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DNA REPLICATION LICENSING

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1. ABSTRACT

The DNA replication licensing system ensures that chromosomal DNA is replicated precisely once before cell division occurs. A DNA helicase must be loaded on origin DNA for replication to initiate. Considerable evidence suggests that the MCM complex acts as a replicative helicase in eukaryotes. When the MCM complex is loaded on the chromatin, the replication origin is formally defined as being licensed for replication. Licensing takes place several hours before origins are activated to undergo replication in S-phase. Genetic and biochemical studies show that the licensing process is well conserved in eukaryotes. Cyclin Dependent Kinases (CDKs), the master regulators of the cell cycle, coordinate the initiation of the two key cell cycle events, replication of DNA and its segregation at mitosis. Eukaryotes have developed complex regulatory mechanisms to ensure that origin licensing is coordinated with these events so that genome integrity is preserved during successive cell divisions.

2. INTRODUCTION

Replication of eukaryotic chromosomal DNA is initiated from several sites on each chromosome, called replication origins. In *Esherichia coli* (*E. coli*) the origin is defined by a single specific replicator DNA, oriC, on which initiator proteins assemble. OriC is bound and marked by DnaA, and then DnaB, a DNA replication helicase, is loaded (57). Studies of DNA replication in eukaryotes during the last decades indicate that similar initiation processes take place on eukaryotic replication origins. Before initiation, origins become competent for replication by the assembly of the pre-replicative complex (pre-RC). The existence of such a complex prior to initiation was first demonstrated by genomic foot-printing analysis of the replication origin ARS1 of *Saccharomyces cerevisiae* (*S.cerevisiae*) (24). The origins thus modified are defined as licensed origins and can be activated for initiation by S-phase CDKs as cells enter S-phase. The first clue in understanding the licensing step at a molecular level came with the discovery of the Origin Recognition Complex (ORC), a complex which binds to *S. cerevisiae* ARS1 (7). In this review, we will describe the licensing factors so far identified and discuss how timely licensing is ensured.

Checkpoint controls regulate cell cycle progression, so that initiation of replication is coordinated

with chromosome segregation (104), thereby ensuring that no DNA segment is left unreplicated nor does it re-replicate before chromosome separation occurs. It is only after completion of chromosome separation that a new round of replication is allowed. Such regulations ensure that identical genetic information is transferred to daughter cells. If cells enter mitosis before completion of replication or re-replicate their DNA, faithful chromosome segregation is interrupted, causing a genomic instability in daughter cells that would result in cell death or malignant cell growth. The control that prevents initiation of mitosis when cells have unreplicated or damaged DNA is known as the S/M checkpoint control, and many checkpoint genes involved in this control have been identified (reviewed in (108)). In this review, we focus on the control that prevents re-replication, and ensures that DNA replication takes place only once per cell cycle.

3. LICENSING CONTROL ENSURES REPLICATION ONCE IN A CELL CYCLE

The DNA replication licensing model, first formulated by Blow and Laskey (8), predicted a positive acting licensing factor for replication. As our knowledge of cell cycle control advances and initiator proteins are being identified, we gain an understanding of replication licensing at the molecular level. Origins undergo a dynamic change during the cell cycle, from a licensed state before initiation to the unlicensed state after initiation has occurred. These states are strictly separated during the cell cycle to ensure a block in re-replication.

Figure 1A illustrates a schematic model describing how regulation of origin licensing prevents re-replication in a cell cycle. At the end of mitosis or at the G1-phase, replication origins become licensed (marked as RED) (note that some potential origins may not be licensed under certain conditions). The licensed origins fall into three categories: early firing origins, where replication initiates early during S-phase; late firing origins; and silent origins, which are licensed but not used. Complete replication results in the production of two identical copies of each chromosome to be segregated into the two daughter cells during mitosis. To prevent re-replication, origins are regulated in a way that 1) once origins fire, they are brought to the unlicensed state, 2) silent origins are converted to the unlicensed state when passively replicated from an advancing replication fork and 3) re-licensing is inhibited until completion of mitosis. When this control is abrogated, re-licensing of replicated chromosomes will occur, leading to re-replication within a single S-phase (Figure 1B1) or during the G2-phase (Figure 1B2). In the following sections, we describe how the licensed state is established and how the two states of origins (licensed and unlicensed) are regulated during the cell cycle.

4. ORIGIN LICENSING FACTORS

4.1. Replication origins in eukaryotes

The *E. coli* origin of replication was recovered as a DNA element that could confer to a DNA fragment the ability to replicate autonomously as an episome. The same strategy was used to identify Autonomously Replicating Sequences (ARSs) in eukaryotes. The yeast *S. cerevisiae* was proven to be a good model system for such analyses. Using a plasmid based assay, various ARS elements were identified. Many ARS elements were shown to act as replication origins on their chromosomal sites, mostly by 2 dimensional (2D) gel analysis. Comparison of *S. cerevisiae* ARSs identified a 11 bp AT rich element, 5' (T/A)TTTA(T/C)(A/G)TTT(T/A)3', called ACS, or ARS Consensus Sequence. The best characterized *S .cerevisiae* ARS is ARS1 that is about 100 bp long and composed of an essential ACS containing A element and three additional elements (B1, B2, and B3) located downstream of the A element (80).

