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MINI-REVIEW

Initiation of DNA Replication in Eukaryotes Is an Intriguing Cascade of Protein Interactions

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Abstract—Initiation of eukaryotic DNA replication is a complex process including the recognition of initiation sites on DNA, multi-step DNA preparation for duplication, and assembly of multi-protein complexes capable of beginning DNA synthesis at initiation sites. The process starts at the late M phase and lasts till the appropriate time of the S phase for each initiation site. A chain of interesting interactions between Orc1p-6p, Cdc6p, Mcm2p-7p, Mcm10p, Cdt1, Cdc45p, Dbf4/Cdc7p, RPA, and DNA polymerase α takes place during this period. The sequence of these interactions is controlled by cyclin-dependent kinases, as well as by ubiquitin-dependent proteolysis in the proteasome. This review summarizes the data on proteins initiating DNA replication and factors controlling their activities.

Key words: initiation of DNA replication, pre-replication complex, replication complex, control over DNA replication initiation

Initiation of DNA replication in eukaryotes is a very important process for dividing cells. It prepares chromatin for replication, simultaneously blocking repeated mitosis, and provides single duplication of DNA during one cell cycle, thus supporting the genome stability. Replication initiation begins well before DNA synthesis and is under the control of a number of factors, among which cyclin-dependent kinases play an important role. This is realized by the interaction among a great number of proteins in the replication initiation sites (origins, *ori*). At present, 25 polypeptides are known to be involved in DNA replication initiation (see table), excluding enzymes controlling their activity. This list will probably undergo amendments with new components. These proteins form sequentially three sorts of complexes in the replication origin during the initiation of replication. These complexes fulfill various functions wherein the activity of each of them is under stringent control in the cell cycle. In the presented review we summarize the regularities of formation and functioning of the complexes between origin and proteins initiating DNA replication in eukaryotes.

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COMPLEX FORMATION BETWEEN THE REPLICATION ORIGIN AND THE REPLICATION INITIATING PROTEIN

Initiation of DNA replication starts from the complex formation between the replication origin and replication initiating protein. This complex is designated as the post-replication complex (post-RC). What is its role in initiation of DNA replication? The post-RC is well-documented to serve as a base for the assembly of higher order structures transforming chromatin into the state competent for replication.

Replication origins. Eukaryotic genomes contain hundreds (yeast) and thousands (metazoan) of origins, from where DNA replication starts, and those are the sites all the preceding steps of preparation to this process are associated with. Replication origins are sufficiently well studied in yeast, whereas the problem of their structure and place in chromatin of higher eukaryotes is too cumbersome and ambiguous. In the present review we are bounded by evidences on origins in budding yeast *Saccharomyces cerevisiae* and fission yeast *Schizosaccharomyces pombe*. In *S. cerevisiae*, origins were first identified as DNA sequences capable of supporting

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Proteins composing post-, pre-, and replication complexes

Complex type	Proteins	Functions
Post-replication (post-RC)	ORC (Orc1p–6p)	binds to *ori* and forms a base for pre-RC assembly
Pre-replication (pre-RC)	ORC (Orc1p–6p)	attaches Cdc6p
	Cdc6p	binds MCM with *ori*
	Cdt1	binds MCM with *ori*
	Mcm10p *S. cerevisiae*	binds MCM with *ori*; facilitates MCM dissociation from pre-RC
	MCM (Mcm2p–7p)	generates a control signal disabling repeated mitosis; transforms chromatin into the state competent for replication; binds Cdc45p and RPA to *ori*
	Dbf4/Cdc7p	participates in binding of Cdc45p and RPA with *ori*; activates helicase MCM
	Cdc45p	releases MCM from pre-RC (?)
	RPA	
Replication (RC)	Cdc45p	attaches DNA-polymerase α to RC
	MCM (Mcm2p–7p)	unwinds DNA; participates in the attachment of DNA polymerase α to RC
	RPA	stabilizes unwound single-stranded DNA sites
	DNA polymerase α	synthesizes RNA-primer, initiates DNA replication

