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Review

DNA replication origins, ORC/DNA interaction, and assembly of pre-replication complex in eukaryotes

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Chromosomal DNA replication in eukaryotic cells is highly complicated and sophisticatedly regulated. Owing to its large size, a typical eukaryotic genome contains hundreds to tens of thousands of initiation sites called DNA replication origins where DNA synthesis takes place. Multiple initiation sites remove the constraint of a genome size because only a certain amount of DNA can be replicated from a single origin in a limited time. The activation of these multiple origins must be coordinated so that each segment of chromosomal DNA is precisely duplicated only once per cell cycle. Although DNA replication is a vital process for cell growth and its mechanism is highly conserved, recent studies also reveal significant diversity in origin structure, assembly of pre-replication complex (pre-RC) and regulation of replication initiation along evolutionary lines. The DNA replication origins in the fission yeast *Schizosaccharomyces pombe* are found to contain a second essential element that is bound by Sap1 protein besides the essential origin recognition complex-binding site. Sap1 is recently demonstrated to be a novel replication initiation protein that plays an essential role in loading the initiation protein Cdc18 to origins and thus directly participates in pre-RC formation. In this review, we summarize the recent advance in understanding how DNA replication origins are organized, how pre-RC is assembled and how DNA replication is initiated and regulated in yeast and metazoans.

Keywords DNA replication origin; origin recognition complex; pre-replication complex; replication initiation

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Introduction

Replication of chromosomal DNA in eukaryotes initiates at multiple DNA replication origins that are distributed on all chromosomes. The budding and fission yeast have ~500 origins in a genome of $\sim 1.4 \times 10^7$bp [1–3], whereas a human cell has $\sim 5 \times 10^4$ origins in 23 chromosomes of 3×10^9bp [4]. Multiple initiation sites remove the constraint to replicate a huge amount of DNA in a limited time. However, a strict regulation system must have evolved to coordinate initiation of DNA synthesis from these hundreds to thousands of initiation sites such that each DNA replication origin fires only once per cell cycle and no segment of chromosomal DNA is ever duplicated more than once or leaves unreplicated. Only in this way, two daughter cells will inherit a complete set of chromosomes after each cell division.

The first step of replication initiation involves assembly of pre-replication complex (pre-RC). Like most biological events in which a major regulation often takes place at the very beginning, assembly of pre-RC is indeed the main target for regulating replication initiation. Although DNA replication is a vital process for cell growth, the assembly of pre-RC as well as its regulation shows a great degree of variety among eukaryotes. The variation is manifested both in the components involved in pre-RC formation and DNA replication origins on which pre-RC assembles. This review summarizes the most recent findings in studying the structures of DNA replication origins and assembling process of pre-RC in the budding yeast *Saccharomyces cerevisiae*, the fission yeast *Schizosaccharomyces pombe* and metazoans.

DNA Replication Origins and ORC/DNA Interaction in Yeast

Over 45 years ago, Jacob *et al.* [5] proposed that an initiator would act on a replicator to facilitate DNA replication. This hypothesis has been proved true from prokaryotes to eukaryotes even though the selection mechanism of replicators in

metazoans appears more complicated and the nature of metazoan replicators is still not yet defined. In *Escherichia coli*, DNA replication origin named *oriC* is a sequence of ~250 bp. *oriC* contains several copies of a consensus sequence of 9 bp that are bound by DnaA, an origin recognition protein. This 9-bp sequence is highly conserved among enteric bacteria (*Escherichia*, *Salmonella*, *Enterobacter*, *Kiebsiella*, and *Erwinia*). Most substitutions in this conserved sequence are not tolerated. Besides this sequence, bacterial DNA replication origins also contain a second conserved element that is AT-rich sequence and is the site for opening the duplex for loading DnaB helicase and subsequent initiation of DNA synthesis. A plasmid harboring *oriC* is able to replicate extra-chromosomally.

By screening for autonomously replicating sequences (ARS) that render a plasmid capable of replicating extra-chromosomally in yeast cells, the budding yeast *S. cerevisiae* origins were identified [6,7]. *Saccharomyces cerevisiae* DNA replication origins have been extensively studied and are probably the best understood eukaryotic origins [8,9]. Much like *oriC* in structure, the size of *S. cerevisiae* DNA replication origins is around 100−150 bp. They contain an essential 11-bp consensus sequence of 5′-(A/T)TTTA (T/C)(A/G)TTT(A/T)-3′ called 'A' element and a non-conserved B region that is also important for origin activity [10]. The 'A' element is the binding site of origin recognition complex (ORC) [11]. Together with the 'A' element, a part of the 'B' region (close to the 'A' element) is used for assembly of pre-RC [12]. The initiation site for bi-directional DNA synthesis is located at the 'B' region. The majority of *S. cerevisiae* DNA replication origins contain only one 'A' element and so only one ORC molecule binds to each origin.