A similar assay allowed the identification of ARS elements in the fission yeast *Schizosaccharomyces pombe*. The *S. pombe* ARSs consist of much longer A/T rich sequences of around 1000 bp. A 11 bp ACS-like sequence was detected in *S. pombe* ARS elements, but deletion of it had only a small effects on ARS activity (17). Rather, *S. pombe* ARSs require several clusters of 50-100bp long A/T rich stretches. ars2004 requires a 40 bp region, a 165 bp region and a 65 bp region which are scattered over a 940 bp long segment, for its full ARS activity (107).

In higher eukaryotes, similar plasmid-based assays for the identification of origin sequences proved unsuccessful. Physical mapping analysis indicates that much longer chromosomal elements are required for origin activity. For example, the human β-globin origin requires a 8 kb DNA segment for origin activity. In the other extreme, almost any DNA can be replicated in *Xenopus* egg extracts.

4.2. Origin licensing factors in eukaryotes

Origin sequences are bound by different protein complexes during the cell cycle. The pre-replicative complex (pre-RC), formed after mitosis, confers to origins the license to replicate and is activated for initiation upon entry into S-phase (23). So far, four key licensing factors essential for pre-RC formation have been identified, the Origin Recognition Complex, Cdc6/18, Cdt1 and the MCM complex. All these factors are conserved from yeast to mammalian cells, suggesting that the basic features of licensing have been conserved in all eukaryotes.

4.2.1. ORC complex

The well-defined origins of *S. cerevisiae* permitted the identification of the Origin Recognition Complex (ORC). A protein complex was biochemically purified that specifically binds to ARS elements *in vitro*. This complex, ORC, is composed of six subunits, Orc1, Orc2, Orc3, Orc4, Orc5 and Orc6 (120, 72, 62, 56, 53, and 50kDa, respectively (7)). Orc1 and Orc5 subunits have an ATP binding motif. Orc4 also has a similar motif. These Orc subunits, as well as licensing factors Cdc6/18 and MCM2-7 (see later) are all members of the AAA+ family of ATPases (6) (98). ORC shows ATPase activity when incubated with ATP (60). This activity is inhibited in the presence of ARS DNA, suggesting that ORC is stabilized on the origin in an ATP bound form (68). It is not yet clarified how ATP hydrolysis is involved in the biological function of the ORC complex. Orc1 is the main subunit that confers ATPase activity and ATP dependent ARS binding.