autonomous replication (autonomous replication sequences, ARSs) [1, 2]. An ARS is 100-200 bp long and contains a specific consensus sequence (ARS consensus sequence, ACS) 11 bp in length, which is necessary to bind with initiating protein, as well as additional elements (B-elements) enhancing the origin function [3]. In particular, ARS1 is the first well-characterized origin, which contains three of those elements: B1, B2, and B3. ACS sequences and B1 have 50 bp and represent the minimum functional region of any origin that is necessary for binding with the initiating protein [4-6].

ORC. ORC (origin recognition complex) is a protein initiating DNA replication in eukaryotic cells, which has been described first for *S. cerevisiae* [4]. ORC-like proteins were found and studied later in other eukaryotic species, such as *S. pombe*, *Xenopus laevis*, *Drosophila melanogaster*, as well as in humans [7-11]. In all eukaryotes ORC consists of six subunits, from Orc1p to Orc6p (120-50 kD). All the six subunits of the complex were shown to be important for *S. cerevisiae* life [12-15]. Two different groups of ORC subunits participate in recognition of origin sequences on binding of this complex with the replication origin [16]. Orc1p, Orc2p, and Orc4p interact with ACS, the other three subunits recognize B1-like elements. Orc5p possibly realizes the binding to nucleotide residues of B1. Bell and Stillman revealed that ORC specifically binds to DNA only in the presence of ATP [4]. Later ORC was found to possess ATPase activity that is regulated by cooperating interaction between the protein with ATP and ARS elements [17-19]. ATP binds to Orc1p subunit and plays a role of cofactor that is necessary for ORC attachment to the origin. A specific sequence of the origin bound to ORC inhibits ATPase activity of Orc1p, whereas single-stranded DNA sites appearing in S phase activate this ATPase activity. ORC conformation thus changes from extended to bent [18]. The binding and hydrolysis of ATP by Orc1p subunit possibly participate in the control of ORC functions in the cell cycle. Recently, Dr. Lee with coworkers described a mechanism of ORC attachment to ARS1 in *S. pombe*. Orc4p protein contains in its N-terminal domain so called "AT-hooks", by means of which ORC binds to several regions of ARS1 enriched by AT-sequences [20]. It is possible that ORC attaches initially to the *ori* of *S. cerevisiae* at the end of mitosis, thus forming the post-RC [21], and remains bound to *ori* in following cell cycles. As this takes place, post-RC exists in the phases S, G2, and M, whereas in the phase G1 it is a constituent of pre-replication complex (pre-RC) [4, 21-23].

PRE-REPLICATION COMPLEX FORMATION

MCM, Cdc6p, and Cdt1. Pre-replication complex is formed on the base of post-RC; the process starts at all origins simultaneously at the phase M and G1 boundary and ends at the late G1 phase in origins that are activated first in going from G1 to S phase. For the origins that have been activated later in S phase, pre-RC formation completes at the period in S phase corresponding to each of them. The Fig. 1 illustrates the scheme summarizing literature data on the processes of post- and pre-replication complex formation in origins, from which the genome replication starts. Interestingly, ORC can interact with

cyclin-dependent kinases (Cdk) in G1 phase during the pre-RC assembly. This interaction may be one of the mechanisms enabling the cell to form pre-RC after mitosis [24]. For a long time it has been thought that cell division cycle protein Cdc6p and a family of six minichromosomal maintenance proteins Mcm2p-7p attach first to post-RC on the border of phases M and G1, the proteins being described for many eukaryotes including mammals [21, 23, 25-32]. The involvement of another protein, Cdt1, in the early stage of pre-RC formation in *S. pombe* cells as well as in higher eukaryotes was demonstrated recently [33, 34]. What functions do Mcm2p-7p, Cdc6p, and Cdt1 fulfill in pre-RC?

