of the six ORC subunits (Table 1)? Most ORC subunits are in the superfamily of AAA^+ ATPases (ATPases Associated with various cellular Activities) with conserved Walker A, B, C, and D motifs (142), except for Orc6 (13). However, only the ATP-binding activity of the Orc1 subunit is required for DNA binding (13). In the frog in vitro system with recombinant human ORC, the ATP binding activity of Orc1, Orc4, and Orc5 subunits is required for replication (94).

The ORC protein ATPase is inhibited when it binds origin DNA. The Orc1 ATPase is activated by the binding of Cdc6 protein (13), which is also an AAA+ ATPase, which then produces a conformational change in the ORC-Cdc6-DNA complex to increase specificity (258,259). On nonorigin DNA, ATP hydrolysis by Cdc6 causes dissociation from the origin. Origin DNA inhibits ATP hydrolysis by Cdc6 and stabilize the complex, i.e, ORC binding to the origin is not specific unless Cdc6 is also bound.

In the presence of ATP, DNA may be wrapped around the ring-like ORC-Cdc6 molecule (Figure 2) (258) similar to initiator DnaA protein in *E. coli* and the oriC origin (87). The ORC ring structure was deduced from EM reconstructions at 25 Å resolution with Cdc6 bound in the presence of the nonhydrolyzable analog ATPγS. Addition of Cdc6 to the ORC produces a more ring-like structure that resembles the MCM complex in size and shape (1,76,96,146, 214). This implies that the ORC-Cdc6 ring binds the MCM ring on this surface to facilitate MCM helicase loading onto the origin.

The proposed structure of Cdc6, which was deduced by comparison with the ORC structure, is similar to the atomic structure of the archaeal homologue, Orc1/Cdc6. Orc1 and Cdc6 proteins are homologues, and archaeal species have one protein Orc1/Cdc6 that does both functions, i.e., origin recognition and the loading of the MCM helicase (12). The winged helix domain (WHD) of the archaeal Orc1/Cdc6 protein has similarity to Cdc6 protein and to a number of DNA-binding proteins. This structure was used to show that the WHD is needed for DNA binding by the *S. pombe* Cdc18 protein, a Cdc6 orthologue (164). Some archaeal species, such as *Sulfolobus solfataricus*, have multiple Orc1/Cdc6 proteins that bind to two different origins (229). All six ORC subunits have WHD motifs (258).

The role of the Orc6 protein is controversial. ORC6 is required for viability in yeast but is not required for DNA binding in vitro (13). In metazoan cells, complexes with lower amounts of Orc6 than the other Orc1–5 proteins still are active (290) and in *Drosophila* all six subunits are needed (13).

In yeast, the ORC is bound to origins throughout the cell cycle. However, in other eukaryotes, ORC binding is regulated. In support of the “ORC cycle” hypothesis (57), Orc1 dissociates from the chromatin-bound Orc2–5 complex and is degraded in cells outside of G1 phase. The process is regulated by CDK1-cyclin A phosphorylation (160). In yeast, phosphorylation of Orc2 and Orc6 by CDK1 is also important for preventing rereplication (198).

Initiative Assembly or Licensing-Load of the DNA Helicase to Form the Pre-RC

The next step is to load the DNA helicase onto the origin (Figure 1*b*). This is accomplished by at least two proteins, Cdc6 and Cdt1 (Table 1). The molecular analogy to this process is “clamp loading” in which the clamp loader loads a ring-shaped molecule onto the DNA by opening of

the ring (Figure 4) (52). The model is based on elegant structural studies in *E. coli* of the pentameric -complex of DNA polymerase III holoenzyme that loads the dimeric β-subunit ring-shaped sliding clamp onto the DNA (52). In eukaryotes, the pentameric Rfc1–5 acts as clamp loader of the trimeric PCNA (proliferating cell nuclear antigen) ring. Clamp loading is an ATP-dependent process and all five RFC subunits are AAA^+-ATPases. Different subunits of the clamp loader act as "wrench," "motor," and "stator" to unlock the ring. In eukaryotes, Rfc1 is the wrench, Rfc2,3,4 acts as the motor, and Rfc5 is the stator. In this model, binding of ATP by the motor produces a conformational change in the wrench, which binds and opens the ring using the stator as a backboard (52).

Cdc6 is also an AAA^+-ATPase, which is required to load on the MCM helicase in G1 phase as first shown in budding yeast (41) and is proposed to act as a clamp-loader (218,302). Using the budding yeast in vitro system, Cdc6 ATPase was shown to be required for the subsequent loading of the MCM complex (225). In this process, the Cdc6 and ORC ATPases act sequentially with Cdc6 required initially. Cdc6 and origin chromatin set off a molecular switch in ORC for pre-RC assembly and determine origin specificity as origin mutations can increase Cdc6 ATPase activity, resulting in a less stable Cdc6-DNA complex (258,259).

Cdt1 protein, like Cdc6 protein, is also required to load the MCM helicase during G1 of the cell cycle of eukaryotes (13). Cdt1 protein (13), which was initially found in fission yeast, was at first missed in budding yeast (201), as it is very divergent (276) but is clearly conserved in eukaryotic evolution. In *Drosophila*, it is called Dup (double-parked) because mutant cells "park" at two points in the cell cycle (305). As Cdc6 ATPase is needed for Cdt1 binding on the origin in vitro, it has been proposed that a Cdt1-MCM complex is loaded onto the ORC-Cdc6-origin complex during initiation (225). Cdt1 and Cdc6 then dissociate and finally ATP hydrolysis by ORC completes the MCM helicase loading reaction (225,258,259).

As stated above, licensing is blocked during S, G2, and M phases of the cell cycle (273). This prevents rereplication. A major level of regulation is catalyzed by CDK, which acts at many redundant levels to block licensing in most eukaryotes (198). These levels include the degradation and localization of several pre-RC components. In fission yeast, it is simpler in that ectopic overexpression of the Cdc6 homologue, Cdc18, is sufficient for rereplication (202). Cdc6 in both yeasts is degraded after CDK phosphorylation (66,120). In contrast, Cdc6 in mammals is exported from the nucleus after CDK phosphorylation (55,217).

Another level of regulation to block rereplication occurs through a protein known as Geminin (Table 1; Figure 1*b*), which was discovered in frog egg extracts (180) and is only found in metazoans, probably because it is important for embryonic development (97,136). Geminin binds to and inhibits Cdt1 and thus prevents replication licensing by blocking the loading of the MCM helicase (180). Geminin performs a similar role in human somatic cells (308) and in mammalian somatic nuclei incubated in frog egg extracts (267). Geminin forms a negative coiled cylinder (239) that acts in a complex with Cdt1 on origin chromatin (168). Geminin is also important for preventing centrosome rereplication in human cells (269).

The MCM DNA Helicase

The *MCM* genes were first identified in a genetic screen for mutants that were defective for the maintenance of mini-chromosomes (Mcm phenotype) in budding yeast (Table 1; Figure 1*b*) (81,156,287). The rationale was that mutations that reduce the activity of proteins important for replication would have more drastic effects on mini-chromosomes, which have only one origin or ARS, are not essential, and can be lost from the cells. Conditional *cdc* (cell division cycle, CDC) mutants of *mcm4*, *mcm7*, and *mcm5* were also isolated in both budding and fission yeasts. A subset of these *mcm* mutations is in a family of six paralogous genes numbered *MCM2–7*, which are conserved in eukaryotes. All six members of the gene family are essential

genes in both budding and fission yeast. All are AAA^+ ATPases with similarity to DNA helicases. In fission yeast, a complex was identified that contained all six subunits in 1:1:1:1:1:1 stoichiometry and had a ring-like structure (1). However, no ATPase or helicase or DNA-binding activity could be found. This fact still represents a major problem in the field and is discussed further below.

Is the MCM complex the DNA helicase needed for DNA replication (147)? The picture was clouded by the fact that the many *mcm*-ts mutants had initiation defects at the restrictive temperature, that is, they could not initiate DNA replication, but replication that had already begun was completed. This is in contrast to most *dnaB* helicase mutants in *E. coli*, which have elongation defects and stop replication immediately because fork progression terminates (140,297). The former are called “slow-stop,” and the latter “fast-stop.” This problem was rectified by the construction of “ts-degron” mutants of five of six *MCM* genes, as the *mcm5*ts-degron mutant was not viable even at permissive temperature (149). Unlike with conventional ts mutants, the protein is degraded at the restrictive temperature. As expected for a mutant with a defective replicative helicase, all five mutants had a fast-stop phenotype. In contrast, both *cdc6ts* and degron mutants affect initiation and are slow stop (31,221). ChIP analyses showed that some Mcm subunits travel with the replication fork (9).

What about DNA helicase activity in vitro? The first breakthrough in biochemical studies was the demonstration that Mcm4/6/7 subcomplexes purified from human cells had ATPase-, ssDNA-, and dsDNA-binding and helicase activity (115,116). However, the activity was weak and not processive. This is problematic as one would expect a replicative helicase to be processive (216). Later studies by several groups confirmed the results using recombinant Mcm4/6/7 proteins from yeast made in insect cells (153,241) or in *E. coli* (127) and also from frog (315) and mouse cells (317). All of these helicase assays are inefficient in that high enzyme:DNA ratios were used, in excess of 5:1, indicating a low turnover number of the enzyme. This fact makes it difficult to perform true processivity assays with low enzyme:DNA ratios.

Although the Mcm4/6/7 complexes had activity, addition of any other subunits inhibited activity, as had been found originally using intact Mcm2–7 complexes (1) or reconstituted fractions purified from cells (115,116). However, Mcm2–7 complexes from frog and budding yeast had ATPase-but not DNA-binding or helicase activity. A model used to explain these results is that Mcm4/6/7 complexes are catalytic and the Mcm2/3/5 complexes are regulatory (241). However, this model predicts a structure of dimers of trimers, which is at odds with a planar structure found for the Archaeal helicase (76,96). Furthermore, it was proposed that two trimer complexes dissociate from one another, thereby activating the Mcm4/6/7 for catalysis (317). This idea is inconsistent with the fact that these proposed “regulatory” Mcm2 and Mcm3 subunits are required for fork elongation (149).