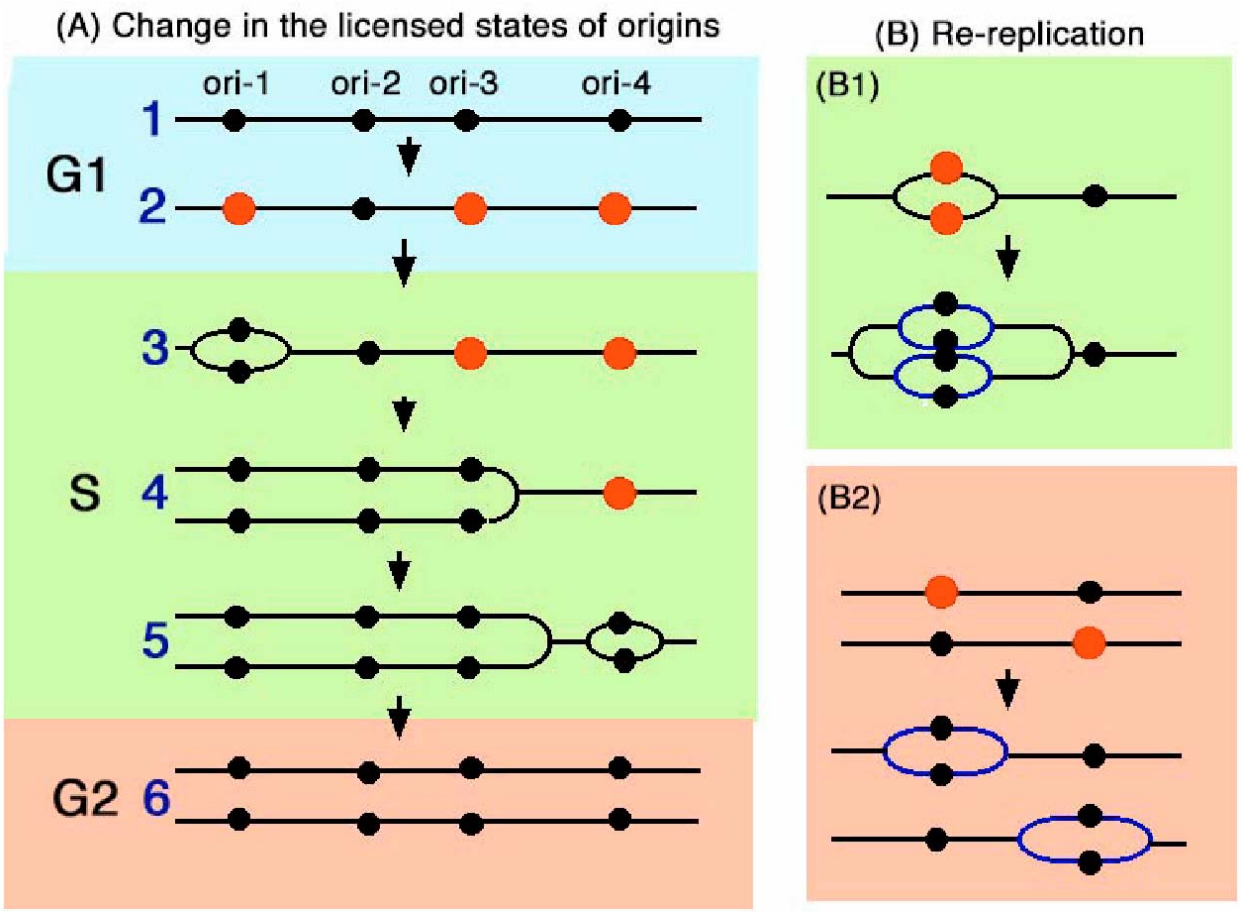

Figure 1. Control of origin licensing in the cell cycle. (A) Changes in the licensed state of origins during the cell cycle prevent re-replication. (1-2); At the end of mitosis or early G1-phase, origins become licensed (turn from black to Red), but some origins (ori-2) may remain unlicensed (black) under certain conditions. (3); Upon entry into S-phase, initiation starts from early firing origins (ori-1). At the same time ori-1 is converted to the unlicensed state. (4); As replication proceeds in both directions, replication forks may pass a licensed origin before it is activated (ori-3). At this time, ori-3 is brought to an unlicensed state. (5); Later in S-phase, initiation starts at late firing origins (ori-4). (6); At the end of S-phase, two copies of chromosome have been produced. (B) Abrogation of the licensing control causes re-replication.(B1); Origins are re-licensed during S-phase. (B2); origins are re-licensed in G2-phase.

Despite the differences in origin organization, homologues of *S. cerevisiae* ORC (ScORC) subunits have been identified in eukaryotes from yeast to men, pointing to an evolutionary conservation of origin–bound complexes in all eykaryotes. Comparison of ORC subunits from different species shows that Orc1, 2, 4 and 5 show higher conservation (22-27% among human, *Drosophila* and *S. cerevisiae*) than Orc3 (18%) and Orc6 (14%).

In *S. pombe,* SpOrc4p has a unique extended sequence in the N-terminal region that contains nine copies of the AT-hook motif. This motif has a high affinity for AT-rich DNA and likely mediates the SpORC complex binding to the AT-rich origins of this organism (15, 63, 70). Consistently, an SpORC complex consisting of five subunits (90kDa Orc1, 68kDa Orc2, 80kDa Orc3, 45kDa Orc5 and 31kDa Orc6), but lacking Orc4 (135kDa) was purified from cell extracts prepared in the presence of lower concentrations of salts. Higher salt extraction was necessary to purify the six subunit complex.

Both ScORC and SpORC complexes appear to associate tightly with origin DNA throughout the cell cycle (74, 76, 106). In contrast, the mammalian ORC complex does not exist as a stable complex in a cell cycle. Orc1 is removed from chromatin and/or degraded in S-phase (73, 90) and Orc6 has a low affinity for other components, while Orc2 is associated with chromatin throughout the cell cycle and likely to form a stable Orc2-5 core complex (22, 89, 134). Thus, it is difficult to isolate the human ORC complex as a six-subunit complex from cell extracts. A human ORC complex composed of six subunits can be isolated using heterologous expression in insect cells. *Drosophil*a and *Xenopus* ORC complexes were purified as six subunit complexes. In *Drosophila*, DmOrc6 is essential for ORC-DNA binding and replication, but most of DmOrc6 does not form a complex with other subunits and is present in the cytoplasm (12), suggesting additional roles

for Orc6 in higher organisms. In this context, it is interesting to note that silencing of human Orc6 causes a failure in chromosome segregation and cytokinesis (114).

4.2.2. Cdc6/Cdc18

In contrast to the discovery of ORC through biochemistry, Cdc6 of *S. cerevisiae* and its *S. pombe* homologue, Cdc18, were isolated through genetic screening as temperature sensitive mutants defective in DNA replication (41, 105), and turned out to play a key role in origin licensing. Cdc6 and Cdc18 have 28% identity, and like ORC subunits, Orc1, 4 and 5, belong to the AAA+ family of ATPase (58). Cdc6/18 and Orc1 are most closely related (ScCdc6 and ScOrc1; 30% identity, SpCdc18 and SpOrc1; 30%, SpOrc1 and ScOrc1; 31%) (35). Both ScCdc6 and SpCdc18 are tightly regulated during the cell cycle, through both transcription and proteolysis, so that they are present only around late M / G1-phase, when licensing occurs. START specific transcription factor complexes (containing Swi6/Mbp1 in *S. cerevisiae* and Cdc10/Res2/Res1 in *S. pombe)*, are responsible for correct cell cycle expression of the Cdc6/18 gene (61). Consistently, the cdc18 gene was also isolated as a target gene that suppressed a cdc10 temperature sensitive mutation when ectopically expressed. The Cdc6/18 protein is degraded through the ubiquitin-proteasome pathway after initiation of replication (5, 26, 50). In contrast to the yeasts, mammalian Cdc6 is regulated differently. A fraction of mammalian Cdc6 remains associated with chromatin in S-phase and G2, with a population exported into the cytoplasm in a CDK-phosphorylation dependent manner (30, 52, 109, 111, 117). At the end of mitosis, Cdc6 is degraded in an APC dependent way (89, 112). In *S. pombe*, ectopic expression of Cdc18 induces over-replication of the genome (96, 101).

