Chapter 1
Molecular Mechanism of DNA Replication

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Abstract Chromosomal DNA must be replicated faithfully and propagated to daughter cells equally. The mechanism of DNA replication is constrained by the characteristics of DNA polymerases, which synthesize chromosomal DNA; i.e., double-stranded DNA must be unwound to serve as a template and 3′-OH (RNA primer in cellular organisms) must be provided to DNA polymerases. Once these two conditions are fulfilled, DNA polymerase can start DNA synthesis everywhere. However, cells regulate this process strictly, mainly at replication origins. DNA replication initiates from replication origins, to which the initiator protein binds. DNA helicase is loaded onto origins and unwinds double-stranded DNA for the syntheses of an RNA primer and subsequent DNA by primase and DNA polymerases. As DNA polymerases elongate the DNA chain in the 5′ to 3′ direction, both strands are synthesized in opposite directions from the initiation site. The synthesis of both DNA strands (leading and lagging) continues in a manner that is coupled with DNA helicase up to its termination. These fundamental mechanisms and regulation of cellular chromosomal DNA replication are outlined using prokaryotic and eukaryotic examples.

Keywords DNA replication • Initiation • Elongation • DNA polymerase • Primase

DNA replication is a fundamental process of organisms. Although the three domains of life, Bacteria, Archaea, and Eukarya, have diverse replication machineries, the characteristics of these machineries are well conserved in cellular organisms. In contrast, virus and plasmids evolved their system more diversely, especially regarding the initiation step. In this chapter, an outline of chromosomal DNA replication in cellular organisms will be provided. For those who would like to know more detailed and comprehensive views, please refer to other reviews, such as Masai et al. (2010).

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1.1 DNA Polymerases for DNA Replication

DNA polymerases synthesize DNA. The elucidation of the characteristics of DNA polymerases is helpful for understanding the mechanistic features of DNA replication.

1.1.1 Replicative DNA Polymerases

There are various types of DNA polymerases in organisms. For example, 15 DNA polymerases have been found in human cells to date. Among them, three DNA polymerases, DNA polymerases α, δ, and ε (Polα, Polδ and Polε), participate in chromosome DNA replication (Johansson and Dixon 2013). In the case of *Escherichia coli*, only one species, DNA polymerase III (PolIII), is involved in DNA replication (McHenry 2011). The number of DNA polymerases that participate in DNA replication varies in prokaryote (e.g., *Bacillus subtilis* requires two DNA polymerases (Dervyn et al. 2001; Sanders et al. 2010)).

Replicative DNA polymerases comprise multiple subunits. The largest subunit is a catalytic subunit, which synthesizes DNA strands. Accumulated evidence suggests that noncatalytic subunits connect the catalytic subunit to other replication factors. For example, the second-largest subunit of Polα connects the catalytic subunit to primase (Johansson and Dixon 2013).

1.1.2 DNA Synthesis by DNA Polymerases

DNA polymerases elongate the DNA strand in the 5′ to 3′ direction on single-stranded templates. This implies two conditions. First, double-stranded DNA must be unwound to expose single-stranded DNA as a template. DNA helicase unwinds the double-stranded DNA at the forefront of DNA replication (called a replication fork; see below). Second, DNA polymerases synthesize DNA from opposite directions at the replication forks. One direction is the same as the direction of fork movement (leading strand), whereas the other is opposite (lagging strand). There might be a mechanism that couples the synthesis of the two strands.

Many DNA polymerases have proofreading $3' \rightarrow 5'$ exonuclease activity: if the DNA polymerase incorporates incorrect nucleotides, it can also digest the DNA strand containing misincorporated nucleotides. Among the replicative DNA polymerases, only Polα lacks this nuclease activity (Johansson and Dixon 2013).

1.1.3 Primers Used to Start DNA Synthesis

DNA polymerases cannot start DNA synthesis without a primer; the 3′-OH of ribose or deoxyribose is required. RNA polymerase starts the de novo synthesis of RNA. For cellular chromosomal DNA replication, primase synthesizes an RNA primer for subsequent synthesis of DNA by DNA polymerases. In eukaryotes, heterodimeric primase associates tightly with Polα to synthesize the RNA primer; subsequently, Polα uses this primer to synthesize short DNA strands (the length of the primer RNA + DNA is 30–35 nt) (Smith and Whitehouse 2012). In the prokaryote, *E. coli*, DnaG, which is a single peptide primase, synthesizes an RNA primer that is used subsequently by PolIII for DNA synthesis (McHenry 2011).