The problem with in vitro helicase assays is that artificial substrates are used (216) (Figure 5*a*). These substrates are composed of a small 40–60-bp primer annealed to a 5-kb ssDNA circle or to a larger primer, which gives a single-stranded tail on which the he-licase translocates in a 3′ to 5′ direction for Mcm4/6/7. In contrast, a large (>100-kb) dsDNA chromosome would have to be unwound at the origin in a eukaryotic cell. In one case, the Mcm4/6/7 complex from fission yeast was more processive (>600 bp) when a substrate containing both 5′ and 3′ tails was used to resemble a true replication fork (Figure 5*a*) (154). In this model, each trimer of a double trimer of Mcm4/6/7, which exists in solution and on the DNA (154), would bind each one of the leading and lagging strands at the fork. Several other models have been proposed and compared (271) (Figure 5*b*) (see below).

The structure of the Mcm2–7 helicase has been deduced by using the archaeal Mth-MCM protein from *Methanobacterium thermoautotrophicum* and the animal virus SV40 T antigen as models (Figure 2) (38,76,78,96,161,214,244). The Mth-MCM is a true homologue whereas the SV40 T antigen is an analogue that results from convergent evolution. Unlike the eukaryotic Mcm2–7 complex, archaeal MCM homo-oligomeric complexes are fully active and processive, even though all studies used >1:1 ratio of double hexamer proteins to DNA (38, 134,250). Originally, EM images showed the Mcm2–7 complex from fission yeast to be a ring, and many isomers were seen including planar hexamers, double trimers, and even tetramers plus dimers (1). Both the atomic structure of the N-terminal domain (76) and the cryo-EM reconstruction of the full-length protein showed the Mth-MCM helicase (96,214) to be a planar, double hexamer in head-to-head conformation (Figure 2).

The N-terminal domain of Mth-MCM is needed for oligomerization and DNA binding through β-fingers or hairpins with positively charged amino acids at the tip that surround the central cavity (76). The C-terminal domain contains the catalytic ATPase and helicase domains (Walker A–D); the structure is yet to be determined. From a comparison with the SV40 T antigen analogue, it has been suggested that dsDNA is pumped bidirectionally into the central opening, unwound, and the ssDNA extruded through the large openings in their C-terminal domains (Figure 5*b*). The resultant ssDNA would be replicated by the replisome as it exits from these openings. As bidirectional replication proceeds, the loops would grow in size. Again by analogy with SV40 T antigen, ATP binding would produce movement of the second set of β-fingers akin to the closing of an iris diaphragm and release the DNA upon ATP hydrolysis (90,161). In Mth-MCM, the large opening is big enough (34 Å) to accommodate dsDNA (d = 20 Å), although it is quite small in SV40 T antigen (15–20 Å), which has suggested that each hexamer only binds ssDNA, as seen in the papillomavirus E1 proteinssDNA structure (71). Another possibility is that dsDNA binding changes the size of the hole to accommodate the helix.

Why have six Mcm2–7 paralogues? During evolution, continued gene duplication and divergence produced different MCM genes with different roles in DNA replication. The order of subunits has been inferred to be Mcm5-Mcm3-Mcm7-Mcm4-Mcm6-Mcm2 in a planar ring by reconstitution of subcomplexes using purified recombinant yeast proteins (Figure 1*b*) (51, 241). This order is consistent with yeast two-hybrid studies of *Drosophila*, mouse, and human MCM proteins (47, 139, 318). As stated above, Mcm2/3/5 may have evolved to be regulatory subunits. Mcm5 is a unique subunit in that it is the only subunit that is very difficult to tag at either its N or C terminus (149, 197), which is why it has not been studied as extensively as the other subunits. As described below, yeast Mcm2, Mcm4, and Mcm6 proteins may be targets of DDK phosphorylation (175, 251) that results in a conformational change in Mcm5 protein and leads to helicase activation and replisome loading (76, 244, 245).

From all these studies, there are several possibilities for the mechanism of MCM helicase unwinding of the DNA during chromosomal replication (Figure 5*b*) (244, 271). If Mcm2–7 is a double hexamer, then the pumping model akin to SV40 T antigen might be right. This model assures coordinated bidirectional replication. Yeast two-hybrid studies have clearly shown that two molecules of the same mouse Mcm subunit can bind to each other (139), which is consistent with a double hexameric structure (244). Also consistent with double hexamers at the origin is the fact that frog Mcm2–7 complexes protect about 80 bp of DNA (69). Similar to SV40 T antigen (5, 176, 256), mutant Mth-MCM single hexamers are less active than wild-type double hexamers (78, 244). Even though mutant SV40 single hexamers are about 5% active on artificial helicase substrates (Figure 5*a*), they are inactive in the SV40 DNA replication system in vitro (11, 303). Furthermore, fission yeast Mcm4/6/7 complexes are dimeric (154). Yet even other archaeal species such as *S. solfataricus* may use single MCM hexamers in which the DNA enters the helicase C-terminal domain (182).

In the “ploughshare” model (271), a double hexamer is loaded onto the origin, then single hexamers translocate in opposite directions along the dsDNA with a ploughshare protein helping to keep the ssDNA unwound as it emerges from behind the helicase (Figure 5*b*). In the “pump-in-ring” or steric exclusion model (127), each hexamer also moves bidirectionally on the DNA, but displaces the opposite strand due to the steric hindrance from meeting the dsDNA at the fork. Finally, the “rotary pump” model has different hexamers twisting the DNA at a distance, resulting in topological strain and unwinding in the center (151). It is based on the fact that MCM2–7 proteins are not located at replication foci in the nucleus. These models will be resolved only when atomic structures of full-length archaeal MCM proteins, eukaryotic Mcm4/6/7, or even Mcm2–7 proteins bound to ssDNA and dsDNA are solved.

In some metazoans, there are two other paralogues, Mcm8 and Mcm9, which are found in human and *Drosophila*, but missing from worm (19,98,123,167,316). Recombinant frog Mcm8 displays both ATPase and helicase activities in vitro, and in reconstituted egg extracts, Mcm8 is required for fork elongation (172). However, it is not known if Mcm8 forms a multimeric complex. In contrast, human Mcm8 is needed for pre-RC assembly and recruits Cdc6, which then loads the Mcm2–7 complex (292). In *Drosophila*, Mcm8 is needed for meiotic recombination (19). It is not yet known whether these discrepancies result from the different systems or the methods used. Mcm9 is most similar to Mcm8 (167,316), but has not been characterized biochemically. Recently, the human MCM-BP protein, a distant homologue that lacks important MCM motifs, has been shown to regulate the Mcm2–7 complex by replacing Mcm2 in the hexamer (231).

Unwinding: Activate the DNA Helicase

In G1 phase, pre-RCs with the Mcm2–7 helicase bound are present on all origins. Nearly 90% of all origins that are bound by ORC also have MCM complex bound (309,310). The next step is to activate the MCM helicase and load on the replisome (Figure 1*b*), with helicase activation being dependent on replisome loading, which both require phosphorylation by CDK and DDK enzymes. In this manner, the cooperation between helicase activation and replisome loading assures coordinated replication. A lack of coordination could allow the helicase to produce unreplicated ssDNA at the fork, which occurs when DNA replication is blocked (32).

The large multiprotein complex that is formed at this step has been referred to as the pre-IC (pre-initiation complex) (326). The original idea was that the pre-IC contains all the proteins needed for DNA replication and then a final activation step is required, which is catalyzed by DDK. The nature of the activation step is unclear as DDK was later shown to be required in the formation of the pre-IC (327). It is possible that the pre-IC does not exist for very long and that replication begins as soon as the pre-IC is formed. Resolution must await an in vitro system that can follow all the events in time.

As described above, intact Mcm2–7 complexes are inactive in vitro. Furthermore, excess MCM complexes are loaded on origins of about 40 MCM hexamers per origin in frog (69) and about 10 in budding yeast (65). Only a subset of these hexamers are selected to become active replication origins by binding a protein called Cdc45. Cdc45 moves with the replication fork (8) and was shown to be needed for both initiation and fork elongation by using a *cdc45*ts-degron mutant (279). Although Cdc45 protein is conserved from yeast to humans, no insight can be gleaned about its biochemical function from primary sequence.

Yet addition of these inactive recombinant Mcm2–7 complexes to frog extracts previously depleted of it restores DNA replication (315). This implies that the complex must be activated in some manner when it is added to the extracts. Mcm2–7 complexes isolated from frog egg extracts displayed helicase activity if bound to Cdc45 protein (177). Cdc45 protein is needed for loading of the replisome, including DNA polymerases and RPA, the eukaryotic SSB (8).

The interaction of Cdc45 protein with the MCM complex was initially inferred from extragenic suppressor studies in which *mcm5*ts mutations were suppressed by *cdc45*cs mutations (108). Both neutralizing antibodies to Cdc45 or a fragment of the Rb (retinoblastoma) protein that binds Cdc45 inhibit MCM helicase activity and further unwinding of the template even after replication has begun in the frog NPE system, which supports the idea that Cdc45 is a helicase cofactor (211). A complex of Mcm2–7, Cdc45, and GINS (see below) purified from *Drosophila* embryos has helicase activity in vitro. From these data, it appears that the Mcm2–7 complex may be activated by the binding of other initiation proteins (189).

Elongative Assembly: Load the Replisome Including DNA Polymerase Holoenyzmes and SSB

CDK and DDK are the two conserved protein kinases required for helicase activation and replisome loading. In budding yeast, there is only Cdk1 or Cdc28 enzyme, but there are six B-type cyclins (Clb1–6) needed for S and M phases (188). Most likely, Cdk1-Clb5 complexes are the most active in regulating DNA replication and Cdk1-Clb2 for regulating mitosis (194). There is little overlap in function in that Cdk1-Clb5 phosphorylates different substrates than Cdk1-Clb2 (48,165,183). Some replication proteins (Table 1) are Cdk1-Clb5 substrates because Clb5 (but not Clb2) has a unique hydrophobic patch that binds the RXL or Cy motif in the substrate (165). This supports the idea that it is substrate specificity by different Cdk-cyclin complexes that drives the cell cycle. However, in other eukaryotes this may not be the case (183). For example, frog mitotic Cdk1-cyclin B1 complexes can promote DNA replication when targeted to the nucleus (187).