By searching for the 'A' element-binding protein, ORC was first identified in *S. cerevisiae* [11]. ORC is a protein complex composed of six different subunits—Orc1, Orc2, Orc3, Orc4, Orc5, and Orc6. The specific interaction between *S. cerevisiae* ORC (*Sc*ORC) and the 'A' element requires the presence of ATP [11]. The Orc1 and Orc5 subunits that contain ATP-binding domain bind ATP, but only Orc1 hydrolyzes ATP [13]. The cross-linking analysis indicates that the five subunits (Orc1−5) of *Sc*ORC contact with DNA and are responsible for *Sc*ORC to bind to the 'A' element (**Fig. 1**) [14]. The Orc6 does not contact with DNA. *Sc*ORC binds to origins throughout cell cycle. There are about 400 DNA replication origins in the budding yeast genome. These origins initiate DNA synthesis at different times in S phase with some firing early and the others firing late [1,2].

The second intensively studied eukaryotic origins are the fission yeast *S. pombe* DNA replication origins [15]. Surprisingly, *S. pombe* origins are quite different from *S. cerevisiae* origins in structure. First, *S. pombe* origins have a size of 500−1500 bp, which are 5−10 times larger than *S. cerevisiae* origins. Second, *S. pombe* origins lack an apparent consensus sequence, but they do contain two or more highly important or essential elements rather than one essential element found in *S. cerevisiae* origins. These highly important or essential elements are often very asymmetric AT-rich sequences with A in one strand and T in the other based on genetic deletion studies in origins ARS1, ARS3002, ARS3001, and ARS2004 [16−19]. Like the budding yeast origins, *S. pombe* origins also have ARS activity and so plasmids harboring a *S. pombe* origin are able to replicate and be stably maintained in fission yeast cells. There are ~500−1000 strong and weak origins distributed on three chromosomes, the majority of which fire early in S phase and only a few initiate DNA replication late [3,20−22].

Compared with *Sc*ORC, *S. pombe* ORC (*Sp*ORC) has an extra domain located in the N-terminal half of its Orc4 subunit. This additional domain contains nine AT-hook motifs that bind to asymmetric AT-rich sequences. Although *S. pombe* origins do not have a consensus sequence, biochemical studies indicate that *Sp*ORC does not bind to its cognate origins randomly [23−26]. Examination of *Sp*ORC/DNA interaction indicates that: (i) the nine AT-hook motifs of S*p*Orc4 is solely responsible for *Sp*ORC to bind to DNA (**Fig. 1**); (ii) *Sp*ORC binds to those highly important asymmetric AT-rich sequences; (iii) ATP is not required for the interaction between *Sp*ORC and DNA; (iv) there are generally two or more ORC-binding sites in each *S. pombe* origin. As stated above, the ORC/DNA interaction is quite different between *S. cerevisiae* and *S. pombe* (**Fig. 1**).

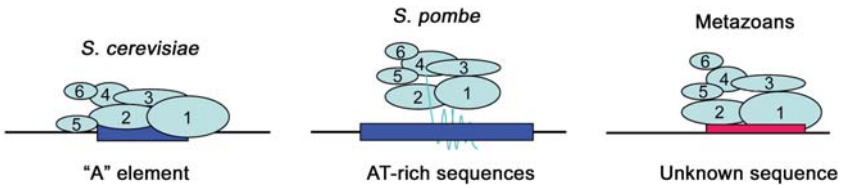

Figure 1 The interaction of ORC and DNA in the budding yeast *S. cerevisiae*, the fission yeast *S. pombe* and metazoans *Sc*ORC uses its five subunits (Orc1−5) to bind specifically to the 'A' element in the presence of ATP. Orc6 does not contact with DNA. *Sp*ORC uses the 9 AT-hook motifs located in the N-terminal half of Orc4 to bind to asymmetric AT-rich sequences. ATP does not affect *Sp*ORC/DNA interaction. The interaction between metazoan ORC and DNA is still not defined. It remains to be determined regarding what sequences metazoan ORC prefers binding to and whether an intrinsic ORC accessory protein guides it to a specific sequence.