RNA primers are removed in several manners. In *E. coli* cells, DNA polymerase I (PolI) $5' \rightarrow 3'$ exonuclease and $5' \rightarrow 3'$ exoIX, both of which exhibit similarity to eukaryotic Flap endonuclease 1 (FEN1; see below), can digest DNA-RNA hybrids and play a role in removing the RNA primer (Fukushima et al. 2007). RNaseH digests DNA-RNA hybrids; however, it cannot cleave the rN-p-dN bond. PolI also fills the gap between DNA strands, and finally, DNA ligase seals the nick. In eukaryotes, one of two pathways works toward this purpose (Balakrishnan and Bambara 2013). Polδ peels off the previous primer, and FEN1 cleaves the junction. If the peeled DNA strand is long, Dna2, which has $5' \rightarrow 3'$ helicase activity as well as single-stranded endonuclease activity, works together with RPA single-strand-binding protein and FEN1. The long peeled-off single-stranded DNA binds to RPA (which is replaced by Dna2) and is first cleaved by Dna2 endonuclease, and the resultant short single-stranded DNA is cleaved by FEN1. The nick is sealed by DNA ligase I.

1.1.4 Sliding Clamps for Processive Synthesis of DNA Polymerases

DNA polymerases use sliding clamps to increase its processivity (number of nucleotide DNA polymerase added per association/dissociation with the template). The prokaryotic sliding clamp, the β-clamp (Fig. 1.1), has a homodimeric ring structure and embraces double-stranded DNA while tethered to PolIII. This increases the processivity greatly. In eukaryotic cells, the sliding clamp is known as proliferating cell nuclear antigen (PCNA) (Fig. 1.1). PCNA has a homotrimeric ring structure and increases the processivity of Polδ and Polε but not Polα (Hedglin et al. 2013). However, Polε has an intrinsic high processivity caused by its structure, even in the absence of PCNA (Hogg et al. 2014). The sliding clamp requires a clamp loader for its loading onto the 3′-OH end of the primer DNA. The *E. coli* $\tau^3\delta\delta'$ complex and the eukaryotic replication factor C (RFC) associate with the 3′-OH primer site and load the β-clamp and PCNA, respectively (Hedglin et al. 2013). According to the structures of ATP- and ADP-bound forms, the

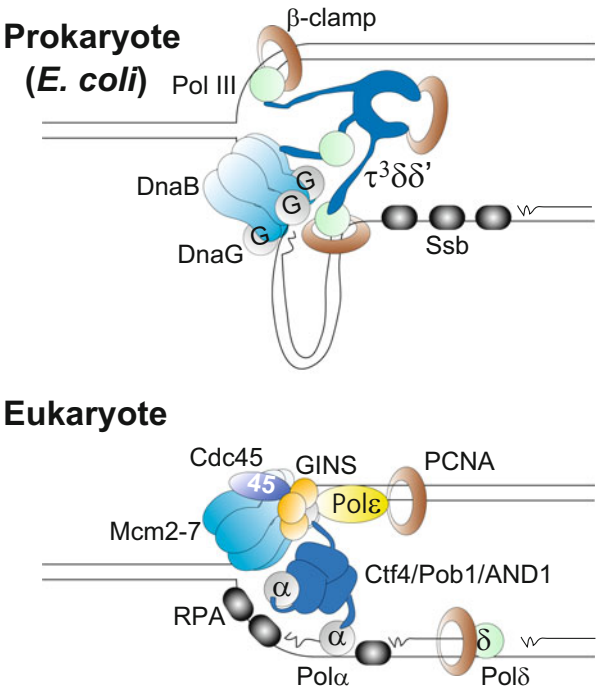

Fig. 1.1 DNA replication forks in prokaryotes and eukaryotes
Main replication machineries are depicted. See text for details

clamp loader opens the ring of the clamp and twists it to embrace the double-stranded DNA (Kelch et al. 2011). *E. coli dnaX* encodes γ and τ proteins. For the production of the τ protein, a programmed frameshift takes place. Various combinations of γ and τ proteins in the complex have been purified, and even $\gamma^3\delta\delta'$ (γ complex) exhibits loading activity for the β-clamp (Hedglin et al. 2013; McHenry 2011). The C-terminal τ-specific extension binds to PolIII for the coupling of the leading and the lagging strands (see below).

RFC is a heteropentamer consisting of Rfc1, Rfc2, Rfc3, Rfc4, and Rfc5. Although this is the major form, there are three additional forms of RFC (alternative forms) in which Rfc1 is replaced by one of Rad17 (Rad24 in budding yeast), Ctf18-Ctf8-Dcc1, and Elg1 (Tsurimoto 2006). Alternative RFCs participate in different DNA metabolism mechanisms. RFC^{Rad17} associates with 5′-OH and loads the 9-1-1 ring complex that is required for the damage checkpoint activation. RFC^{Ctf18} loads or unloads PCNA (reverse reactions are always possible) and is required for the accurate transmission of chromosomes. RFC^{Ctf18} forms a complex with Polε by binding to the catalytic subunit Pol2 (Murakami et al. 2010). The physiological meaning of the formation of this complex remains unknown. RFC^{Elg1} seems to play a role in the unloading of PCNA. A lack of RFC^{Elg1} increases the amount of PCNA in the chromatin fraction, and addition of partially purified RFC^{Elg1} releases PCNA from the chromatin fraction (Kubota et al. 2013).