In addition to its role in licensing, Cdc6/18 is implicated in another important cell cycle control. A *S. pombe* strain lacking the cdc18 gene, in spite of being unable to initiate replication, enters catastrophic mitosis with a “cut” phenotype (58). This was the first evidence that initiator proteins are important not only for replication but also for the checkpoint control which arrests mitosis when replication is defective. High-level expression of cdc18 in *S. pombe* blocks entry into M-phase by checkpoint dependent and independent manners (36). Also, in mammalian cells, Chk1 dependent G2 arrest was observed when human Cdc6 was over-expressed in G2-cells (16). Additionally, in *S. cerevisiae*, Cdc6-dependent inactivating of the mitotic CDK-cyclin complex is important for mitotic exit (10).

4.2.3. Cdt1

During a screen for targets of the Cdc10 containing transcription factor in *S. pombe*, cdt (Cdc10 dependent transcript) genes were isolated by immunoprecipitating and PCR-amplifying Cdc10 bound DNA fragment (42). A novel gene, called cdt1, showed a strict regulation by Cdc10 and suppressed a cdc10 temperature sensitive mutation when ectopically expressed. Genetic and biochemical analysis of SpCdt1 showed that it is required for origin licensing in this organism (100). Due to the low level of conservation of Cdt1 across species, the identification of Cdt1 homologues from metazoa had to await their discovery in independent screens. *Drosophila* Cdt1 was isolated as a mutation (dup, or double parked) which showed DNA replication defects. The Dup protein was shown to colocalize with ORC proteins and to be 20% identical to SpCdt1 (138). *Xenopus* Cdt1 was isolated during a screen for genes expressed early in embryogenesis and shows 24% identity to SpCdt1 (79). ScCdt1 has only 10% identity to SpCdt1 (128). Similar to the cdc18 gene, Spcdt1 is transcribed around late M/G1-phase and its protein levels peak at this stage (42, 100). Human Cdt1 also accumulates in G1-phase, and is degraded in S-phase (102, 141). In contrast to these observations, ScCdt1 is present throughout the cell cycle and is regulated by nucleocytoplasmic shuttling: ScCdt1 accumulates in the nucleus in G1-phase, but is excluded from nucleus in a CDK dependent way after S-phase onset (128). The Cdt1 protein does not have known functional motifs, except a coiled-coil domain.

4.2.4. The MCM complex

The MCM complex consists of six related proteins of around 70-120 kDa, Mcm2, 3, 4, 5, 6 and 7, collectively named MCM2-7. Mcm (mini-chromosome maintenance) genes were first isolated by a genetic screen for mutations that caused a loss of mini-chromosomes in *S. cerevisiae* (78). Later, the MCM complex was purified from *Xenopus* egg extracts as a factor that is essential for licensing of chromosome DNA in G1-phase and is lost from chromatin after replication at G2-phase (13, 64, 77). All six Mcm proteins are essential and belong to the AAA+ family of ATPases. The overall similarity between the members of MCM2-7 is 20-30%. A region of 250 amino acids that contains the ATP binding region is highly conserved (reviewed in (131)). The six proteins interact with each other and form a hexameric complex with a molecular weight of around 600 kDa. Electon Microscopy (EM) analysis of purified *S. pombe* MCM complexes shows a globular structure with a central cavity (2). Considerable evidence suggests that MCM2-7 acts as the replicative DNA helicase. Ishimi first showed that a purified human trimeric complex composed of Mcm4, 6, and 7 (Mcm4-6-7) possesses ATPase activity and a helicase activity with a 3’ to 5’ direction (46), though the helicase activity was low and non-processive. When a template with a forked structure of 5’ and 3’ single strand-exposed DNA was used in the presence of single-strand binding protein, highly processive helicase activity was detected, capable of unwinding more than 500 bp (69). Molecular size analysis and EM observations indicate that two MCM4-6-7 heterotrimers form a hexameric toroidal structure with a central channel (118). Addition of Mcm2 or a Mcm3-5 complex to the MCM4-6-7 complex inhibits its helicase activity, probably by changing the hexameric structure (47). In spite of these observations, all six Mcm proteins are required to complete DNA replication (66). Inactivation of any of the components results in defects in DNA replication, though inactivation of Mcm2, 3, and 5 together may restore MCM function. Recently, a model was presented that catalytic MCM4-6-7 and regulator MCM2-3-5 sub-complexes co-operate to hydrolyse ATP, predicting a