Besides the essential ORC-binding site, *S. pombe* origins contain another type of essential site that is bound by Sap1 protein (**Fig. 2**). Sap1 was initially identified as a protein bound to a sequence involved in mating-type switching [27]. However, it has also been found to be essential for cell viability; thus, it must perform other function(s) essential for cell growth [28]. Some studies suggest that Sap1 plays a role in replication fork stability [29,30]. It also shows that Sap1 binds to the origin ARS2004 [29]. We recently demonstrate that Sap1 binds to an essential element in *S. pombe* origin ARS3001 (Yang *et al.*, submitted). ARS3001 contains two essential elements, Δ3 and Δ9 [16]. The footprinting assay indicates that Δ3 is bound by ORC and Δ9 is bound by an unknown protein throughout cell cycle [31]. Later, the Δ9-binding protein was isolated and identified to be Sap1. The finding that Sap1 binds to the essential Δ9 element implied that Sap1 might play an essential role in replication initiation. The further studies of Sap1 indicates that Sap1 binds to all origins and is required to load the replication initiation protein Cdc18 to DNA replication origin and thus it directly participates in assembly of pre-RC and replication initiation. Therefore, Sap1 is a replication initiation protein (Yang *et al.*, submitted). Sap1 prefers to bind to a sequence of $(A/T)_{4+n}$(**C/G**)$(A/T)_{10-11}$(**C/G**)$(A/T)_{4+n}$ (*n* could be 0 or a number; the bold bases **C/G** are important for Sap1 binds to this sequence). Like ORC, there are probably more than one Sap1-binding site in each origin with various binding affinity. In conclusion, *S. pombe* origins contain two types of essential elements that are bound by ORC and Sap1, respectively (**Fig. 2**). The presence of two types of essential elements explains the large size of *S. pombe* origins (Yang *et al.*, submitted).

Assembly of pre-RC

Assembly of pre-RC takes place at late M and early G1 phase when the cyclin-dependent kinase (CDK) activity is low. The high CDK activity prevents pre-RC assembly by phosphorylating components of pre-RC and inhibiting their activities [32]. In budding yeast, ORC acts as a loading pad to recruit Cdc6 to origins at early G1 phase. Then, ORC and Cdc6 together load Cdt1–MCM to origins to form a pre-RC (**Fig. 2**) [12,33,34]. In this process, the hydrolysis of ATP by both ORC and Cdc6 is required. The mutations that eliminate the ATPase activity of ORC or Cdc6 are lethal to cells and show defects in pre-RC formation [35,36]. Moreover, the loading of MCM to origins occurs repeatedly, it means that several MCM molecules are loaded onto DNA per origin. Theoretically, only two MCM hexamers are needed to establish bidirectional replication forks at each origin. Why are multiple MCM molecules loaded to DNA at each origin? A possible reason is that additional MCM complexes may act as back-up when cells are at replication stress and some replication forks collapse when replication forks encounter DNA lesions or replication barriers. Then, these additionally loaded MCM will be activated to initiate DNA synthesis to complete replication for each segment of chromosomal DNA. After MCM is loaded onto DNA, ORC, and Cdc6 are no longer needed for MCM to bind to DNA. Moreover, MCM is resistant to high salt extraction because it forms a ring around DNA. MCM hexamer has a globular shape with a central cavity which DNA passes through. At G1 phase,