PCNA interacts not only with replicative DNA polymerases but also with translesion DNA polymerases; this polymerase switch is regulated by the ubiquitination of PCNA. PCNA also interacts with many proteins that are related to replication (ligase 1, FEN1, Dna2), repair (Msh2-Msh3-Msh6, XPG, Ung), DNA methylation enzyme (Dnmt1), and histone chaperone (CAF). These interactions help the coordination of DNA replication and related reactions (Tsurimoto 2006).

1.2 Unwinding at Replication Forks

At replication forks, DNA helicase moves at the front (Fig. 1.1). Replicative helicases in cellular organisms comprise hexameric subunits, circle single-stranded DNA, and thus unwind double-stranded DNA by steric hindrance using energy of ATP hydrolysis. In *E. coli*, the replicative helicase, homohexameric DnaB, encircles the lagging-strand template and moves in a $5' \rightarrow 3'$ direction on the template DNA (LeBowitz and McMacken 1986; Itsathitphaisarn et al. 2012). In contrast, eukaryotic replicative helicase consists of the heterohexameric subunits, mini-chromosome maintenance 2–7 (Mcm2-7), circles the leading-strand template, and moves in a $3' \rightarrow 5'$ direction on the template DNA, in an opposite fashion to the prokaryotic helicase DnaB (Bell and Botchan 2013). Mcm2-7 has all conserved Mcm domains spanning AAA^+ -type ATP-binding domains. Moreover, auxiliary factors, GINS and Cdc45, and Mcm2-7 need to form a Cdc45-Mcm-GINS (CMG) complex for robust helicase activity (Ilves et al. 2010). Helicase activity is also enhanced by the association with the DNA polymerases, PolIII in *E. coli* (τ protein mediates its association; see below) (Kim et al. 1996) and Polε in human cells (Kang et al. 2012). Polδ does not accelerate the helicase activity of the CMG complex, as does Polε (Kang et al. 2012), which suggests that DNA polymerase loaded on the same template as DNA helicase enhances the activity. Moreover, the CMG complex of budding yeast recruits the leading-strand polymerase, Pol ε, preferentially (Georgescu et al. 2014).

The single-stranded DNA produced by helicase is covered by a single-stranded DNA-binding protein (SSB) (Fig. 1.1). Prokaryotic SSB has a homotetrameric structure and eukaryotic SSB, which is called replication protein (or factor) A (RPA or RFA), has a heterotrimeric structure. SSB and RPA are removed for the synthesis of DNA by DNA polymerase.

DNA replication occurs over all chromosomes. There might be obstacles such as DNA damage and large-protein-associated templates. DNA replication may suffer from shortage of precursors (dNTP). In these situations, DNA replication forks stall or are arrested. To be stalled or arrested, forks require three proteins, Mrc1/Claspin, Tof1/Swi1/Tim, and Csm3/Swi3/Tipin, on Mcm2-7 helicase. Tof1 and Csm3 form a fork protection complex (FPC) and associate with Mcm2-7. At the replication fork barrier sites in r-DNA clusters (see Chap. 10), cells require FPC to stall replication forks (Leman and Noguchi 2012). Based on this observation, it is suggested that FPC regulates Mcm2-7 helicase activity. Claspin is required for

efficient replication in *Xenopus* egg extracts. Mrc1 associates with Mcm2-7 in an FPC-dependent manner and is required for the full activation of the Rad53 checkpoint kinase in budding yeast. Because the checkpoint mediates fork stalling, Mrc1 is also required for fork stalling when the checkpoint is activated (Katou et al. 2003). Moreover, the absence of these three proteins seems to uncouple Mcm2-7 helicase and DNA polymerase. Although the mechanism underlying this phenomenon has not been uncovered, it seems likely that the activity of the replicative helicase, Mcm2-7, is regulated to adjust to conditions.

1.3 Coupling of the Leading and Lagging Strand Syntheses

DNA polymerases synthesize the leading and lagging strands at the replication forks. DnaB helicase and the clamp loader, $\tau^3\delta\delta'$, couple the synthesis of the leading and lagging strands (Fig. 1.1). The τ subunit binds to the catalytic subunit of PolIII so that three DNA polymerases are tethered to a single clamp loader, one for the leading and two for the lagging strands, to promote the coupling of the synthesis of both strands and efficient synthesis of the lagging strand (Reyes-Lamothe et al. 2010; McInerney et al. 2007). The τ subunit also interacts with DnaB helicase. Therefore, the DNA polymerases that synthesize the leading and lagging strands are placed in close vicinity, and their syntheses are coupled. Without PolIII, DnaB helicase activity is reduced, as described above. Moreover, the single hexameric DnaB helicase has the capacity binding to three DnaG primases (Corn and Berger 2006), which further helps the coupling of DNA syntheses.