In mammals, there are many different CDKs and cyclins with at least four CDKs (Cdk1–4) and four classes of cyclins (A, B, D, and E) required for cell cycle progression (188). The Cdk2 homologue is probably used in DNA replication, although Cdk1 (also known as Cdc2) can substitute (4,266), accounting for the viability of Cdk2 knockout mice (208). By analogy, Cdk2-cyclin E and Cdk2-cyclin A act as yeast Cdk1-Clb5 for DNA replication, whereas Cdk1-cyclin B act as yeast Cdk1-Clb2 for mitosis.

CDK and DDK enzymes are regulated independently of each other, but by similar mechanisms (174,188,243). Both kinase subunits are inactive in monomeric form and are activated by the binding of an unstable activating subunit, Cyclin and Dbf4/Drf1 protein for CDK and DDK, respectively. Thus, CDK is Cyclin-dependent kinase and DDK is Dbf4-dependent kinase. Cell cycle regulation of the unstable subunit assures cell cycle regulation of activity. With CDKs, other levels of regulation occur including protein inhibitor binding, phosphorylation by other kinases, and cyclin subcellular localization (188). With DDK, it is simpler in that Dbf4 protein is absent in G1 phase because it is targeted for proteosomal degradation by the APC (anaphase promotion complex) (174,243). As cells enter S phase, the APC is inactivated by CDK phosphorylation and Dbf4 is stabilized. In vertebrates, a Dbf4 paralogue has been identified called Drf1 (185,270,278,314). In frog, Cdc7-Drf1 is mainly the embryonic form found in egg extracts, while Cdc7-Dbf4 functions in somatic cells (270).

How do the two protein kinases activate the MCM helicase and load on the replisome (Figure 1*b*)? CDK might have evolved later to coordinate the cell cycle with DNA replication, while DDK is simpler in having only a very specific role in DNA replication (191). Evidence indicates that the Mcm2–7 complex is a target of phosphorylation by DDK, which is needed to load on the Cdc45 protein (245, 327). Initially, Mcm2 was shown to be a preferred substrate of DDK in vitro and in vivo in budding yeast (157), in fission yeast (29), and in human (237, 285). Genetic studies also demonstrated an interaction between DDK and the MCM complex. The *mcm5-bob1* allele (P83L) in budding yeast bypasses the role of DDK in replication (103) and a *dbf4* mutation suppresses the defect in a *mcm2*ts mutant (157). Later studies confirmed that all Mcm2–7 subunits except for Mcm5 protein are DDK substrates in vitro in several

eukaryotes [reviewed in (13)]. Recently, phosphorylation sites in the N terminus of budding yeast Mcm4 were mapped and shown to be important for formation of the pre-IC and for DNA replication by using nonphosphorylatable serine/threonine-to-alanine substitution mutants (251). However, phosphorylation of these sites is important but not essential. In similar studies in fission yeast, it was proposed that the N terminus of Mcm4 or Mcm2 or Mcm6 could be phosphorylated by DDK to allow for replication (175). The hypothesis is that there is considerable redundancy in the system with any of 3 different subunits of the MCM complex acting as targets for DDK. Support for this hypothesis would show that the phenotype of any of these phosphorylation mutants is suppressible by *mcm5-bob1*, which can bypass DDK function (103).

Phosphorylation of the MCM complex by DDK leads to the loading of Cdc45 protein in frog egg extracts (121,294), budding yeast (245,327), and in fission yeast (312). In budding yeast, there is a low amount of Cdc45 bound to the origin detectable by ChIP studies in G1 phase when both CDK and DDK are inactive (8,245,327). In another ChIP study, Cdc45 protein binding to early origins was appreciable even in G1 phase cells (124). Similarly, Cdc45 protein binding to bulk chromatin was found to be independent of DDK (326). It is possible that Cdc45 protein may be weakly bound to origin chromatin in G1 phase that is later stabilized by combined CDK and DDK action. In the *mcm5-bob1* mutant, Cdc45 loading at early origins occurs in G1 phase and *mcm5-bob1* bypass is dependent on Cdk1-Clb5 activity (245). Although DDK is bypassed, it was hypothesized that the cells are alive because DNA replication is still regulated in the cell cycle by CDK (245). Recently, the role of CDK in replication was also bypassed, which led to synthetic lethality with DDK bypass by the *mcm5-bob1* mutation and to replication occurring in G1 arrested cells (321). Therefore, CDK and DDK regulate similar events independently, i.e., helicase activation and loading of the replisome (24). This explains why both kinases are needed for replication in all eukaryotes examined.

However, the order of action by the two kinases is different in different systems. In budding yeast, CDK acts before DDK (205). In frog egg extracts, DDK acts first (121,294). This discrepancy may be due to the different experimental systems used or it may be that there is really a difference between replication in the two organisms. Recently, the order of action in fission yeast was found to be similar to that in frog (312), indicating that these differences may indeed be organism specific. Budding yeast, like most somatic cells, coordinates cell size and division by CDK in G1 phase, whereas fission yeast coordinates size and division mainly in G2 phase and embryos lack the coordination completely (188). Thus, budding yeast CDK is already being used in G1 phase and would act before DDK.

DDK may have a relaxed specificity of phosphorylation in that there is a weak consensus phosphorylation site that consists of serine or threonine residues with nearby acidic residues or serine and threonine residues that are phosphorylated by CDK and are also acidic (37). In fact, the seven DDK phosphorylation sites in the N terminus of yeast Mcm4 could be replaced by a synthetic sequence with serines and acidic amino acids or the N terminus of Mcm2 (251). However, efficient phosphorylation by DDK in vivo and in vitro requires a second region that docks the Mcm4 substrate to the DDK.

How would phosphorylation of Mcm2, Mcm4, or Mcm6 by DDK activate the helicase and load the replisome? One hypothesis is that DDK phosphoryation results in a conformational change in the Mcm5 protein that activates the helicase and is a signal for the binding of Cdc45 protein (Figure 1*b*) (76, 244, 245). This hypothesis is based on structural modeling of the budding yeast *mcm5-bob1* P83L mutant that bypasses DDK function, using the structure of the archaeal MtMCM N terminus with a similar mutation, P62L (76). The N terminus of MthMCM has three domains called A, B, and C (Figure 2*a*,*b*). Amino acids with large side chains such as leucine at the P83 residue of Mcm5 would “push-out” the A domain, which is

an alpha-helical domain at the very N terminus. The assumption is that this structure would normally be caused by phosphorylation by the other subunits in the hexameric complex. Using high-resolution genomic footprinting, the structure of ARS1 origin chromatin in S phase was shown to be more accessible to permanganate cleavage than in G1 phase and was dependent on DDK function. Similar origin accessibility was also seen in the *mcm5-bob1* mutant arrested in G1 phase (92). Thus, structural changes of origin chromatin in the *mcm5-bob1* mutant mimic the effect of DDK phosphorylation. These changes correlate with the binding of Cdc45 protein that occurs in G1 phase in the *mcm5-bob1* mutant (245).

Recently, CDK's role in promoting origin activation has been determined (277,312,322). In order to understand this role, a number of important replication proteins must be described. The GINS complex, which is based on the numbers 5, 1, 2, and 3 in Japanese (*Go*, *Ichi*, *Nii*, *San*), is composed of the Sld5, Psf1, Psf2, and Psf3 proteins, and is required for replication (Table 1). The budding yeast GINS complex was identified using a combination of yeast two-hybrid approaches and extragenic suppressor analyses, coupled with biochemical purification studies (272). The approach centered on Dpb11 (DNA Polymerase B possible subunit), a subunit of DNA polymerase ε holoenzyme, which is also called Pol2 or PolB (179). *DPB11* was isolated as a high-copy suppressor of *pol2*ts mutants (10). Sld mutants are Synthetic Lethal with Dpb11, while Psf proteins interact or Partner with Sld5 protein in two-hybrid analyses. The Sld2 and Sld3 proteins were also identified in this way in that a *cdc45*ts mutant is suppressed by high-copy *SLD3*, and *SLD4* is allelic with *CDC45* (124). The GINS complex has also been found in frogs (146) and in a large-scale degron screen for yeast DNA replication mutants (125). In both yeast cells and frog egg extracts, GINS functions interdependently with Cdc45 protein in the loading of the replisome (146,272) and has a ring-like structure in the EM (146). Sld2 and Sld3 proteins are also important for replisome loading with Cdc45 protein in both budding and fission yeasts, but Sld3 is absent in metazoans (124,192). Thus, a large number of proteins are needed in addition to Cdc45 to load on the replisome. These proteins may also help to activate the MCM helicase in that Mcm2–7 complex can be activated by the binding of Cdc45 and GINS (189), thereby coupling helicase activation and replisome loading (24).

The role of CDK in this process is to phosphorylate Sld2 (178) and Sld3 (277,322), causing them to bind to the BRCT repeats in Dpb11, which then binds origin chromatin and recruits the replisome with Cdc45. The role of CDK in replication can be bypassed by using phosphomimetic mutation of the phosphorylation site *sld2*-T84D and fusion of Sld3 to Dpb11. Similar to DDK bypass, bypass of CDK is not sufficient for DNA replication in cells arrested in G1 phase because DDK is inactive as Dbf4 protein is absent due to proteosomal degradation (243). However, overexpression of Dbf4 protein, which can overcome the degradation and produce active DDK (243), together with CDK bypass, allow for DNA replication in G1 arrested cells (277,322). Similarly, this CDK bypass and DDK bypass by *mcm5-bob1* are synthetically lethal (322). In both cases, lethality occurs because the cell cycle regulation of DNA replication has been completely ablated. Because CDK is inhibited in these cells by the overexpression of a nondegradable Sic1 mutant, CDK cannot prevent Mcm2–7 loading and rereplication occurs. Thus, both papers demonstrate positive and negative roles for CDK in DNA replication. In fission yeast, Sld3 loading is not dependent on CDK and GINS but only on DDK (312,313). In this case, Sld3 may load first followed by GINS, Cdc45, and finally the replisome, perhaps explaining why DDK acts before CDK in fission yeast. In frog, the Sld2 (RecQ14) protein acts very late, does not need CDK, and is required only for RPA SSB protein loading (234).