The proteins Mcm2p-7p are the most known among the family of minichromosomal maintenance proteins that have been first identified in *S. cerevisiae* in mutants devoid of the ability to maintain minichromosome stability [31]. Mcm2p-7p proteins form a hexameric complex (minichromosomal maintenance proteins, MCM), the key component of pre-RC. MCM generates a control signal on the non-replicated chromatin to inhibit precocious mitosis in G1 phase and is necessary for cell advance to S phase [35]. On one hand, MCM attachment to replication origin is controlled by phosphorylation–dephosphorylation of distinct subunits in this complex. For instance, partial dephosphorylation of Mcm4p subunit hyperphosphorylated in phase M facilitates the formation of pre-RC, whereas the complete dephosphorylation of Mcm3p subunit inactivates the complex and hinders its binding to chromatin [36]. On the other hand, the MCM attachment to origin depends on Cdc6p and Cdt1 proteins that, being cooperating, load MCM on chromatin [33, 37]. In the absence of Cdc6p *S. cerevisiae* cells loss their ability to initiate DNA replication and undergo "truncated" mitosis, in the course of which non-replicated chromosomes segregate accidentally on the spindle poles. The ability of Cdc6p to prevent "truncated" mitosis before DNA replication is accomplished is provided by its interaction with Cdk [24]. Cdc6p activity in phase G1 is probably regulated also by its interaction with ATP, since mutations in conservative sequences of ATP-binding Cdc6p motif resulted in the loss of chromatin ability to attach MCM in *S. cerevisiae* cells [29] and inhibition of DNA replication in human cells [38]. Mcm10p protein associated with chromatin and interacting with Mcm2p-7p components was demonstrated recently to participate in MCM binding with pre-RC in *S. cerevisiae* [39]. The fact that the MCM binding to origin is controlled by so numerous factors evidences for the cell requirement of a complex of regulatory mechanisms to avoid the repetition of mitosis with non-replicated chromatin, thus providing genome stability. MCM binding with replication origin takes the chromatin to the so-called "replication-licensing" state. MCM binding to origins makes possible another cascade of protein interactions in replication initiation sites, which results immediately in the beginning of DNA synthesis. MCM plays an important role in this interaction chain as well.

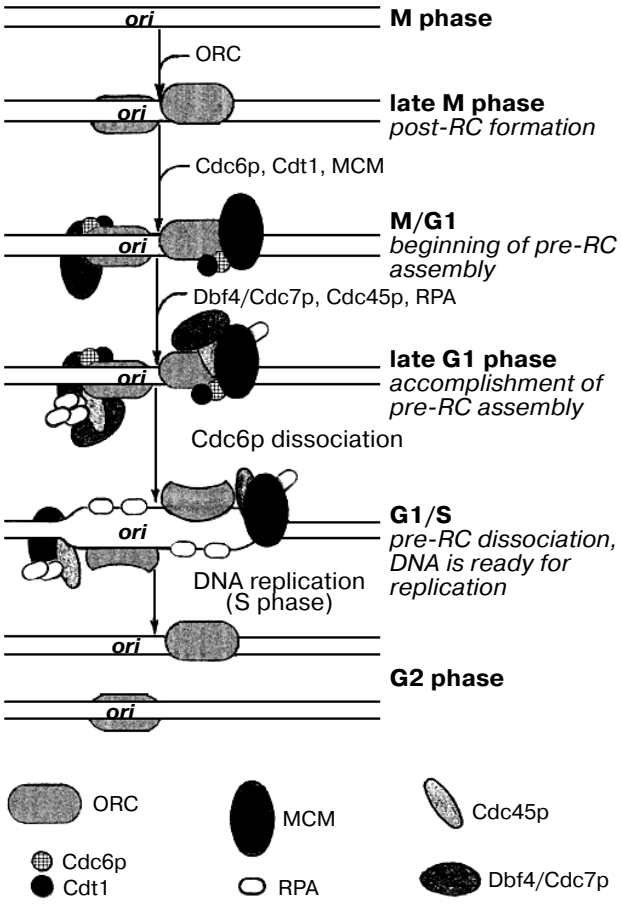

Fig. 1. Post-RC and pre-RC formation in *Saccharomyces cerevisiae* origins beginning DNA replication in early S phase represented schematically. ORC, being attached to *ori*, remains bound to it in consequent cell cycles.