In eukaryotic cells, Polδ and Polε mainly synthesize the lagging and leading strands, respectively (Fig. 1.1). During lagging-strand synthesis, Polα is frequently recruited because it tightly associates with primase (every ca. 165 bases [short Okazaki fragments in eukaryotes] (Smith and Whitehouse 2012)). In contrast to the prokaryotic system, the eukaryotic clamp loader, RFC, has not been implicated in the coupling of leading- and lagging-strand DNA syntheses. Rather, GINS, which is a component of active replicative helicase, and Ctf4 may work toward the coupling (Tanaka et al. 2009; Gambus et al. 2009). GINS, which is a heterotetrameric complex, consists of the Sld5, Psf1, Psf2, and Psf3 subunits (Kubota et al. 2003; Takayama et al. 2003). The N-terminal portion of the Sld5 subunit binds to Ctf4/Pob1 (AND1 in mammalian cells) in yeast, which in turn binds to the N-terminal portion of the catalytic subunit of Polα. A recent study revealed that Ctf4 forms a homotrimeric complex, each subunit of which has the ability to bind to either GINS or Polα (Simon et al. 2014). Thus, it is proposed that two Polα molecules are tethered to the helicase via one GINS molecule. As described for the τ protein in *E. coli* cells, this tethering may promote the efficient synthesis of the lagging strand and the coupling of lagging-strand synthesis with the helicase. Conversely, the Psf1 subunit of GINS binds to Dpb2, which is the second-largest subunit of Polε (Sengupta et al. 2013). This suggests that leading-strand polymerase Polε is

tethered to the helicase via GINS. Therefore, the leading- and lagging-strand polymerases seem to be tethered to the GINS component of the active replicative helicase CMG.

1.4 Establishment of Replication Forks

1.4.1 Loading of DNA Helicases onto Replication Origins

Replication forks form at replication origins, which are specified by the initiator protein or origin-binding proteins. In *E. coli,* DnaA binds to a specific DNA sequence termed DnaA box. DnaA binds to ATP and has ATPase activity, while the ATP form of DnaA binds to the DnaA box with higher affinity than does the ADP form. Binding of DnaA to multiple DnaA boxes located at replication origins melts double-stranded DNA partially with Fis and IHF proteins. The replicative helicase, DnaB hexamer, is loaded onto this single-stranded DNA, to circle single-stranded DNA (Costa et al. 2013; Bell and Kaguni 2013) (Fig. 1.2).

Eukaryotic cells have a heterohexameric origin recognition complex (ORC; Orc1-6) that binds to replication origins (Fig. 1.2). In general, a single ORC binds to one replication origin, although origins are clustered at a locus and, thus, multiple ORCs bind to the limited region. Orc1, Orc4, and Orc5 bind to ATP to associate with origins. The architecture of the ORC is suggested to be similar to that of the binding of DnaA to multiple DnaA boxes at origins (Clarey et al. 2006; Ozaki et al. 2012). The budding yeast ORC recognizes a short specific DNA sequence (ARS conserved sequence, ~10 bp) at replication origins and associates throughout the cell cycle. In the case of fission yeast, Orc4 has an AT hook and ORC binds to the AT-rich region, which can be predicted by a computer program. In mammals, Orc1 is degraded or dissociated from chromatin during the G2 and M phases. Moreover, ORC does not have binding specificity, so that replication origins are determined by the chromatin environment rather than by DNA sequence. In contrast with DnaA of *E. coli*, melting of origin DNA by binding with ORC has not been reported (Costa et al. 2013; Bell and Kaguni 2013).

Replicative DNA helicase is loaded onto the replication origins that are associated with initiator proteins (e.g., DnaA and ORC) (Fig. 1.2). In *E. coli*, one hexameric DnaB helicase forms a complex with six DnaC proteins (the 6 DnaB:3 DnaC form has also been isolated (Makowska-Grzyska and Kaguni 2010)) and is loaded onto the molten single-stranded DNA by DnaA. DnaC is an ATP-binding AAA^+ protein that opens the ring of hexameric DnaB to encircle the single-stranded DNA (Arias-Palomo et al. 2013). The loading of DnaB seems to occur one by one for leading- and lagging-strand templates using the DnaB-DnaA interaction (Costa et al. 2013). In some prokaryotes, this step requires additional factors (Li and Araki 2013), although their function has not been elucidated well.