Also identified in the original *mcm* screen was the *MCM10* gene, which is not a Mcm2–7 homologue (81,156,287). Mcm10 is needed for the loading of the Cdc45 protein after pre-RC formation and for stabilizing the replisome as shown in human cells (118), frog egg extracts

(307), budding (228,238) and fission yeast (228). Mcm10 protein may act by stimulating DDK (155) and DNA polymerase α activities at the fork. Fission yeast Mcm10 protein also has primase activity in vitro, which is required in vivo (75).

In summary (Figure 1*b*), activation of the helicase and loading of the replisome are regulated by the combined action of DDK and CDK. In this proposed model, DDK phosphorylates any one of Mcm2/4/6 proteins, resulting in the "pushing out" of the A domain of Mcm5 in the hexamer. The Mcm5 protein structural change together with CDK phosphorylation of Sld2 and Sld3, Mcm10 and the binding of Cdc45 and Dpb11 proteins load the replisome, which then activates the Mcm2–7 helicase. The DNA is unwound by the helicase and the DNA is replicated by the replisome. It is not clear what the exact role of Mcm10 is in this model but it is known to be required for Cdc45 loading and replisome stability (see above). Perhaps, the loading complex falls apart without it.

PREMEIOTIC DNA REPLICATION

During meiosis, a diploid cell undergoes one round of DNA replication followed by two successive nuclear divisions to yield four haploid products. After DNA replication, homologous chromosomes pair, recombine, and synapse. The first division (MI) is reductional; the newly recombined homologous chromosomes segregate from one another. The second meiotic division (MII) is a mitotic, equational division, during which sister chromatids segregate [reviewed in (230)]. As in the mitotic cell cycle, meiotic cells exhibit controls that ensure DNA replication occurs once and only once throughout meiosis. Unlike in the mitotic cell cycle, however, additional mechanisms must function during meiosis for chromosomes to segregate properly into four haploid progeny. First, meiosis-specific features are included within chromatin during meiotic S phase to promote meiotic recombination. Meiotic recombination creates a physical connection and tension between homologues prior to MI, thus promoting proper segregation of homologues during MI. Additionally, meiotic cells must also inhibit an additional round of DNA replication between MI and MII.

In every model organism studied, the duration of the meiotic S phase is longer than that of the mitotic S phase (17,33,112). In budding yeast, meiotic S phase is two to three times longer than S phase in mitotic cells (35). The explanation for a prolonged meiotic S phase is unclear, because origin usage and the rate of replication fork progression appear to be similar in both mitotic and meiotic S phases. For example, in *S. cerevisiae* DNA replication initiates at the same ARS elements during mitosis and meiosis (42,111), and replication fork progression from these ARS elements occurs at similar rates during both S phases (42). In fission yeast, meiotic S phase requires the same genes for progression of DNA replication as in the mitotic S phase (80). It has been proposed that a prolonged meiotic S phase allows for the establishment of meiosis-specific features of chromatin to promote homologous recombination (35,112). Two meiosis-specific proteins, the chromatid cohesin Rec8 (138) and the transesterase Spo11 that catalyzes formation of DNA double-strand breaks (DSBs) to initiate recombination, are thought to play a role in controlling meiotic S phase progression (35,130). This model is supported by the fact that at least in *S. cerevisae*, DNA replication and homologous recombination are directly related to one another. Chromosomal regions where DNA replication is blocked or delayed are also blocked or delayed for formation of DSBs. Furthermore, the time between replication and DSB formation is constant (1.5 to 2 h) for all regions of the genome studied (23).

Regulation of the initiation of meiotic S phase utilizes much of the same cellular machinery (Table 1) as in the mitotic S phase [reviewed in (80,263)]. It has been difficult to determine genetically if mitotic replication factors are also required for meiotic S phase because meiosis is an inherently temperature-sensitive process. Because the restrictive temperature is often

lower for meiosis than for mitosis, temperature-sensitive alleles that display a strong S-phase defect in mitosis may display a leaky intermediate phenotype when incubated at a lower restrictive temperature for meiosis. Furthermore, meiotic cells appear to be able to function with lower levels of some replication factors than mitotic cells (82). For instance, in *S. pombe* temperature-sensitive *cdc18/cdc6*, *mcm2*, and *mcm4* mutants were proficient in meiosis and meiotic S phase in conditions that caused arrest in the mitotic cell cycle, suggesting that these replication initiation proteins were not required for meiotic replication (82). However, this conclusion was disputed in later studies. In one study, the same mutants of *S. pombe* displayed a meiotic replication defect when sporulated at higher, more restrictive temperatures (190). Additional work in fission yeast has shown that MCM proteins are chromatin-associated during meiotic S phase, suggesting a possible role in DNA replication, and a double temperature-sensitive degron *mcm4* mutant does not replicate its DNA in meiosis (163). In this case, both the degron cassette at the N terminus of *mcm4* and a hypomorphic *mcm4*ts mutation were required to completely block DNA replication. Taken together, licensing in the meiotic S phase requires Cdc18 (or Cdc6 in *S. cerevisiae*) and the Mcm proteins as found in mitotic cells.

As in the mitotic cell cycle, CDK and DDK also play vital roles during meiotic S phase for helicase activation and replisome loading. Earlier works in *S. cerevisiae* with temperature-sensitive alleles of Cdc28/Cdk1 suggested this protein was dispensable for initiation of meiotic S phase (254). However, an analogue-sensitive mutant, *cdc28-as1*, is blocked for DNA replication during meiotic S phase in the presence of the analogue inhibitor (16). Cdc28/Cdk1 may also play additional roles in meiosis downstream of DNA replication such as regulating DSB formation (107). In *S. cerevisiae*, *clb5*Δ *clb6*Δ mutants deleted for both S-phase Clb5 and Clb6 cyclins that are used for initiation in the mitotic S phase (242) do not replicate their DNA during meiosis (264). This is because in mitotic cells the remaining four Clb cyclins in the cell are functionally redundant with Clb5 and Clb6 (242).

The precise role of DDK during meiosis is unclear, but studies involving temperature-sensitive and analog-sensitive DDK mutants suggest that this complex plays a role in meiosis beyond DNA replication. In *S. cerevisiae*, *cdc7* temperature-sensitive mutants arrest in meiosis after DNA has been replicated but before DSB formation and recombination (111,240), suggesting that *CDC7* plays a unique role in meiosis beyond its mitotic replication functions. An analogue-sensitive mutant, *cdc7-as3*, exhibits a similar meiotic defect in the presence of inhibitor, though DNA replication takes about 4 h longer than wild-type cells in the presence of inhibitor (295). Temperature-sensitive *cdc7* (*hsk1*) mutants of *S. pombe* also display prolonged meiotic S phase and defects in DSB formation (207). Dbf4, the regulatory subunit of DDK, may also have multiple meiotic functions. In budding yeast, depletion of Dbf4 before meiosis greatly delays meiotic S phase, and cells in which Dbf4 has been depleted after initiation of S phase arrest before anaphase I, and this arrest is independent of defects in DNA replication or recombination (289). Thus, DDK regulates both DNA replication during meiotic S phase and DSB formation after meiotic S phase.

Despite overlap between proteins that regulate DNA replication in mitosis and meiosis, one gene is specifically required for meiotic S phase. Mutants of *S. cerevisiae* lacking *MUM2* fail to completely replicate their DNA and arrest prior to MI. Though *MUM2* is expressed throughout the mitotic yeast cell cycle, *mum2*Δ mutants do not appear to have any defects in vegetative growth (72). *MUM2* genetically interacts with *ORC2* and with *POL1* (Table 1), which encodes the catalytic subunit of the DNA polymerase α-primase complex, suggesting that Mum2p affects the DNA replication machinery (53), though its precise function is unknown.

Another unique aspect of meiotic DNA replication is that a second round of DNA replication must be inhibited between the two nuclear divisions. In *S. cerevisiae*, three CDK-dependent and nonredundant mechanisms inhibit rereplication of DNA during the cell cycle: CDK inhibits Cdc6 activity and expression, promotes nuclear exclusion of Mcm2–7, and downregulates ORC ac through inhibitory phosphorylation (99,198). Work with *Xenopus* oocytes and *S. pombe* indicates that similar mechanisms prevent rereplication of DNA in meiosis. Degradation of Cdc6 protein and repression of its synthesis prevents rereplication of DNA in immature *Xenopus* oocytes (159,304). Exclusion of Orc proteins from the nucleus of immature *Xenopus* oocytes also prevents pre-RC formation, thus providing a second mechanism for inhibiting DNA replication (304). In fission yeast, Mcm4p is not associated with chromatin during the time between MI and MII (163), even though Mcm4p levels are constant throughout meiosis (82). Although the precise mechanisms inhibiting DNA rereplication during meiosis may not be identical to inhibition of mitotic DNA rereplication, both processes involve modification, degradation, and/or relocation of components of the pre-RC.