Dbf4/Cdc7p, Cdc45p, and RPA. The attachment of Cdc7p kinase with its regulatory subunit Dbf4 (Dbf4/Cdc7p) to partly formed pre-RC occurs in G1 phase [23, 40], whereas the attachment of Cdc45p protein [21, 41, 42] as well as single-stranded DNA-binding protein (replication protein A, RPA) [42, 43] occurs in the late G1 phase or on the border of G1/S phases. The interaction between Cdc45p and Dbf4/Cdc7p in the late G1 is necessary for subsequent "switch on" replication in origins after the pre-RC assembly [44]. The binding of Cdc45p with chromatin depends on Cdc6p and Mcm2p proteins and on activities of Dbf4/Cdc7p kinase and cyclin-dependent kinases of S phase (Cdk-S) [41, 42]. Evidence exists that Cdc45p may serve as a substrate for Dbf4/Cdc7p kinase, but only after Cdk-S activation [45]. Cdk-S activity, in turn, is controlled by Sic1p

inhibitor that undergoes ubiquitin-dependent proteolysis in the end of G1 phase [46]. The attachment of Cdc45p protein to pre-RC occurs in the late G1 phase of the cell cycle probably only in sites first starting DNA synthesis at the transition to S-phase. Cdk- and Dbf4/Cdc7p-dependent Cdc45p binding with other origins is realized at a distinct time for each of them during the whole S phase [41, 42, 47]. Lei and Tye proposed the following mechanism for Cdc45p attachment to pre-RC. After Cdk-S action Dbf4/Cdc7p phosphorylates Mcm2p in each origin at a distinct moment in S phase, which is determined individually for each the origin. As a result of Mcm2p phosphorylation, the conformation of MCM complex changes in a distinct way endowing it with the capacity to bind Cdc45p [40]. At the same time, RPA binds with pre-RC. Similar to Cdc45p, RPA binds with origin by Cdk-S-dependent way involving Mcm2p [42]. Cdc45p and RPA accomplish pre-RC assembly. Interestingly, Dbf4/Cdc7p kinase binds to origin some time before it starts its functioning. After the binding with pre-RC Dbf4/Cdc7p "waits for" the activation of Cdk, and then it phosphorylates its substrate [40]. The role of such "early" binding of this kinase with *ori* still remains unclear.

In the wake of the pre-RC assembly, its partial dissociation occurs with the appearance of post-RC in origins and the beginning of RC formation [21, 32] (Fig. 1). Cdc6p dissociates first from pre-RC on the transition from G1 to S phase or earlier, being phosphorylated by Cdk, followed by ubiquitinylation and degradation [21, 48, 49]. This way of Cdc6p inactivation was demonstrated for lower eukaryotes [49] as well as for some higher eukaryotic cells [48]. Interestingly, in human cells Cdc6p level remains constant during the whole cell cycle. However, Cdc6p is transported from the nucleus to the cytoplasm on the transition into S phase. Probably, this process is also controlled by Cdk [50, 51]. Cdc6p removal from the nucleus (by hydrolysis or transportation into cytoplasm) is one of the mechanisms preventing multiple replications during one cell cycle.