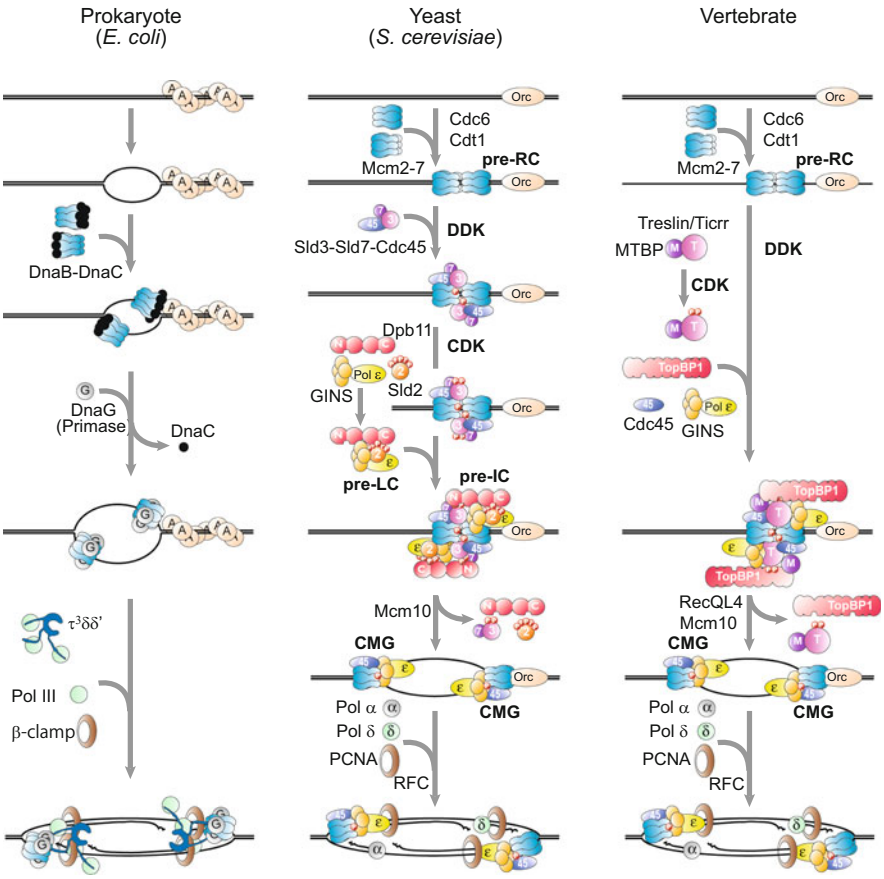

Fig. 1.2 The initiation step of chromosomal DNA replication in prokaryote and eukaryotes Counterparts of *E. coli* $\tau^3\delta\delta'$ in yeast and vertebrates shown in Fig. 1.1 are not depicted. See text for details

In eukaryotes, a pair of Mcm2-7 helicase cores (head [N terminus] to head orientation) is loaded onto replication origins to form the prereplicative complex (pre-RC: Fig. 1.2). This reaction requires three additional factors, ORC, Cdt1, and Cdc6. Budding yeast Cdt1 associates with Mcm2-7 and keeps the ring open. Cdc6 is an ATP-binding AAA^+ protein that associates with ORC. The ATP form recruits the Mcm2-7-Cdt1 complex onto ORC-bound origin DNA (Bell and Kaguni 2013; Costa et al. 2013). An in vitro reaction for the formation of the pre-RC revealed fast recruitment of the first Mcm2-7 core and slow recruitment of the second Mcm2-7 core onto origin DNAs (Riera et al. 2014). The first Mcm2-7 forms a transient intermediate on origins and is sensitive to high salt. If the loaded Mcm2-7 is crippled, Mcm2-7 is dissociated. Once the second Mcm2-7 is recruited successfully, a pair of Mcm2-7 on origins is stabilized. The stably loaded Mcm2-7 encircles the double-stranded DNA and is resistant to high salt. The in vitro reaction

further showed that the C-terminal portion of Mcm3 is essential for the recruitment of the Mcm2-7 complex (Frigola et al. 2013) and that the C-terminal portion of Mcm6 is inhibitory and masked by Cdt1 (Fernández-Cid et al. 2013). To date, the exact mechanism underlying the formation of a stable loaded complex by two Mcm2-7 molecules has not been described. Because Orc5 has two Cdt1-binding sites (Takara and Bell 2011), ORC-(Mcm2-7-Cdt1)^2 formation may be the signal that allows stable pre-RC formation. ORC-Cdc6 is similar to RFC structurally (Sun et al. 2013).

1.4.2 Activation of DNA Helicase and Formation of the Replication Forks at Origins

In *E. coli* cells, dissociation of DnaC from DnaB, which is enhanced by the DnaG primase (Makowska-Grzyska and Kaguni 2010), allows DnaB helicase activity (Fig. 1.2). τ-PolIII further enhances this helicase activity. Thus, once DnaB helicase is activated, replication forks form automatically. Moreover, *Helicobacter pylori* does not have the *dnaC* gene and its *dnaB* gene complements defective *dnaB* and *dnaC* of *E. coli* (Soni et al. 2003, 2005), suggesting that DnaB of *H. pylori* is loaded without DnaC.