CHECKPOINT REGULATION OF DNA REPLICATION

DNA checkpoint mechanisms evolved to monitor the successful completion of cell cycle events involving DNA replication and mitosis. If DNA replication is blocked or the DNA is damaged, a signal transduction pathway (Figure 6) is activated, resulting in a block to further initiation events in S phase and to entry into mitosis (206). This pathway consists of sensors (RFC, RPA, PCNA, 9-1-1, Polε), amplifying mediators (Rad9, Mrc1/Claspin), transducers or transmitters of the signal (Mec1, Tel1, ATM, ATR, Chk2/Rad53, and Chk1 protein kinases) and effector target proteins (Dun1 and Cdk1 protein kinases, Cdc45, p53 transcription factor, MCM helicase, Polζ) that produce the response (cell cycle arrest, transcription, DNA repair, translesion synthesis, etc.). The pathway may not be linear in that replication forks may be both sensors and effectors (280). These checkpoint responses are important medically as defects in human Chk2, ATM, and p53 lead to human cancer by increasing genomic instability (128). [For reviews on checkpoint response see (27,84,128,206,265).]

The checkpoint response was initially discovered in budding yeast using the following rationale: If replication is blocked, cells are prevented from entering mitosis by an active process. Checkpoint mutants are defective in the process, do not sense that replication has stopped, enter mitosis with unreplicated chromosomes, and die (106,299,301). For example, if wild-type cells are treated with HU (hydroxyurea), an inhibitor of RNR (ribonucleotide reductase), they will arrest in S phase, but retain viability and are alive after HU is removed. In contrast, *mec1* or *rad53* checkpoint mutants are more sensitive to HU because they die rather than arrest in its presence (301). Another example is with DNA polymerase α mutants (*cdc17*ts), which arrest with a G1-content of unreplicated DNA at 38°, but with a nearly complete G2-content of DNA at the partially restrictive temperature of 34°(300). Only the arrest at 34° was dependent on known checkpoint genes because the cells have incompletely replicated DNA.

Why do the *cdc17*ts mutants arrest in G1 phase at 38°? Another checkpoint must have prevented the cells from entering mitosis with completely unreplicated DNA. Many other *cdc* replication mutants from the Hartwell collection, such as cdc7 and cdc6, also do not make DNA at the restrictive temperature and arrest at the G1/S boundary (31,105,243). It has been suggested that *cdc* mutants of this type arrest because they are “leaky” and produce some replicated or damaged DNA that activates the known DNA checkpoint (221,280). However, in precise measurements of DNA replication by heavy isotopic labeling of *cdc7*ts mutants at the restrictive temperature, most cells make very little DNA, with a small percentage of the cells (<30%) that replicate only chromosome III (226), which is only about 2% of the genome. It is

unlikely that there is enough “leakiness” to account for the arrest that occurs in the 70% of cells that do not initiate DNA replication.

Another explanation is that some strains of budding yeast may lack a novel checkpoint mechanism that detects unreplicated DNA. These strains enter mitosis with un-replicated DNA (148,221,279,280,284) and undergo a “reductional anaphase” (221). This novel checkpoint, the “G1/M-phase checkpoint” (284), is based on the fact that *cdc7*ts and *dbf4*ts mutants arrest in G1 phase only in some genetic backgrounds and independent of allelic “leakiness.” Strains without this checkpoint still retain the known DNA replication, damage, and mitotic checkpoint pathways. This checkpoint is independent of known checkpoint genes such as *MEC1* and *RAD53*, but it has not been genetically defined as it is dependent on too many unknown genes (284,301). Fission yeast replication mutants, e.g., *cdc18*, also lack this undefined checkpoint and will actually septate or cut the unreplicated DNA after mitosis to yield a “cut” phenotype (132).

In budding yeast, late origins did not fire when cells were released from G1 into S phase in the presence of HU (235). In *mec1* or *rad53* checkpoint mutants, replication occurs at the late origins. The rationale is that HU lowers the level of dNTPs to slow replication, which activates the checkpoint. Obviously, there still must be enough dNTPs in HU for replication as it does occur at the late origins if the checkpoint is mutated. In fact, a considerable amount of replication occurs in HU, producing ssDNA birectionally from origins, which has been used to map origins in budding and fission yeast using microarray technology (73). With *rad53* checkpoint mutants in HU, replication forks stall at the origins (73) because the replisome dissociates and forks are reversed, producing a “chicken-foot” morphology (166). Therefore, the checkpoint response is important for stabilization and restarting of stalled replication forks (27).

How does the checkpoint response prevent firing at late origins? In yeast, frog, and human systems, ATM/ATR (Mec1/Tel1), Chk1, and Chk2 (Rad53) kinases are important (44,235, 248,268). In fact, all origins in these studies fire earlier even under normal conditions. The hypothesis is that ssDNA generated during normal replication activates ATR/ATM pathway to inhibit initiation at late origins. The inhibition occurs by preventing CDK- and DDK-dependent Cdc45 protein loading, thus blocking helicase activation and replisome recruitment (8,248,268).

The role of DDK in the checkpoint response is controversial in that different results have been obtained even in the same experimental system (13,63,68,122,220,243). In frog egg extracts, DNA damage by etoposide, which produces ssDNA breaks, inhibits DDK by dissociating the Cdc7-Dbf4 complex (44). Similar results were obtained in human BCR-ABL transformed cells (60). Dbf4 protein also becomes phosphorylated by Rad53 (Chk2) kinase in response to replication arrest in both yeast species (257,302). In this scenario, DDK would be inhibited when the checkpoint is activated.

However, the major form of DDK in frog egg extracts is Cdc7-Drf1 and not Cdc7-Dbf4 (270), and Cdc7-Drf1 is not down-regulated in response to dsDNA breaks or replication inhibition with aphidicolin, a polymerase inhibitor (220,314). In a number of human cell lines including the same BCR-ABL transformed cells used above, both Cdc7-Dbf4 and Cdc7-Drf1 DDK complexes were unaffected by either replication blocks or DNA-damaging agents (278). Assays of DDK were increased in HU-arrested budding yeast cells (210), but reduced in another study (302). It is difficult to compare these two studies as the former used Mcm2 and the latter used Mcm7 as a DDK substrate. Yet phosphorylation of known DDK physiological substrates Mcm2 and Mcm4 is unaffected by replication inhibitors in human (186,278) and in budding yeast cells in vivo (157,251).

DDK may also be required for the checkpoint, which is difficult to reconcile with inhibiting it during the checkpoint response, as discussed above. In fission yeast, DDK (Hsk1) is required for the checkpoint response (68,274). However, in *cdc7*Δ *mcm5-bob1* budding yeast cells in which DDK is deleted and bypassed by the *mcm5-bob1* mutation, both DNA damage (219) and replication (302) checkpoints are intact and Rad53 (Chk2) kinase becomes activated (63, 219). In fact, *cdc7*Δ *mcm5-bob1* budding yeast cells are sensitive to DNA-damaging agents because they have a defect in the Rev3-Rev7 (DNA polymerase)-dependent translesion synthesis subpath-way (219) of the Rad6 epistasis group that also affects mutagenesis (84). As expected, *cdc7* mutants are also defective in both UV-induced and chemical mutagenesis (243). In addition, CDK but not DDK is needed to activate the checkpoint during dsDNA break repair by homologous recombination in budding yeast (114). Thus, it is possible that DDK may be required for some aspects of the checkpoint in some organisms, but clearly not in budding yeast. DDK is also not required for maintaining the checkpoint (280).

A possible explanation for some of these conflicting results is that DDK may be needed for replication restart after the checkpoint response has decayed (68). This is based on finding that *dbf4* (*dfp1*) mutants of fission yeast are sensitive to damage by MMS, yet retain the intra-S phase checkpoint (86). This is consistent with the fact that *cdc7*Δ *mcm5-bob1* mutants are sensitive to HU because they may not be able to adapt or recover from HU and restart replication (302). In checkpoint adaptation, cells continue in the cell cycle even in the presence of DNA damage (233). Adaptation is regulated by the budding yeast Cdc5 protein, a polo-like protein kinase (91), which also has a role in the initiation of DNA replication and interacts with DDK (102). This effect on adaptation may also explain the Rev3-Rev7 epistatic relationship (219) if one assumes that DDK is needed to restart replication with DNA polymeraseζ. Furthermore, inhibition of late replication to slow S phase during replication stress may not be as important for viability as replication restart, thus obviating the need to inhibit DDK (68). In support of this idea, the *mec1–100* hypomorphic mutant of budding yeast is defective in late origin firing but is still resistant to DNA damage by stabilizing replication forks (212,280).

The Mcm2–7 helicase may be a target of the checkpoint response (249). Budding yeast Mcm2–7 complex is important to establish but not maintain the checkpoint, but pre-RCs do not play a direct role in checkpoint control during chromosome replication (148). Replication forks are also important for checkpoint response (280). In human cells, Mcm2 and Mcm3 are phosphorylated by the ATM/ATR kinases during checkpoint activation, and lowered Mcm7 levels inhibit the intra-S-phase checkpoint (43). In frog egg extracts, if DNA polymerase progression is inhibited by aphidicolin or by impassible DNA lesions in the template, the Mcm2–7 helicase still unwinds and produces ssDNA, which is coated with the RPA and activates the checkpoint as a sensor (Figure 6) (32). This may explain why reduced Mcm7 inhibits the checkpoint (43).

Other replication proteins important for the checkpoint include Mrc1 (Claspin), Dbp11, PCNA, RFC, and DNA polymeraseε. Mrc1 acts as a mediator for amplifying the Rad53 and Chk1 signal when replication is blocked in both yeast species (3) and in frog it is called Claspin (152). Mrc1 is part of the replisome and monitors the progress of replication fork (152,209) and interacts with checkpoint proteins Tof1and Csm3 when forks are arrested (129). Dpb11 and its loader Sld2 (Drc1) are also important for the replication checkpoint and activating Rad53 (Chk2). (152). In frog, the N terminus of xRTS protein is similar to Sld2 and also loads the replisome (234). However, the remaining nonconserved C-terminal domain of xRTS is a RecQ helicase family member (234), which is an orthologue of the gene mutated in human Rothmund-Thomson syndrome, a familial degenerative skin disorder with increased susceptibility for bone cancer (150). DNA polymerase ε has an unknown function in the checkpoint and in DNA replication because its polymerase activity is not required for DNA replication, but the C terminus is needed for the checkpoint and for viability (67,74,137).