Cdt1 is also inactivated after pre-RC formation. Thus, the activity of Cdt1 is regulated in different ways in lower and higher eukaryotes. Cdt1 in the yeast *S. pombe* is expressed periodically and accumulates in phase G1 in the nuclei, whereas in G2 phase its level decreases [52]. In higher eukaryotes, Cdt1 activity is under the control of protein geminin [53, 54], which is absent in cells in phase G1, accumulates during the phases S, G2, and partially M, and disappears in M phase on the border of metaphase and anaphase [55].

REPLICATION COMPLEX FORMATION

Unlike Cdc6p and Cdt1, the proteins Mcm2p-7p, RPA, and Cdc45p, after dissociation from pre-RC, remain bound to chromatin in S phase during DNA synthesis. Fast MCM release from pre-RC in yeast cells resulted from the interaction between Mcm10p and Mcm7p [39]. Cdc45p possibly facilitates MCM detachment from Mcm10p [10].

MCM. MCM complex is known to possess helicase activity associated with Mcm4p, 6p, and 7p subunits [21], but it does not manifest till it is stimulated under the action of Cdk-S and Dbf4/Cdc7p [40, 56]. Mcm2p-7p were clearly demonstrated to be essential not only for initiation, but for the elongation phase of replication as well, namely for the progression of replication forks [57]. These data indicate that MCM is a constituent of RC and participates in DNA duplex unwinding in replication forks. In human cells, MCM binding to the origin on RC formation is realized with the participation of Mcm10p protein, which accumulates and forms a complex with chromatin in S phase, in contrast to Mcm10p in *S. cerevisiae*. In going from G2 phase to M phase, Mcm10p dissociates from chromatin due to its hyperphosphorylation, whereas on the border of M and G1 phases it is subjected to proteolysis via a proteasomal mechanism [58]. Thus, in human cells a negative control of MCM activity after DNA replication is realized via Mcm10p phosphorylation and proteolysis. Besides, a negative control of MCM activity may result from direct phosphorylation of distinct components of this complex. In particular, a phosphorylation of specific Mcm4p sites in M phase, probably under the action of Cdk in HeLa cells, results in a loss of helicase activity in Mcm4p, 6p, and 7p [59], whereas a high concentration of Cdk–cyclin E complex in *Xenopus* embryo nuclei hinders Mcm3p binding with DNA, thus preventing MCM reassociation with chromatin after the replication [60]. MCM complex is involved not only in early stages of replication initiation on the cell transition into G1 phase, but in accomplishment of this process in S phase as well. At the late stage of replication MCM performs two functions: it melts DNA in the origin and serves as a base for attachment of other components on RC formation. Hence, multiple mechanisms of regulation, which seem to be excessive at first glance, are easily explainable. In phase G1 these mechanisms are directed to the realization of cell progression to S phase without repeated mitosis, whereas in S and G2 phases they prevent repeated replication of already replicated chromatin.

RPA. Since RPA is a single-stranded DNA-binding protein, its presence in RC on replication initiation is necessary for stabilization of an unwound DNA site [43, 61]. RPA is composed of three subunits of 70, 34, and 11 kD, wherein the 70-kD subunit expresses the single-stranded DNA-binding activity, and the 34-kD subunit is a regulatory one. The latter is phosphorylated by Cdk, which is possibly necessary for the initiation of DNA replication [62].

Cdc45p. Cdc45p is also involved in RC formation. It is obligate for DNA-polymerase α attachment to replication origins [47]. Cdc45p was shown to bind with human Mcm7p as well as with p70 subunit of DNA-polymerase α *in vitro* [63]. The authors hypothesize that Cdc45p attaches DNA-polymerase α to RC by Mcm7p binding. Cdc45p attaches DNA-polymerase α to chromatin involving Mcm2p in *S. cerevisiae* yeast cells [42].