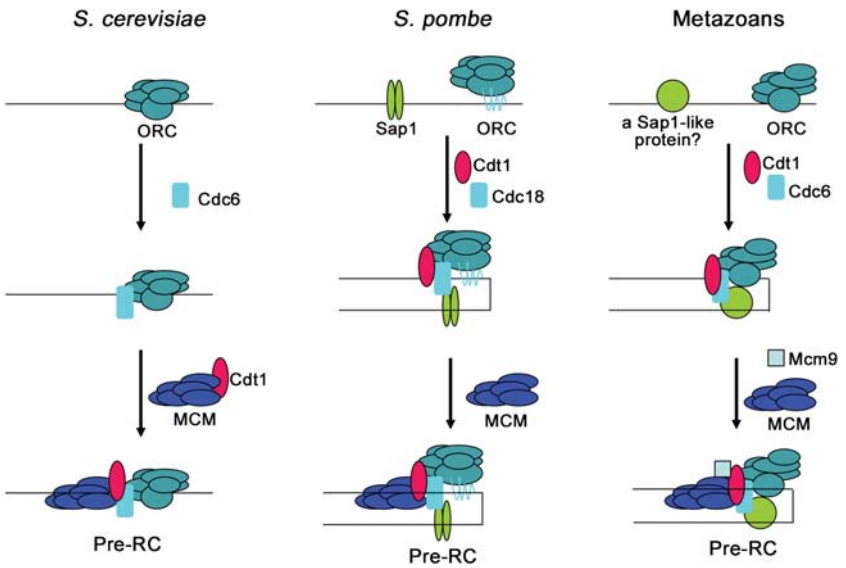

Figure 2 Assembly of pre-RC *Sc*ORC binds to DNA throughout cell cycle and loads Cdc6 to origins at G1 phase. ORC and Cdc6 together load MCM–Cdt1 complex to origin to complete pre-RC formation. In *S. pombe*, ORC and Sap1 bind to DNA replication origins during cell cycle. Cdt1 is loaded to origin by ORC alone, but ORC and Sap1 are both required to load Cdc18 to origins. After MCM is recruited to origins by Cdc18 and Cdt1, pre-RC is assembled. In metazoans, ORC is essential for loading Cdc6 and Cdt1 to origins. It is currently unknown whether a Sap1-like protein also exists and is required to load Cdc6 to origins. After Cdc6 and Cdt1 bind to origins, they act together to load Mcm9 and MCM to origins.

these loaded MCM is not active as a DNA helicase. They are activated when additional components of Cdc45 and GINS are loaded and form a complex with MCM at the G1 to S phase transition. The separation of loading and activating MCM makes it easier to coordinate DNA synthesis with multiple origins in each chromosome and to prevent duplication of a DNA segment more than once in a cell cycle. In budding yeast, assembly of pre-RC has been achieved with purified ORC, Cdc6, Cdt1, and MCM *in vitro*, indicating that ORC, Cdc6, and Cdt1 are sufficient to load MCM to DNA [37–39].

In the fission yeast, assembly of pre-RC also requires ORC, Cdc18 (*S. pombe* homologue of Cdc6), Cdt1, and MCM. But, an additional protein, Sap1, is recently demonstrated to be an essential component of pre-RC (Yang *et al.*, submitted). Similar to *S. cerevisiae*, only ORC is needed to recruit Cdt1 to origin. However, it requires two proteins, ORC and Sap1, to load Cdc18 to origin in *S. pombe* (**Fig. 2**). Recently, we demonstrate that Sap1 is essential for the transition from G1 to S phase during the cell division cycle. When Sap1 dissociates from chromatin in a *sap1* temperature-sensitive (ts) mutant at restrictive temperature, initiation of DNA replication is significantly inhibited at 30 examined origins. Moreover, Cdc18-overexpression-induced DNA re-replication is dramatically reduced in $sap1^{ts}$ mutant cells. Finally, we demonstrate that Sap1 is required to load Cdc18 to chromatin for assembly of pre-RCs. It is proved that Sap1 does not function at replication forks for fork progression. Thus, Sap1 is a newly discovered replication initiation protein and directly participates in the assembly of pre-RCs by loading Cdc18 to origins. After Cdt1 and Cdc18 are loaded onto origins, these two proteins act together to load MCM to origins to complete pre-RC formation (**Fig. 2**).

By database searching, a Sap1 homologue is not found in the budding yeast *S. cerevisiae*. Why do *S. pombe* cells use an additional protein to load Cdc18 to origins? The reason is probably due to the difference in ORC/DNA interaction between the two yeasts. The ORC in *S. cerevisiae* uses five (Orc1−5) of its subunits to contact with DNA. In contrast, *S. pombe* ORC just uses its extra 9 AT-hook motifs (located in the N-terminal half of the *Sp*Orc4 subunit) to bind to asymmetric AT-rich DNA sequences. The difference in the interaction of ORC and DNA between the two yeasts may cause the difference in loading Cdc6/Cdc18 to origins (**Fig. 2**).

DNA Replication Origins and Assembly of pre-RC in Metazoans

Like unicellular organisms of bacteria and yeasts, DNA replication in higher eukaryotes also initiates at specific sites. Moreover, these initiation sites are inheritable from one cell division to the next and can still direct replication initiation if they are moved to ectopic sites in the genome [40–42]. This indicates that these initiation sites are genuine replication origins. However, the characteristics of metazoan origins are still elusive. In some aspects, metazoan origins are quite similar to the fission yeast DNA replication origins. First, both of them lack an apparent consensus sequence. Second, both of them are large and range from one to a few kilobases in length [40,43]. Third, some studies have indicated that metazoan origins probably are also AT-rich sequences [44–46]. But, unlike yeast or bacterial origins, metazoan origins appear to have very low ARS activity in general even though some origins show ARS activity [47–49]. It is found that the size rather than a particular DNA sequence presented in a plasmid has more effect on plasmid stability [50–52]. On the basis of the previous studies, it suggests that metazoan origins are not solely determined by DNA sequences, other factors such as chromatin structure and gene transcription may have significant influence on the selection of DNA replication origins and the timing when origins are activated to initiate DNA synthesis during S phase [53, 54]. Certainly, more studies are required to define the nature of metazoan origins.