PCNA and RFC are clamps and clamp loader, respectively (52) (see above). PCNA is a trimer of three identical subunits, whereas RFC is a pentamer with five different subunits, Rfc1–5. In replication, PCNA is the processivity factor that clamps the replicative DNA polymerases δ and ε on the DNA. When DNA replication is blocked, alternative clamps or clamp loaders are used. The hypothesis is that alternative clamps are loaded during DNA damage to recruit DNA repair proteins or to activate the checkpoint or for other responses such as chromosome cohesion (173). With the clamp loader, the Rfc1 subunit is replaced by another protein such as Rad17 to form a Rad17-Rfc2–5 complex during DNA repair (18,70). This alternative clamp would load on an alternative clamp such as the 9-1-1 complex instead of PCNA. 9-1-1 is composed of three different subunits called Rad9, Rad1, and Hus1 from fission yeast and is conserved in other eukaryotes. There are at least four different clamp loaders and two different clamps. In some cases, PCNA is used as the clamp but is modified by mono-ubiquitination (110,288) to load on DNA polymerase η for translesion synthesis during polymerase switching (126). Ubiquitination is catalyzed by the Rad6-Rad18 E2:E3 conjugating:ligase complex (110), which explains why translesion synthesis by polymerase η (RAD30) in yeast is in the *RAD6* epistasis group (see above) (84). The combination of different clamps and clamp loaders could produce a variety of molecules on the DNA to elicit a number of different cellular responses (173). Indeed, the clamp and clamp loader analogy can explain many of the interactions of proteins with long molecules such as DNA.

Some checkpoint proteins also have positive roles in DNA replication such as Mrc1, Dpb11, PCNA, and Drc1 (discussed above). Yeast *mrc1*Δ mutants have a reduced rate of replication (209). The replication and checkpoint functions of Mrc1 are separable in that only the checkpoint response is affected if the Mec1 phosphorylation sites in Mrc1 are mutated.

Similar results are found with Rad53 protein kinase (63,101,323). In budding yeast, *RAD53* or *MEC1* are essential genes that can be rescued by overexpression of *RNR1*, or by down-regulation of the Sml1 protein, which is an inhibitor of Rnr1 (59,232,323–325). However, these mutants are still checkpoint defective. Yet *rad53*Δ *sml1*Δ strains grow slower than *mec1*Δ *sml1*Δ strains (101,323). Similarly, only the growth defect of the *rad53*Δ *sml1*Δ strains can be suppressed by deletion of the *HHT2 HHF2* genes, the major histone H3/H4 genes (101). Therefore, Rad53 has a role in DNA replication that does not involve the checkpoint function and may involve regulation of histone levels during the S phase (63,101). In support of this idea, Rad53 protein binds to origins (63,129) and interacts with two important replication genes, *CDC7* and *MCM5* (Table 1) (62,63). Again, the same is not true of *mec1* mutants or mutations of any other checkpoint genes. Rad53 protein kinase is likely involved in the initiation of DNA replication as *rad53* mutants display reduced origin firing using 2D-gel analysis (166) or the Mcm assay (63). Rad53 kinase also may regulate origin firing based upon growth conditions to optimize the rate of DNA replication (255). *SLD6* was found to be allelic with *RAD53* (124), indicating a possible interaction with *DPB11*, which is important in replisome loading (Figure 1*b*) (see above).

Regions of the genome such as the yeast rDNA repeats that are difficult to replicate because of compact chromatin structure and high levels of transcription in the opposite direction to the replication fork require Rrm3, a DNA helicase (117,282). The Tof1 and Csm3 checkpoint proteins regulate the process by inhibiting Rrm3 and produce a pause site at the Ter termination site in the rDNA (184). Surprisingly, Mrc1 protein is not needed in this case. Similar results are seen at replication “slow zones” in *mec1* and *rad53* mutants (34). Thus, the checkpoint proteins can help regulate replication through difficult genomic regions.

SUMMARY POINTS

1. The mechanism of DNA replication has been conserved in evolution. Because replication proteins for initiation have been conserved between eukaryotic and archaeal organisms, archaeal proteins are being used as simpler, structural models.
2. Most eukaryotes except for budding yeast have ill-defined origins of replication that rely on epigenetic mechanisms for molecular recognition by initiator proteins. In this regard, budding yeast may have become streamlined during evolution in both origin and centromere recognition.
3. Replication is initiated at multiple origins along the DNA using a conserved mechanism that consists of four steps: origin recognition, assembly of a prereplicative initiation complex, followed by activation of the helicase and loading of the replisome.
4. Cell cycle regulation of DNA replication occurs through protein phosphorylation by two protein kinases, CDK and DDK. Both kinases are needed for helicase activation and for loading of the replisome in S phase. CDK also inhibits initiation by preventing pre-RC assembly during S phase. Thus, pre-RC formation can only occur in G1 phase and helicase activation can only occur in S phase. In Boolean terms, there is never a phase in which both events are true as this would result in rereplication due to reloading and subsequent activation.
5. The same proteins are used in premeiotic replication that occurs during gamete production, but in reduced activity. DDK has an additional function after DNA replication but before DSB formation during the meiotic cell cycle.

FUTURE ISSUES

1. Most of the progress in this field has been made by combining the knowledge gained from yeast genetic in vivo studies with in vitro studies in the frog. These studies have identified the important regulatory molecules and their functions. Important contributions have also been made from genetic studies in *Drosophila* and mice and from human cultured cells using siRNA approaches. These studies have revealed more complex regulation in metazoan cells. Structural insights have been made by solving atomic structures of simpler, archaeal proteins that are easier to produce and are more stable at ambient temperatures (Figure 2). A working model of DNA replication under cell cycle control has resulted (Figure 1*b*). However, a fully reconstituted in vitro replication system in which both initiation and elongation occur with all purified components is still lacking. Given all the tools of modern, recombinant DNA technology, this issue should be resolved in the future.
2. In most eukaryotes, origin recognition is still a thorny issue. We do not know the type of chromatin or DNA structure the ORC recognizes. The fact that it is not sequence dependent makes this hard to analyze. Resolution will require higher-order chromatin structure analysis. Another issue is the importance of the temporal program. What is the physiological significance of the fact that origins are activated at different times during the S phase? The connection with gene expression is unclear (203). In fission yeast, it is random every generation (227). Perhaps, late origins exist to be a “fail-safe” mechanism ensuring complete DNA replication before mitosis. In this model, late origins would only be used if that part of the replicon was still unreplicated at the end of S phase. Even this model may be doubtful as a recent report suggests that yeast cells do not know if normal replication is actually completed before entering mitosis (283,298).

3. The role of protein phosphorylation is becoming clear (Figure 1*b*), even though there is a fair amount of redundancy in the process. Both CDK and DDK are activated in late G1 phase using different regulatory subunits. Yet they regulate the same process. This ensures that replication cannot occur in a haphazard manner, as it requires two independent events. Before the coordination of growth and division was optimized during evolution, only DDK may have been used (191). Then CDK evolved to ensure coordination and to prevent rereplication. DDK is rate limiting for initiation in both yeasts (25, 64; N. Rhind, personal communication). Future studies will focus on defining how phosphorylation of important serine and threonine residues in substrates by the two kinases produces the biochemical and structural changes needed to ensure function.
4. Checkpoint regulation is very important in cancer progression by regulating genome stability (128). It is clear that DNA replication is at the heart of checkpoint regulation (Figure 6). Replication proteins are important regulators of the checkpoint and replication itself is needed to generate the signals needed. Tumor suppressors such as p53, BRCA1/2 (Breast Cancer), BLM (Bloom's syndrome), and FANC (Fanconi's anemia) genes are all important players in the response (135). Knowledge of DNA replication is important for cancer therapy also in that most modalities either block or damage DNA replication. Future direction will be on designing drugs that intercept the checkpoint. For example, resveratrol, which is a natural compound found in red wine and used in chemoprevention, activates the checkpoint only in cancer cells and not in normal cells (286).
5. Although phosphorylation by CDK and DDK is also needed for DNA replication in the meiotic cell cycle, their exact roles are unknown. In addition, both kinases have roles after DNA replication. Only identification of the physiological substrates that are phosphorylated by these kinases during meiosis will reveal the molecular nature of these roles.

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Glossary

MCM
: mini-chromosome maintenance

pre-RC
: prereplication complex

CDK
: cyclin-dependent kinase

DDK
: Dbf4-dependent kinase

Licensing
: process by which pre-RC has been assembled on origins

Replicon
: DNA replicated from a single origin

RF

replication factor (A or C)

PCNA
proliferating cell nuclear antigen

ARS
autonomously replicating sequence

ACS
ARS consensus sequence

Checkpoint
process in which cells monitor the completion of important events such as DNA replication and mitosis