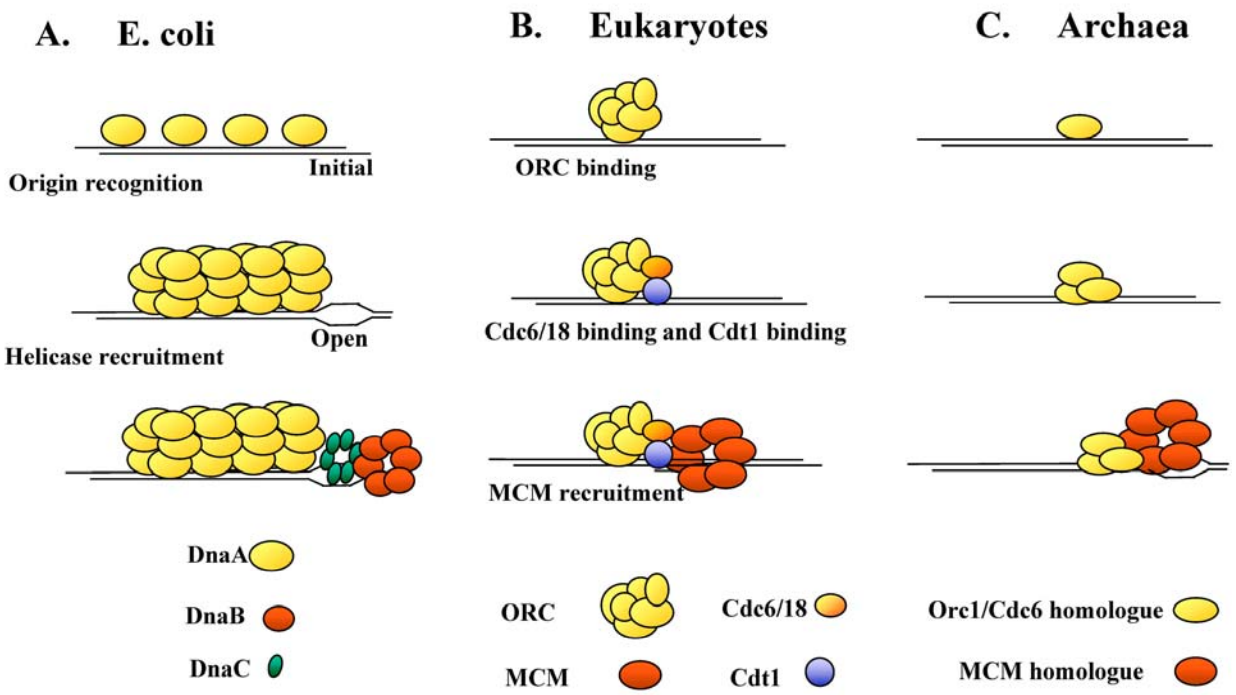

Figure 2. Origin licensing in the three domains of life. (A) Origin activation in Bacteria. The initiator protein DnaA is bound to *cis* acting elements on oriC forming the initial complex. Oligomerization of DnaA forms the open complex and recruits to the origin the helicase DnaB, together with the helicase loader DnaC. (B) Licensing in eukaryotes. ORC binds to *cis* acting elements on replication origins. Binding of Cdc6/18 leads to a conformational change which loads the MCM helicase onto chromatin. An additional factor, Cdt1 is essential for MCM loading. (C) Most Archaea possess a simplified repertoire of licensing factors consisting of one protein equally related to both Orc1 and Cdc6/18 and one MCM family member. A model for the loading of these proteins on the origins is shown.

mechanical similarity to F1-ATPase (119). F1-ATPase rotates a centrally located gamma subunit and the MCM complex was proposed to use a similar mechanism to rotate DNA. So far, helicase activity of a purified MCM2-7 complex has not been observed. The MCM 2-7 complex is not active in G1 phase and a modification at the onset of S-phase, such as phosphorylation, may cause a structural change producing an active helicase. Recent results suggest that MCM2-7 purified from S-phase extracts is associated with Cdc45 and has both ATPase and helicase activity (83).

4.3. Glimpses from the evolutionary past: licensing factor homologues in Archaea

Archaea constitute the third domain of life and are believed to be related to the evolutionary ancestor of all modern day eukaryotes. Despite possessing origins of replication which appear more bacteria-like in overall organization, decoding of several archaeal genomes revealed homologues of licensing factors in this domain. Several species of Archaea appear to contain a single gene almost equally related to both Orc1 and Cdc6 and a single member of the MCM family. Orc1/Cdc6 and MCM homologues from *Pyrococcus abyssi* were found associated *in vivo* with the proposed single replication origin of this species (86), while the MCM homologue of *Methanobacterium thermoautotrophicum* was shown to form a double hexamer exhibiting processive DNA helicase activity *in vitro* (14, 59, 121). Archaea may thus possess a simplified version of the eukaryotic licensing machinery.

5. ESTABLISHING THE LICENSED STATE

In prokaryotes and eukaryotes alike, as well as many viruses, origin associated proteins bind to *cis* DNA elements and process the origin DNA to allow the assembly of the replication machinery. Loading of the replicative helicase is a key step in this process. In eukaryotes, formation of the pre-replicative complex on origin DNA, by the sequential assembly of ORC, Cdc6, Cdt1 and the MCM proteins, takes place after mitosis and gives to origins the license to replicate. Figure 2 compares the processes which lead to origin activation in *E. coli*, Archaea and eukaryotes.

5.1. Lessons from *E. coli*

E. coli has one circular chromosomal DNA that is replicated from a single site, called oriC. oriC DNA has two functional elements; one comprises five DnaA boxes, to which initiator protein DnaA binds, and the other has three A/T rich 13 mer sequences. DnaA belongs to the AAA+ family of ATPases. Initial binding of DnaA monomers to the DnaA boxes of oriC is followed by oligomerization of 20-40 subunits of DnaA in the ATP-bound state. This causes a local unwinding of the A/T rich repeats and allows the loading of two hexamers of the

replicative helicase, DnaB, assisted by the helicase loader DnaC. DnaB extends the single stranded unwound region and DNA synthesis is initiated by primase and polymerase III. At the same time, the β-clamp subunit of polymerase III loaded onto the origin stimulates ATP hydrolysis by DnaA and inactivates the oriC (reviewed in (57)).