DNA-polymerase α. No wonder that DNA-polymerase α is one of the components of the complex triggering DNA replication. It is this polymerase that expresses, among numerous DNA-polymerases, the primase activity that is able to synthesize a short primer from ribonucleoside triphosphates. This RNA-primer generates a signal triggering the replication mechanism [64]. DNA-polymerase α is found in all eukaryotes investigated, and it is well studied [65-73]. It is composed of four subunits: a large one of 165-180 kD possessing DNA-polymerase activity, a subunit of 66-70 kD probably possessing a regulatory function, and two small subunits of 58-60 and 48-56 kD combining into a primase. One of the ways for the control over the replication initiation was shown to be associated with phosphorylation of any of two DNA-polymerase α large subunits by Cdk [74-78]. Cyclin E and Cdk therewith stimulate the replication initiation on the transition of cell into S phase, and cyclin A and Cdk inhibit it in G2 phase [77].

As pointed out above, activation of replication origins takes place over the entire S phase [42, 79, 80]. For instance, ARS1 from *S. cerevisiae* is activated in early S phase, whereas ARS501 activation occurs in the late S phase [81]. Most origins are activated in the middle of the S phase [82]. Interestingly, the regions on *S. cerevisiae* chromosomes replicating in early and late S phase are arranged in a mosaic ("mixed") pattern [81]. The central part of *S. cerevisiae* chromosome VI is replicated in the early S phase, whereas its telomeres are replicated in the late S phase [83]. A DNA region of size 67 kb adjacent to the telomere on the right end of chromosome V and containing ARS501 is replicated in late S phase [84]. It seems likely that the late replication of this chromosome region is a consequence of its vicinity with the telomere. Besides, at the end of S phase, "silencing genes" are known to be activated, for instance, the loci HML and HMR, which are non-expressed in distinct cell types, are located in sub-telomere regions [85]. In contrast, actively expressing genes, such as the locus MAT, are replicated in the first half of S phase. There is no doubt that activation of the above mentioned "early- and late-active" origins in *S. cerevisiae* depends on Dbf4/Cdc7p kinase [42, 79, 80]. Why does this kinase not trigger the replication in all origins simultaneously? In each origin probably a local regulation of its activity by additional protein kinases, such as Rad53, takes place [86, 87]. The Rad53 kinase blocks the start-up of "late-active" origins in early S phase. When this block is eliminated, Dbf4/Cdc7p kinase activates MCM, resulting in several events: stimulation of MCM helicase activity, RPA and DNA polymerase α binding to replication origins. DNA polymerase α attachment to RC, DNA unwinding in the origin, and RNA-primer synthesis accomplish the process of DNA replication initiation in eukaryotes (Fig. 2). Note that the role of DNA polymerase α is only limited by the start-up of DNA replication. This DNA polymerase cannot perform the processive DNA synthesis and does not possess a correcting activity. Hence, in further replication it adds ~20 nucleotides to a primer and is substituted by DNA-polymerases δ or ε [43].

Recent reports indicate that post-RC (the whole chain of events of DNA replication initiation begins from its formation) undergoes changes in the S phase of the cell cycle. These alterations are concerned the affinity of ORC to DNA in metazoan cells. For an example, the protein Orc1p in *Xenopus* forms a tight complex with chromatin in early interphase until the pre-RC assembly finishes. Then the affinity of Orc1p to DNA decreases in S phase, and it can be removed from chromatin by either treatment with a salt of high concentration or elevation