POL
DNA polymerase

AAA$^+$
ATPases associated with various cellular activities

CDC
cell division cycle

Replisome
replication complex that polymerizes or copies the DNA

Origin
site on the chromosome at which DNA replication begins

Helicase
enzyme that converts dsDNA into ssDNA

ORC
origin recognition complex

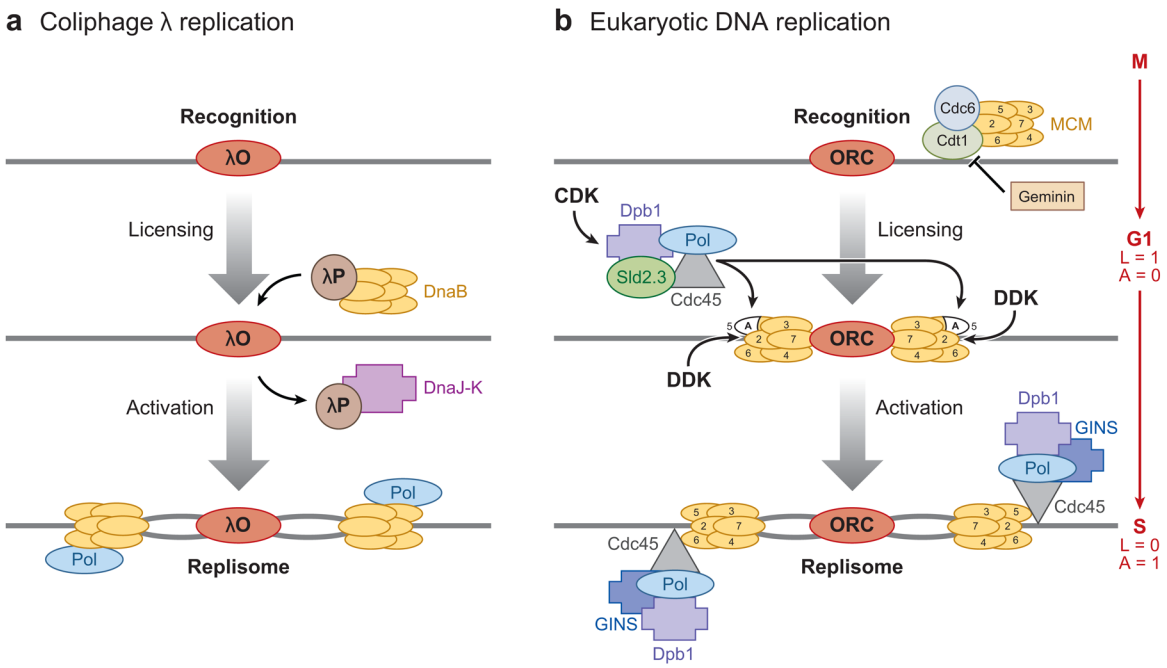

Figure 1.

Models of the regulation of DNA replication. (*a*) In coliphage λ replication, origin is recognized by λO protein, then λP protein loads hexameric DnaB helicase. λP protein is removed by DnaJ-K protein, which activates the helicase and allows replisome to replicate the DNA. (*b*) In eukaryotic DNA replication, origin is recognized by ORC, then Cdc6 and Cdt1 protein load the hexameric MCM helicase to form the “licensed” (L) pre-RC in G1 phase (L = 1, A = 0). Geminin inhibits Cdt1 and pre-RC formation. CDK and DDK become active in late G1, activate (A) the MCM helicase and load on the replisome that contains the DNA polymerases. In addition, CDK inhibits any further licensing (L = 0, A = 1). Toward this end, CDK phosphorylates Sld2 and Sld3 proteins and DDK phosphorylates MCM proteins, which “pushes out” the “A” domain of Mcm5.

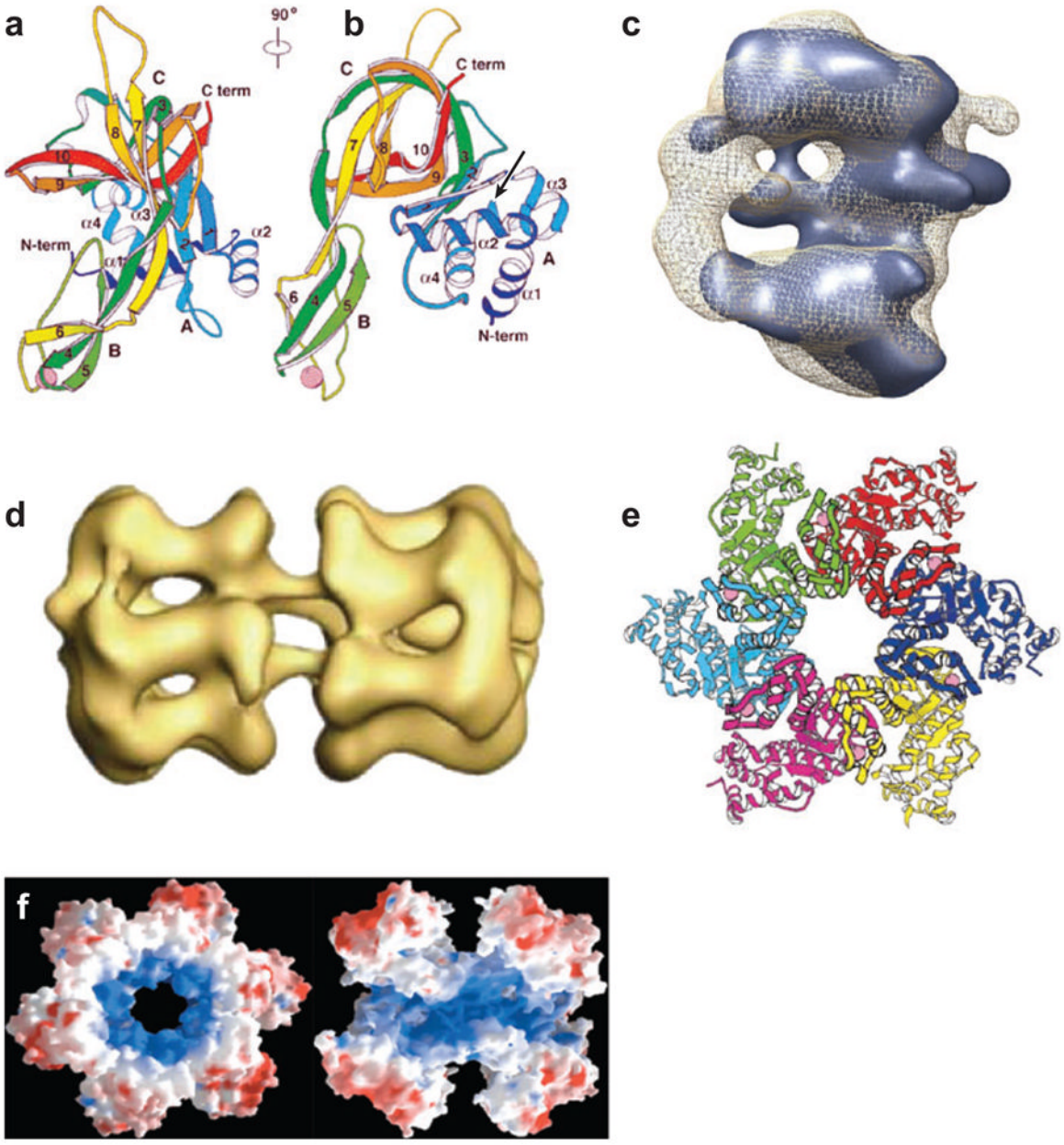

Figure 2.

Structures of ORC/Cdc6 and DNA helicases. (*a*) Ribbon diagram of the atomic structure of the N-terminal fragment of a single archaeal Mth-MCM subunit (*b*) rotated 90°. A, B, and C domains are indicated. Arrow indicates the position of the P62 residue (76). (*c*) EM reconstruction of yeast Orc/Cdc6 complex with ORC in blue (258) (*d*) EM reconstruction of the full-length archaeal double hexameric Mth-MCM complex (96). (*e*) Ribbon diagram of the atomic structure of a single hexamer of SV40 T antigen (161). (*f*) Space-filling diagram of the atomic structure of the N-terminal fragment of a single hexamer of the archaeal Mth-MCM complex (*left*) and a cut side-view (*right*) with two subunits removed for clarity; blue indicates positively charged amino acids, red indicates negatively charged amino acids (76).

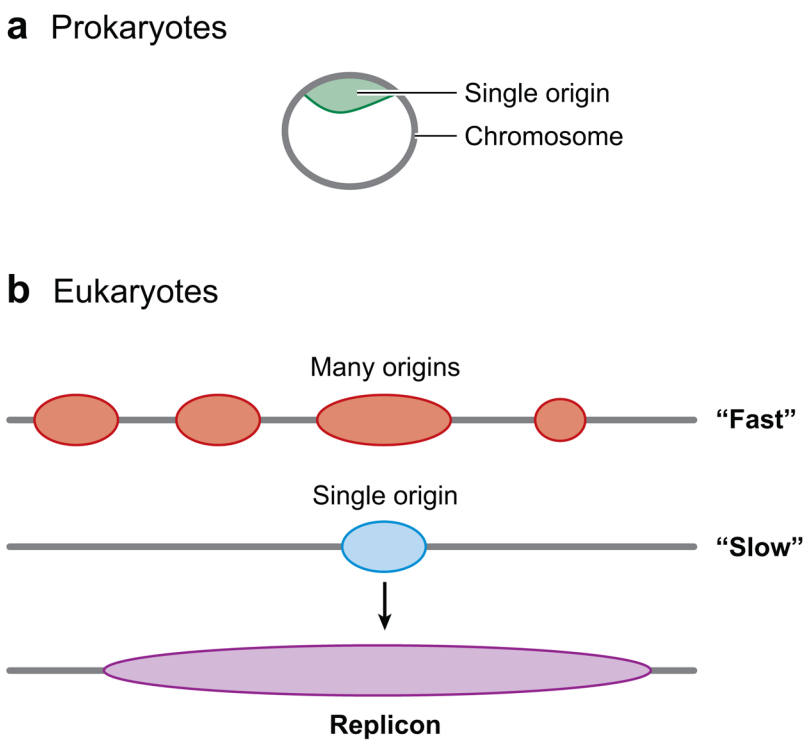

Figure 3.
Regulation of DNA replication by origin usage. (*a*) Prokaryotes have a single origin on a circular chromosome (*above*). (*b*) In eukaryotes, multiple origins are found on a single chromosome. When replication is "fast," many origins are used, whereas only one origin is used in this region when replication is "slow". Replication proceeds bidirectionally from an origin to form a replicon (*below*).