5.2. Licensing in eukaryotes

Based on studies using *Xenopus* egg extracts and the yeast systems, the following model has emerged for the steps leading to licensing of eukaryotic origins (Figure 2). The ORC complex binds to origins and marks the position of initiation sites on the chromatin. In yeast, all ORC subunits remain associated with origin throughout the cell cycle, though in mammalian cells re-assembly into the six complex ORC is required at the end of mitosis. The origin bound ORC permits the association of Cdc6 and Cdt1, which are recruited independently of each other. These factors then load the MCM complex on the origins (3, 18, 25, 79, 100, 129). Loading of the MCM complex marks the completion of DNA licensing. Since the unwinding of duplex DNA is a crucial step for initiating DNA replication, loading of the replicative helicase on the origins is a key point that must be carried out before replication starts. A crucial difference in origin activation between Bacteria, Archaea, and Eukarya is that in eukaryotes, origin licensing is temporally separated from initiation in the cell cycle, for example in mammalian cells licensing is established several hours before initiation starts. Unwinding of origin DNA to initiate replication requires activation of kinases (see next section).

The molecular steps that lead to the assembly of the pre-replicative complex on origin DNA have remarkable similarities to origin activation in the *E. coli* oriC (Figure 2), except that MCM loading appears to proceed in the absence of a melted structure of origin DNA such as found in oriC. Origin recognition and binding by ORC can be paralleled to the initial binding of DnaA monomers to oriC DNA. We postulate that Cdc6 binding leads to formation of a complex which may be likened to the complex formed by oligomerization of DnaA. Complex formation may be facilitated by interactions between adjacent AAA+ domain containing subunits, as reported for other AAA+ type ATPases. Several ORC subunits as well as Cdc6 belong to this family of ATPases. The fact that most Archaea have a single protein almost equally related to both Orc1 and Cdc6 and that this Orc1/Cdc6 protein is structurally related to DnaA supports this analogy (28, 75). Separation of the initial origin DNA recognition (ORC function) from the conformational change which recruits the helicase (Cdc6 function) during evolution may have permitted tighter cell cycle control of the timing of helicase recruitment and may have allowed the ORC complex to resume more divergent roles linked to specific DNA binding and chromatin remodeling. In addition, the temporal separation of helicase loading from origin unwinding in eukaryotes may have been important for allowing the coupling of licensing to passage through mitosis together with a tight control over S-phase onset. *In vitro* analysis of ORC and Cdc6 interactions with ARS1 indicates that Cdc6 binding to ORC induces a conformation change that increases the affinity of ORC to origin DNA (94). Cdc6 mutated at its ATP binding site is unable to induce such a structural change. Additionally, this mutant Cdc6, though it can still bind to ORC, is unable to load MCM on chromatin. The conformational change induced by Cdc6, as well as the presence of Cdt1, is essential for the subsequent recruitment of the MCM complex onto chromatin.

How is MCM loading accomplished? *In vitro* reconstitution experiments show that chromatin association of ORC and Cdc6 occurs in the presence of ATP or ATP-γ-S, but loading of MCM does not occur in the presence of ATP-γ-S, suggesting that ATP hydrolysis by Cdc6 and/or ORC is required for MCM loading (34). Based on structural similarities of Cdc6/18 and Orc1 to loader proteins such as RF-C (a ring shaped loader of the sliding clamp PCNA) and DnaC, a similar mechanism for MCM loading has been proposed (110). DnaB loading by DnaC requires ATP hydrolysis, and at the same time DnaC is brought to an inactive ADP bound form. If ATP hydrolysis inactivates Cdc6 and ORC, similar to DnaC, this would prevent further MCM loading once an origin is licensed. In fact, ORC, Cdc6 and Cdt1 loose their affinity for chromatin after loading the MCM complex (44, 116).

Why is the presence of Cdt1 required for MCM loading? Cdt1 was shown to be transported into the nucleus together with the MCM complex in *S. cerevisiae* (128); mouse Cdt1 was shown to bind Mcm6 directly and to possess a non-specific DNA binding activity (146); while *S. pombe* Cdt1 was found in a complex with Cdc6/18 when overexpressed. Cdt1 may therefore deliver the MCM complex to origins. Alternatively, Cdt1 may have a role to induce changes on origin bound complexes which are essential for MCM loading. An understanding of the action of Cdt1 at the molecular level would have to await a better characterization of its structure and function.