In eukaryotes, Cdc45 and GINS associate tightly with Mcm2-7 to exhibit helicase activity (Bell and Botchan 2013; Costa et al. 2011; Ilves et al. 2010; Moyer et al. 2006; Tanaka and Araki 2013) (Fig. 1.2). This phenomenon occurs at replication origins with the aid of many replication factors and is highly regulated by the cell cycle. Sld3 functions as the hub for the recruitment of Cdc45 and GINS to yeast replication origins (Kamimura et al. 2001; Nakajima and Masukata 2002). Budding yeast Sld3 forms a complex with Sld7 (Tanaka et al. 2011b). The Cdc45-Sld3 association occurs throughout the cell cycle (Kamimura et al. 2001); however, it seems to be disrupted by the activation of the cell cycle checkpoints (Kanemaki and Labib 2006). This complex associates with the pre-RC formed origins in a DDK-dependent manner (Tanaka et al. 2011a; Yabuuchi et al. 2006; Heller et al. 2011). DDK is a Dbf4-dependent protein kinase or Cdc7 protein kinase that is required for DNA replication in eukaryotes. DDK phosphorylates the N-terminal stretches of Mcm2 and Mcm6 heavily (Sheu and Stillman 2006, 2010), and this phosphorylation may promote the recruitment of the Sld3-Cdc45 complex. Cyclin-dependent kinase (CDK), which is essential for the onset of the S phase (initiation of DNA replication), phosphorylates two replication proteins, Sld2 and Sld3, in budding yeast to initiate chromosomal DNA replication. Phosphorylated Sld2 and Sld3 bind to another replication protein Dpb11. Dpb11 has two pairs of tandem Brca1 C-terminal repeats (BRCT), which is a phosphopeptide-binding domain. The N-terminal and C-terminal pairs bind to CDK-phosphorylated Sld3 and Sld2, respectively (Tanaka et al. 2007; Masumoto et al. 2002; Zegerman and Diffley 2007). The CDK-dependent association between Sld2 and Dpb11 lures GINS and

Polε and forms the pre-loading complex (pre-LC), which includes Sld2, Dpb11, GINS, and Polε (Muramatsu et al. 2010). Subsequently, the interaction between Dpb11 and CDK-phosphorylated Sld3 recruits GINS via the pre-LC (Tanaka and Araki 2010; Araki 2010). In this scenario, two protein kinases, DDK and CDK, participate in the recruitment of Cdc45 and GINS, and Polε functions as a protein scaffold at the initiation step, rather than as a DNA polymerase (Muramatsu et al. 2010; Handa et al. 2012). Sld2, Dpb11, and Sld3 function only at the initiation step and not at the elongation step. The association of GINS with the spacer region located between pairs of BRCT domains is important for efficient replication. This interaction is conserved in vertebrates GINS and TopBP1 (Dpb11 homologue in vertebrates; see below) (Tanaka et al. 2013). The Mcm10 protein functions in the late step of initiation, because although Cdc45 and GINS associate with replication origins and form a tight complex in the absence of Mcm10 origin, DNA is not unwound (Kanke et al. 2012; van Deursen et al. 2012; Watase et al. 2012; Thu and Bielinsky 2013). However, the molecular function of Mcm10 remains unknown.

The mechanism underlying the formation of replication forks seems to be conserved, to some extent, in Metazoa (Fig. 1.2). TopBP1 or its relatives, which is a probable counterpart of Dpb11 in Metazoa, have multiple BRCT domains (Makiniemi et al. 2001; Hashimoto and Takisawa 2003). *Xenopus* and human TopBP1s have nine BRCT domains (Rappas et al. 2011; Huo et al. 2010), and the peptide spanning the first four N-terminal BRCTs (BRCT0, BRCT1, BRCT2, and BRCT3) supports DNA replication in *Xenopus* egg extracts (Kumagai et al. 2010). This peptide binds to CDK-phosphorylated Treslin/Ticrr (Kumagai et al. 2011, 2010; Sansam et al. 2010; Boos et al. 2011), which is a counterpart of Sld3. Among the four BRCTs, BRCT1 and BRCT2 contain the phosphopeptide-binding patches, and the fourth BRCT (BRCT3) from the N terminus is dispensable for Treslin binding. Treslin has homology to the Sld3 central region, which binds to Cdc45 and conserved CDK phosphorylation sites (Sanchez-Pulido et al. 2010). The N-terminal Treslin binds to MDM2 binding protein (MTBP), which is also required for DNA replication (Boos et al. 2013). The N-terminal portion of Sld3 also binds to Sld7 (Tanaka et al. 2011b). RecQL4 of Metazoa has similarity with Sld2 in the N-terminal portion that precedes the helicase domain and is required for DNA replication (Matsuno et al. 2006; Sangrithi et al. 2005). However, the interaction between TopBP1 and RecQL4 does not depend on CDK phosphorylation (Matsuno et al. 2006). Treslin associates with chromatin in a manner that depends on the pre-RC, but not on TopBP1. TopBP1 chromatin binding depends on the pre-RC but not on Treslin. Thus, it is suggested that TopBP1 and Treslin form a complex on chromatin in a CDK-phosphorylation-dependent manner and then stably associate with chromatin. This is consistent with the fact that CDK facilitates the association of TopBP1 with chromatin (Hashimoto and Takisawa 2003). In the absence of recQL4, TopBP1, Cdc45, GINS, and Polε associate with chromatin, whereas Polα and RPA do not (Sangrithi et al. 2005; Matsuno et al. 2006). These observations suggest that recQL4 functions at the late initiation stage, before the unwinding of origin DNA, unlike Sld2. Nematoda do not have recQL4, unlike other Metazoa; instead, they express SLD-2, which does not possess a helicase domain but exhibits

homology to Sld2 of budding yeast. SLD-2 binds to Nematoda TopBP1 (Mus101) in a CDK-phosphorylation-dependent manner, and mutations of the CDK phosphorylation sites of SLD-2 confer warm lethality (Gaggioli et al. 2014). Thus, Nematoda may initiate DNA replication via a mechanism that is similar to that of yeast. Future studies will reveal the details of the initiation step of DNA replication in Metazoa.