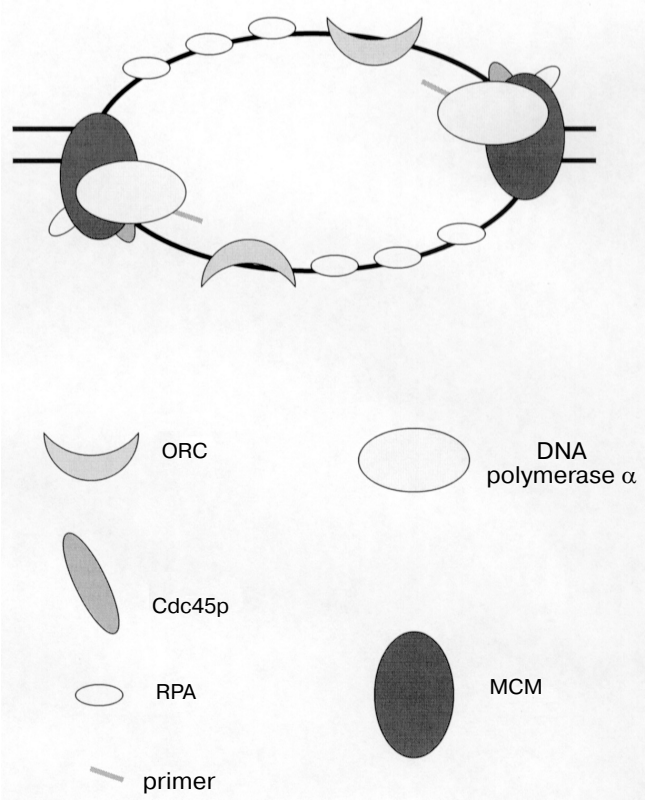

Fig. 2. DNA replication initiation in the closing stage represented schematically. Replication complex formation in S phase and synthesis of RNA-primer.

of cyclin-dependent kinase levels [88]. Mammalian Orc1p dissociates from chromatin in S phase and then evolves into mono- or diubiquitinylated form, then deubiquitinylates and binds DNA again on the transition from M to G1 phase. In contrast, Orc2p remains bound to chromatin during the whole cell cycle, and it is not a substrate for ubiquitinylation [89]. A protein complex containing Orc1p and Orc2p reassociating at the end of mitosis dissociates from human cell chromatin in S phase [90]. ORC undergoes post-translational alterations in the cell cycle in *S. pombe* yeast cells: phosphorylation of one of its subunit, Orc2p, begins in S phase and achieves maximum in G2 and M phases [91]. Thus, one way preventing repetitive replication of already replicated chromatin is connected with post-RC inactivation either by Orc1p dissociation from the complex (in higher eukaryotes) or by Orc2p phosphorylation (in lower eukaryotes).

Thus, at present much attention is attracted to genetic and biochemical investigation of proteins involved in DNA replication initiation in eukaryotes. Mutant protein form production facilitates revealing their functions in a given step of replication initiation. In metazoan cells all the proteins are found corresponding to the proteins initiating DNA synthesis in lower eukaryotes (ORC and MCM hexamers, Cdc6p, Mcm10p, Cdt1, Cdc45p, RPA, Dbf4/Cdc7p kinase, and DNA polymerase α). Although those proteins (particularly, Mcm10p) differ in their roles in lower and higher eukaryotic cell cycles, the accumulated data indicate that initiation of replication is conservative in eukaryotes. In recent years an important success was achieved in understanding the regulation mechanisms in virtually all the stages of replication initiation. Those are realized on the level of functioning of all the components forming post-, pre-, and replication complexes. These components are subjected to the action of more than one factor. An example of such factors is found in regulation of MCM complex, whose activity depends directly on Cdc6p, Mcm10p, Cdt1, cyclin-dependent kinases, Dbf4/Cdc7p kinase, and phosphatases. Taking into account the factors influencing the proteins listed above, the picture would be considerably complicated. Although progress in the investigation of DNA replication initiation is obvious, some questions remain unclear. Thus, the nature (logic) of the DNA replication initiation process is poorly known in embryogenesis. Some regulation mechanisms at particular stages of DNA synthesis initiation are in the opening stage of the search and require further investigation. The role of Cdc6p protein in the attachment of Cdc45p to replication origin is unclear. It is unknown why Dbf4/Cdc7p kinase attaches to *ori* some time before it begins to carry out its functions. To take into considerations the intensity of the experimental works in this field, we expect new and interesting results in the near future.

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