Does the loaded MCM complex encircle the double stranded DNA? Where on the origin are MCM complexes positioned with relation to ORC? And how many MCM complexes are loaded from one ORC bound origin? In *S. cerevisiae*, both ORC and MCM antibodies precipitated a 270bp fragment of ARS1 by CHIP (chromatin immunoprecipitation) (129), indicating that the MCM complex is loaded near the ORC complex. In addition, whole genome CHIP analysis of ORC and MCM binding sites on *S. cerevisiae* chromosomes showed that MCM binding sites cluster near ORC bound sites in this organism. In *S. pombe*, CHIP analysis over the 1000 bp-long ars2004 (which contains three functionally important regions, I, II, and III) indicates that the ORC complex binds to Region I and III, which are about 600bp apart (it is not known if two ORC complexes bind to each region or a single ORC binds to both region), while MCM complexes are enriched in the middle, between regions I and III, about 300bp away from ORC bound sites (125). This suggests that the loaded MCM complex is not located in close vicinity to the ORC complex. In mammalian cells, the distance between the bound ORC and MCM is suggested to exceed 500-1000 bp. In *Xenopus* egg extracts, the number

of MCM complexes loaded is 20-40 times more than the number of chromatin bound ORC complexes (45). This indicates that several MCM complexes are loaded from a single ORC bound site. A similar observation was reported when linear DNA fragments coupled to beads were incubated with *Xenopus* egg extracts. ORC and MCM complexes were detected at a 1:1 ratio on 80 bp fragments. The ratio changed to 1: 20-40 when a 6 kb DNA fragment was used. MCM complexes are believed to be in excess over ORC complexes on DNA in several species, including yeast and mammals.

Laskey and Madine recently proposed an elegant model to explain current evidence for the loading and function of the MCM complex (67). This model postulates that MCM complexes are loaded onto DNA at the origins of replication but then move away from the origin by rotation along the helical thread of DNA. After being anchored to an immobile nuclear structure, an identical rotary action would pump the DNA back towards the origin, causing it to unwind at anchored sites of DNA replication.

5.3. Other factors involved in or regulating licensing

In vitro reconstitution experiments using purified proteins demonstrate that a substantial level of *Xenopus* sperm DNA can be licensed when incubated with purified ORC, Cdc6/18, Cdt1 and the MCM complex, together with nucleoplasmin that promotes decondensation of sperm DNA (34). Does this mean that these four factors are enough for licensing *in vivo*? Recent results indicate that at least one more factor is involved in establishing and maintaining licensing *in vivo* in *S. cerevisiae*. Noc3, first identified as a protein required for ribosome maturation and transport, was isolated as a multicopy suppressor of a mcm5 mutant (149). Noc3 is associated with origins, but not with non-origin DNA throughout the cell cycle. In the absence of Noc3, though ORC is detected on the chromatin, Cdc6 and MCM chromatin association is inhibited. In addition, when Noc3 was inactivated after licensing was completed, MCM proteins were removed from chromatin. Since Noc3 is a HLH protein and co-precipitates with Orc1 and Mcm5, Noc3 may facilitate MCM loading by modulating the chromatin structure around the origin. Further biochemical characterization will be required to clarify the involvement of Noc3 in licensing. It will also be interesting to investigate whether Noc3 connects replication to ribosomal RNA regulation, since this work suggests a link between protein synthesis and cell growth and DNA replication. A similar connection was proposed for Yph1, a yeast pescadillo homologue. Yph1 was isolated as one of the proteins that co-precipitated with *S. cerevisiae* Orc1 (27). It is required for S-phase progression, is mostly located in the nucleolus and forms complexes with ribosomal proteins.

Mcm10 was originally isolated in the same genetic screen used for isolating the Mcm2-7 genes in *S. cerevisiae*. It shows genetic interactions with Mcm and Orc genes (91). Interestingly, in mcm10-mcm7 double mutants, defects of each single mutant are suppressed. The ScMcm10 protein is associated with origins throughout the cell cycle. When Mcm10 was inactivated, the MCM complex dissociated from chromatin, suggesting that Mcm10 is required for keeping origins in the licensed state (43). Experiments with the fission yeast, *Xenopus* and human cells however, suggest that in these organisms, Mcm10 may function after licensing and before S-phase onset ((140) (49), (37), see also later).

6. ACTIVATION OF LICENSED ORIGINS

6.1. S-phase promoting kinases; CDKs and DDKs

When cells are in an appropriate growth condition, they establish replication licensing and initiate DNA replication. The key regulator that drives cells into S-phase is S-CDKs (Cyclin Dependent Kinases bound with S-phase specific cyclins) such as Cdc2-Cig2 in *S. pombe*, Cdc28-Clb5 and 6 in *S. cerevisiae*, and Cdk2-cyclinE and A in higher eukaryotes (122). In addition, cells require another kinase, Cdc7/Dbf4 (Cdc7 kinase bound with the regulatory subunit Dbf4, originally isolated in *S. cerevisiae* also called DDK for Dbf4 Dependent Kinase) (81). It is important that activation of CDKs beyond a certain threshold only takes place after licensing is completed, since premature activation of CDK blocks licensing (see later section).

6.2. Molecular events at origin unwinding

At the G1/S transition, phosphorylations by CDKs and DDKs are believed to cause a change in the structure of the pre-RC that leads to recruitment of additional factors to the licensed origins, unwinding of origin DNA, and finally loading of the DNA polymerases. In the *S. cerevisiae* ARS1, a melted chromatin structure was detected after completion of the Cdc7 function and before Cdc8 (a thymidylate kinase) functions (33).

Cdc45 is a key factor that converts the inactive pre-RC complex to a pre-initiation complex ready for replication. It appears that Cdc45 association with the MCM complex is a key step in origin unwinding. MCM 2-7 complex immunoprecipitated from *Xenopus* egg extracts is free of Cdc45 and does not have helicase activity. On the contrary, MCM2-7 complexes purified from S-phase chromatin are tightly associated with Cdc45 and show high helicase activity (83). Though the possibility of contaminating helicases in the precipitates remains, it is conceivable that a conformational change at the MCM2-7 complex, following association with other factors such as cdc45, leads to an increase in its helicase activity.