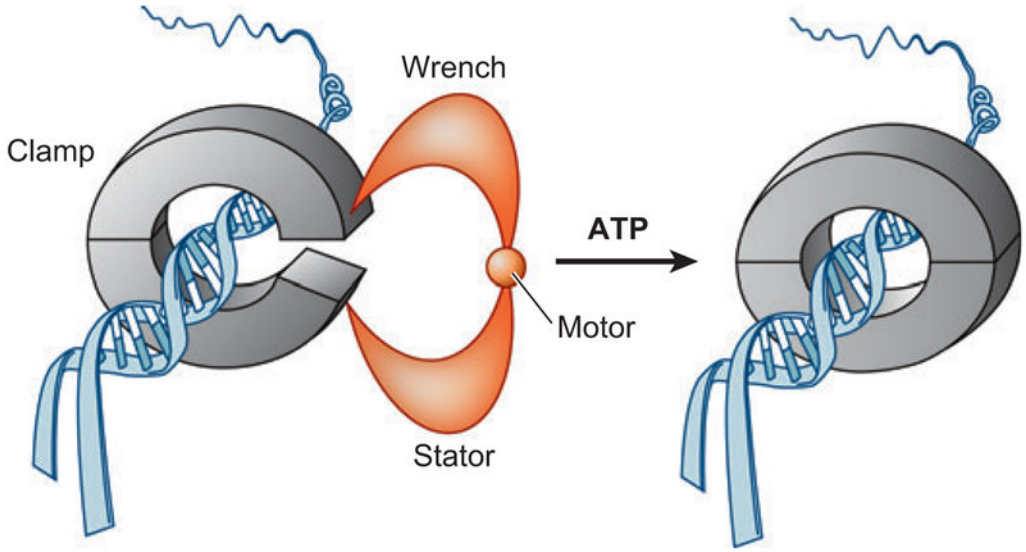

Figure 4.
The clamp loading mechanism. Clamp loader (*orange*) opens clamp using the energy from ATP hydrolysis. Clamp loader is composed of "stator," "wrench," and "motor" functions. Clamp loader fixes clamp onto the "stator" while opening the clamp with the "wrench" and "motor." Open clamp is bound to DNA and then closed. Adapted from (52).

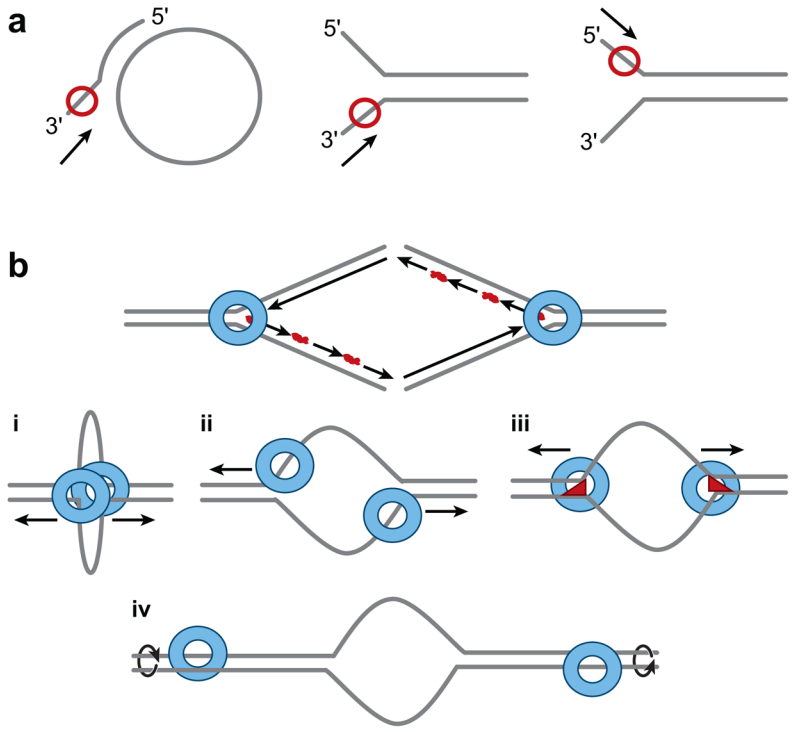

Figure 5.

Helicase substrates and models. (*a*) In vitro helicase substrates that are used frequently have small ssDNA (50 bp) annealed to ssDNA circle (5 kb) with nonhomologous 3′ tail. Helicases (*red circle*) such as SV40 T antigen or Mcm4/6/7 complex translocate 3′ to 5′ on the tail to unwind DNA and release oligonucleotide from the larger circle. Other substrates used resemble replication forks that are produced by annealing small ssDNA oligonucleotides with nonhomologous ends. Helicases can translocate 3′ to 5′ as above or 5′ to 3′ (DnaB). (*b*) A single hexameric helicase is depicted as a ring (*blue*) at the ends of a conventionally drawn replication fork. Lagging strand Okazaki fragments are shown with RNA (*red*) primers at their 5′ ends. (*i*) The SV40 T antigen model (161) is made by putting the two rings together forming a loop. In this model, the DNA is pumped into the channel of the double hexamer and then extruded out the holes in the outside C-terminal domains (Figure 2*d*). (*ii*) In the “pump-in-ring” model, each single hexamer translocates on a different strand of DNA (127). (*iii*) In the “ploughshare” model, the ploughshare (*red*) acts as a wedge and keeps the ssDNA unwound as it emerges from behind each single hexamer (271). (*iv*) In the “rotary pump” model, different single hexamers twist the DNA at a distance resulting in topological strain and unwinding in the center (151).

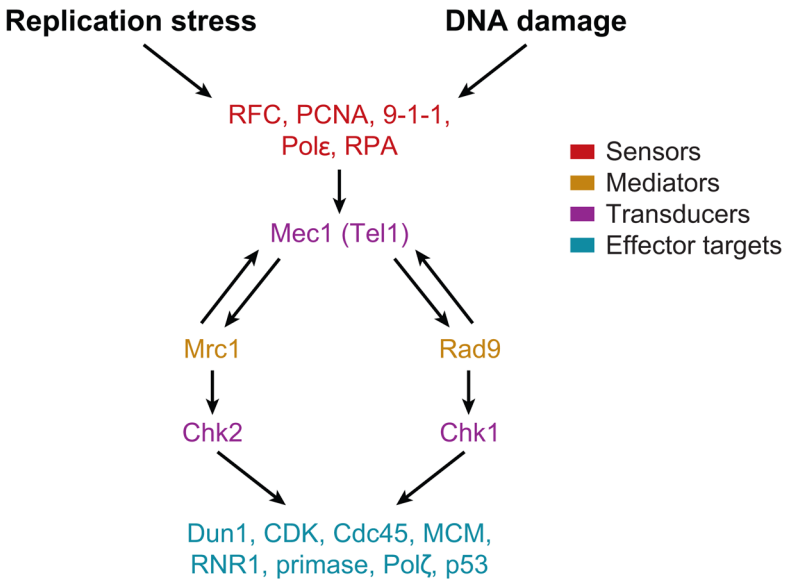

Figure 6.
DNA replication and damage checkpoint regulation. Replication stress or blockage or DNA damage induces activation of a signal transduction pathway of many different proteins. The proteins are in different classes indicated as sensors, mediators, transducers, and effector targets. For example, if DNA replication is blocked, ssDNA coated by RPA sends a signal to activate Mec1 protein kinase. Mec1 binds to Mrc1, which amplifies the signal by binding to and activating Chk2 (Rad53) protein kinase. Chk2, in turn, inhibits Cdc45 helicase activation and loading of the replisome.

Table 1

The function of eukaryotic replication proteins

Protein Name[a]	Alias/Homologues[b]	Function(s)[c]
Orc1	Orp1 Cdc6	ATPase for DNA binding
Orc2	Orp2	ATPase
Orc3	Orp3	ATPase
Orc4	Orp4	ATPase
Orc5	Orp5	ATPase
Orc6	Orp6	?
Initiative Assembly-Helicase loading		
Cdc6	Cdc18 Orc1	ATPase helicase clamp loader
Cdt1	Tah11 Dup	Helicase clamp loader
Geminin		Inhibits helicase clamp loader
Initiative Assembly-Helicase unwinding		
Mcm2	Nda1 Cdc19	ATPase helicase DDK substrate
Mcm3		ATPase helicase
Mcm4	Cdc54 Cdc21 Dpa	ATPase DDK substrate
Mcm5	Cdc46 Nda4 Bob1	ATPase helicase regulation
Mcm6	Mis5	ATPase DDK substrate
Mcm7	Cdc47 Prolifera	ATPase helicase
Mcm8		ATPase helicase
Mcm9		ATPase helicase
Mcm10	Dna43 Cdc23	Helicase Regulation DNA Primase DDK activator
Helicase Unwinding/-Elongative Assembly		
CDK-kinase	Cdc28 Cdc2 Cdk2	Protein kinase for helicase activation and replisome loading. Inhibits helicase loading to block re-replication
DDK-kinase	Cdc7 Hsk1	Protein kinase for helicase activation and replisome loading
CDK-regulatory	Clb1-6 CycA CycE	Activate Cdk1 and Cdk2 kinases
DDK-regulatory	Dbf4 Dfp1 Drf1 Chiffon Ask Him1 Rad35	Activate Cdc7 Kinase
Cdc45	Sna41	Helicase activation and replisome loading
Sld2	Drc1	Cdk substrate; loading of Dpb11 and Cdc45
Sld3		Cdk substrate; loading of Dpb11 and Cdc45
Dpb11	TopBP1 Rad4 Cut5 Mus101	Helicase activation and replisome loading
GINS complex		Helicase activation and replisome loading
Sld5	Cdc105	Helicase activation and replisome loading
Psf1	Cdc101	Helicase activation and replisome loading
Psf2	Cdc102 CG18013	Helicase activation and replisome loading
Psf3	Cdc103	Helicase activation and replisome loading
Replisome		
Polα primase holoenzyme	Pol1 Cdc17	Polymerase priming; replication of lagging strands
Polδ holoenzyme	Pol3 Cdc2	Bidirectional replication
Polε holoenzyme	Pol2 Cdc20	Bidirectional replication
Rfc1-5 complex		PCNA clamp loading
PCNA	Pol30	Polymerase clamp
RPA1-3 complex	SSB	SSB coats ssDNA

[a] Refers to the name of the protein most commonly used.

[b] Refers to other names that are used.

[c] Also refer to Figure 1*b* for description of protein function(s).