Different from the budding or fission yeast ORC that binds to the specific ‘A’ element or asymmetric AT-rich sequences, purified ORC molecules from human and drosophila have little sequence specificity and ATP hardly affects the interaction of ORC/DNA (**Fig. 1**) [55,56]. DNA topology is reported to affect the binding of metazoan ORC to DNA [56, 57]. It is also reported that some other proteins may affect the interaction of metazoan ORC and DNA [58–61]. Certainly, the interaction mechanism between metazoan ORC and DNA is not determined; neither is the selection mechanism of DNA replication origins in metazoans. Assembly of pre-RC in metazoans also includes ORC, Cdc6, Cdt1, and MCM proteins. Mcm9 is recently discovered to be a component of pre-RC in *Xenopus* (**Fig. 2**) [62]; it interacts with Cdt1 and MCM and is required to load MCM to origins. Does a Sap1-like protein exist in metazoans? It is highly possible that a Sap1-like protein exists and is required to load Cdc6 and subsequently MCM to origins; further studies will solve this question (**Fig. 2**).

Regulation of pre-RC Assembly

It is crucial to maintain genomic integrity in eukaryotic cells. Errors in pre-RC assembly often result in substantial changes in the amount of genetic material, which will cause cell death, or transform cells to become malignant tumors. Through tens of millions of years of evolution, eukaryotic cells have evolved a regulation system to strictly

control pre-RC formation in order to guarantee precise duplication of each segment of chromosomal DNA per cell cycle. Pre-RC is assembled only in late M and early G1 phases when CDK activity is low. When CDK activity reaches to a certain level, pre-RC is activated and other replication proteins are loaded onto origins to initiate DNA synthesis. A high level of CDK activity in S, G2, and early M phases strictly inhibits assembly of pre-RC. But CDK activity is also required for replication initiation by activating pre-RC and some other replication proteins such as Sld2 and Sld3 [63,64]. By separating the pre-RC assembly and its activation to different cell phases, origin firing more than once in a single cell cycle is prevented. Different organisms have different mechanisms in controlling pre-RC formation. In budding yeast, cdc6 is expressed only at G1 phase and degraded in the other time of cell cycle [65,66]. Although Cdt1 and MCM are present throughout cell cycle, but they are in nucleus only at late M and G1 phase. After replication is initiated, Cdt1 and MCM are exported into cytoplasm [67–69]. Moreover, ORC also appears to lose activity due to phosphorylation of Orc2 and Orc6 subunits by CDK during pre-RC activation because DNA re-replication occurs in G2/M period only when these two subunits could not be phosphorylated by abolishing their CDK sites [32]. In the fission yeast, both Cdc18 and Cdt1 are present only at G1 phase and degraded at the other phases of cell cycle [70–72]. In metazoans, besides regulating functions of ORC, Cdc6, Cdt1, and MCM proteins by CDK [73–79], there is an extra regulation way to inhibit pre-RC formation in S and G2 phases. It is geminin that accumulates during S and G2 phases and binds to Cdt1 and blocks its function to load MCM for pre-RC formation [80–82]. As discussed above, eukaryotic cells use multiple ways to prevent pre-RC assembly outside G1 phase, including proteolysis, nuclear export, chemical modification, and the inhibiting protein geminin, indicating that the prevention of re-initiation of DNA synthesis from any individual origin is crucial to maintain genomic integrity.

Summary and Perspective

It appears clear that DNA replication in all eukaryotes is initiated at specific sites no matter whether the genomes are from single cellular organism yeast or multiple cellular organisms such as human. The structure of DNA replication origins in yeast is quite clear, but the nature of metazoan origins is still elusive. The elucidation of the interaction between metazoan ORC and genomic DNA will help us to understand how metazoan origins are selected. It has been reported that some proteins may influence the binding of metazoan ORC to DNA as described above. It seems that there is still one or more intrinsic ORC interacting protein(s) waiting to be identified. The question how pre-RC is assembled in *S. cerevisiae* should have been solved. But the recent identification of an initiation protein Sap1 in *S. pombe* implies that the mechanism of pre-RC formation in metazoans has not yet been determined. An obvious question is whether there exists a homologue of Sap1 in higher eukaryotic cells. A regulation system to restrict pre-RC formation to G1 phase is certainly different among eukaryotes. The details of the regulation mechanisms need to be further elucidated.

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