1.5 Regulation of the Initiation Step of DNA Replication

DNA replication efficiency is mainly regulated by the initiation step of DNA replication. Once replication starts, the replication is completed unless the replication forks stall because of DNA damage and shortage of precursors (see below).

In *E. coli*, the association between DnaA and origin DNA is regulated (Katayama et al. 2010; Skarstad and Katayama 2013). The protein level of DnaA increases at the initiation step of DNA replication. Moreover, an increase in the level of ATP in good nutrient conditions increases the ATP-DnaA form and enhances the initiation of DNA replication (multiple initiations occur in bacteria). Conversely, the association between DnaA and origin DNA is inhibited once replication starts. The system termed regulatory inactivation of DnaA (RIDA) regulates the DnaA nucleotide form. The Hda protein, which is homologous to DnaA, binds to ADP and forms a complex with the β-clamp that is loaded on DNA and is released from DNA polymerase. This complex binds to ATP-DnaA and promotes the hydrolysis of ATP on DnaA, leading to a decrease in the ATP-DnaA form. Origin DNA is also protected from the reassociation of DnaA by the SeqA protein. Origin DNA contains many GATC sequences, which are methylated by Dam methylase. During DNA replication, hemimethylated DNA spanning the GATC sequences appears. The SeqA protein binds to hemimethylated GATC sequences and prevents the immediate association of DnaA.

In eukaryotic cells, the cell cycle regulates the formation of the active helicase, as described above. The protein levels of several replication proteins fluctuate during the cell cycle, via transcription and degradation of the proteins. Moreover, some of the replication proteins are modified for regulation (Siddiqui et al. 2013).

ORC association with origins is regulated in mammals, whereas its association is observed throughout the cell cycle in budding yeast. The pre-RC forms mainly in G1 phase (late M phase is also possible in budding yeast), a time at which CDK activity is low. At the G1/S boundary of yeasts, CDK phosphorylates Sld2 and Sld3 to promote the initiation of DNA replication, as described above. Concomitantly, CDK phosphorylates ORC, Mcm2-7, Cdt1, and Cdc6, all of which function at the step of pre-RC formation. CDK-phosphorylated Mcm2-7 and Cdt1 are excluded from the nucleus, and CDK-phosphorylated Cdc6 is degraded. These proteins are regulated by different mechanisms in different organisms. In fission yeast, Mcm2-7 stays in the nucleus throughout the cell cycle, and Cdt1 and Cdc6 are degraded. In

mammals, Cdc6 is excluded from the nucleus and Cdt1 is also degraded. In later period, geminin binds to residual Cdt1 to inactivate it (Siddiqui et al. 2013).

All origins are not fired in a single cell cycle (some are dormant), although the pre-RC forms at all origins. The temporal regulation of origin firing occurs at the step of the CDK-dependent formation of replication forks in budding yeast, because increased dosages of either combinations of Sld3-Sld7 and Cdc45 (Tanaka et al. 2011a) or Dpb11, Sld2, Sld3, and Cdc45 (Mantiero et al. 2011) diminish temporal regulation; all origins fire almost at the same time. In *Xenopus* egg extracts, increased CDK activity facilitates origin firing in mammalian nuclei (Thomson et al. 2010). This is consistent with budding yeast regulation (see Chap. 2 for details).

1.6 Alternative Pathways for the Formation of Replication Forks

DNA replication starts at origins. However, cellular organisms have alternative pathways to start DNA replication outside conventional origins, probably as a backup system. Two characteristics of DNA polymerases are important; DNA polymerases need single-stranded DNA as a template and a 3′-OH from the primer. The stable DNA replication or restart mechanism of stalled replication forks in *E. coli* has been long known (Kogoma 1997). PriA and PriC work toward this purpose (Gabbai and Marians 2010). PriA binds to single-stranded DNA and has helicase activity in the $3' \rightarrow 5'$ direction. It functions to remodel the lagging-strand template to expose single-stranded DNA to load the helicase for restart of stalled forks. PriB and DnaT are recruited to PriA bound to single-stranded DNA; in turn, they recruit the DnaB to the site. Replication forks are then formed. The PriA-PriB-DnaT system prefers a short DNA gap that occurred at replication forks. In contrast, PriC recruits DnaB to a long DNA gap. PriC binds to Rep, which possesses helicase activity in the $3' \rightarrow 5'$ direction and remodels the lagging-strand template, and recruits DnaB-DnaC. Moreover, when the DNA strand invades double-stranded DNA and forms a D-loop via the action of recombination proteins, the PriA-PriB-DnaT system recruits DnaB to form replication forks. Furthermore, in RNaseH-deficient cells, the conventional origin, oriC, is repressed; therefore, replication starts from oriK. At this origin, transcription and DNA polymerase I (PolI) are essential for the initiation of DNA replication. Thus, an R-loop model is proposed: RNA polymerase transcribes, and the resulting transcript is taken over by PolI, while PriA-PriB-DnaT recruits DnaB helicase. This initiation requires the recA recombination protein but not other recombination proteins (Kogoma 1997). The recA protein may facilitate R-loop formation.

In yeast, break-induced replication (BIR) has been described (Anand et al. 2013). In this replication, similar to the *E. coli* system, recombination proteins transfer single-stranded DNA to parental double-stranded DNA, and the resultant

D-loop is used for initiation. Pif1 helicase is loaded onto this D-loop, and Polδ synthesizes DNA using it as a primer, together with RFC and PCNA (Saini et al. 2013). The BIR reaction is reconstituted from the Rad51 (a counterpart of recA in eukaryotes), Pif1, Polδ, RFC, and PCNA proteins (Wilson et al. 2013). BIR is also observed in human cells (Costantino et al. 2014).

In the archaea *Haloferax volcanii*, prominent replication origins are dispensable and other origins are not detected in the cells that lack the origins. Moreover, the cells that lack the origins require RadH, which functions for homologous recombination (Hawkins et al. 2013). Therefore, recombination-dependent initiation may take place in this organism.

1.7 Termination of Replication

In eukaryotic cells, DNA replication initiates at many replication origins in both directions. When the replication forks moving in opposite directions meet, they are postulated to terminate. However, a topological problem remains. At the front of replication forks, the replicative helicase unwinds the double-stranded DNA, which leads to the accumulation of helical stress (positive supercoils and/or precatenanes); this can be relaxed by topoisomerases. This helical stress is not easily relaxed at the termination region, and catenated molecules appear. This structure is solved by topoisomerase II (Baxter and Diffley 2008).

In the case of *E. coli*, the termination region is predetermined on circular chromosomal DNA. This is caused by the binding of a termination protein, termed Tus. The Tus protein binds to multiple sites in the terminal region and blocks one direction of DNA replication (Neylon et al. 2005). Topoisomerase IV, a type II topoisomerase, separates the catenane caused by termination, and the site-specific recombination system, XerCD/dif, partially compensates for this function (Duggin et al. 2008).

When replication forks reach the end of the linear chromosomes (telomere), the RNA primer of the lagging strand at the end is removed; however, conventional DNA polymerases cannot fill out the remaining single-stranded DNA region. Telomerase is recruited to the end, and a telomere repeat sequence is synthesized and added to the end of the strand.

1.8 Chromatin in DNA Replication

Chromosomal DNA binds to various proteins, which affects its structure. Although histones are well-known protein in eukaryotes, various chromosome-associated proteins contribute to DNA metabolism in prokaryotes. To initiate DNA replication, origin DNA binds not only to DnaA but also Fis and IHF proteins, which coordinately facilitate the DnaA-mediated melting of origins (Kaur et al. 2014). HU

proteins bind to DNA nonspecifically and enhance the melting of replication origins (Chodavarapu et al. 2008).

Eukaryotes have a nucleosome structure consisting of histone octamers. Histones are also modified (e.g., acetylated, methylated, or phosphorylated) and bind to other chromatin proteins. Most origins do not have nucleosomes in budding yeast. At the initiation, nucleosome structure affects the binding of ORC. In the case of yeast, the nucleosome stabilizes the bound ORC (Hizume et al. 2013). In Metazoa, ORC does not have binding specificity and probably binds to nucleosome-free regions (MacAlpine and Almouzni 2013).

During the elongation steps, nucleosome formation is very dynamic. To synthesize DNA, nucleosomes are first removed. After DNA synthesis, nucleosomes are reconstituted (MacAlpine and Almouzni 2013). The replisome progression complex that is formed at the replication forks contains FACT complex (Gambus et al. 2006), which is a histone chaperone that is required for the reassembly of histones to nucleosome. To disassemble the nucleosomes, Mcm2-7 encounters these structures and Mcm2 seems to function to manage the nucleosome (Foltman et al. 2013). Histones are modified at a specific locus, to repress transcription or to form a tight structure. These modifications are conserved during replication. Although the manner via which these modifications are inherited after replication is unknown, many DNA replication proteins are suggested to be involved in this inheritance.

1.9 Perspectives

The outlines of chromosomal DNA replication have been described at the molecular level. However, the coordinated replication of chromosomal DNA and the regulation of DNA replication have not been well elucidated. Eukaryotic DNA replication, especially in multicellular organisms, has not been well described. In addition, DNA replication with other aspects related to DNA metabolism, such as recombination, repair, chromatin, and epigenetics, has not been well documented. Moreover, higher phenomena, such as development and neurogenesis, may be related to DNA replication. In future studies, although the fundamental aspects of DNA replication will not change, this process will be described from the perspective of wider biological phenomena or reactions.

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