Several factors in addition to Cdc45 interact with origin bound complexes at the G1/S transition, before initiation can occur. In *Xenopus*, Mcm10 associates with Pre-RC independently of DDK activity, and stimulates origin binding of Cdc45 (140). Similarly, experiments in *S. pombe* indicate that Mcm10, while bound to chromatin throughout the cell cycle, is required for the chromatin association of Cdc45 (37). Dpb11 of *S . cerevisiae*, isolated as a multicopy suppressor of pol ε, is required for loading of both pol ε and pol α to origins (4, 85). Sld genes (Sld1 - Sld6) were isolated as synthetically lethal mutations with dpb11 (54). Sld2 forms a complex with Dpb11 (see later), Sld3 forms a complex with Cdc45 (55), while Sld5 and its *Xenopus* homologue were recently shown to form a novel

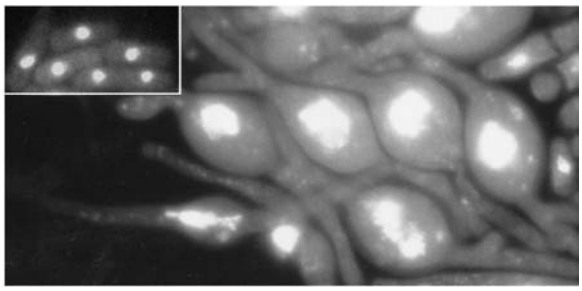

Figure 3. Over-replicating *S. pombe* cells. The photograph shows DAPI staining of nuclear DNA. Insert shows that of wild type cells.

complex (called GINS) which binds to chromatin in a manner mutually dependent with Cdc45-Sld3 and is required for the initiation of replication (56, 65, 126). Xmus101, the *Xenopus* Dpb11 homologue, was recently shown to be required for the loading the GINS complex onto chromatin (65) and, somewhat surprisingly, for the loading of Cdc45 (132).

Dpb11, as well as several of the Sld gene products appear to connect DNA replication with checkpoint control. Cut5, the *S. pombe* Dpb11 homologue, is also involved in checkpoint regulation. Human TopBP1 and *Xenopus* Xmus101 are homologous to Dpb11/Cut5 and both have BRCT1 domains, a domain often detected in checkpoint proteins (132).

Building of the pre-initiation complex leads to origin unwinding. Once the origin is unwound, RPA binds to the single stranded DNA, and pol α-primase and pol ε are recruited to initiate DNA synthesis (51, 93, 136, 151) (reviewed in (127)). Where does unwinding of origin DNA occur in relation to the position of ORC and MCM? Unwinding of ARS1 DNA occurs in the B2 element located about 30 bp downstream of the B1 element (to which and to element A ORC binds) and the first sites of leading strand synthesis for both orientations are mapped between B1 and B2 elements (1). Linker scanning analysis suggests that the MCM complex is associated with element B2 (139). In the *S. pombe* ars2004, 2D gel analysis indicates that initiation sites within this origin occur close to region II, where the MCM complex is located and about 300 bp away from the region where ORC associates (125). These observations indicate that unwinding occurs where MCM is located. Evidence in higher eukaryotes however, suggests that MCM proteins may be located away from sites of replication (reviewed in (67)).

6.3. Targets of CDKs and DDKs for origin activation

Though the order to action of CDKs and DDKs may differ between species, activation of both CDKs and DDKs is essential for S-phase onset. Many of the components of ORC and MCM complexes, such as Orc2, Mcm2 and Mcm4 undergo S-phase specific phosphorylation. Much evidence suggests that MCM proteins are phosphorylated by the DDK, which leads to interactions with Cdc45 and to an active helicase. Specifically the Mcm2 protein is an excellent substrate for DDK, both in a monomeric form and complexed with other Mcm proteins (72, 81, 82). Genetic evidence shows an interesting interaction between Cdc7 and MCM proteins. A mutation in the mcm5-bob1 gene in *S. cerevisiae* bypasses the requirement for Cdc7 kinase and a mutation in dbf4 complements a mcm2 mutation (39). Consistently, in mcm5-bob1 cells, melting of the origin region was observed in G1 arrested cells, while such melting is normally detected after the execution step of Cdc7 (33). In fission yeast, Mcm10 stimulates MCM phosphorylation by DDK (71).

ORC, Cdc6/18, Cdt1 and MCM all contain consensus sites for phosphorylation by CDKs. It is not known at the moment whether phosphorylation of any of these proteins is required for origin firing. Rather, phosphorylation of these proteins by CDKs is interpreted as having a negative role to prevent re-replication (see later section), though it is possible that phosphorylation is required for activation, and at the same time it causes the inactivation of licensing factors. The first protein whose phosphorylation by CDK was shown to be required for DNA replication is the Sld2/Drc1 protein (84). Sld2 was isolated as one of the genes that showed synthetic lethality with dpb11, while Drc1 as a multicopy suppressor of dpb11 (54, 137). Dpb11 is required for the recruitment to origins of pol ε and pol α. Phosphorylation of Sld2 occurs at the onset of S-phase, and this phosphorylation is essential for Sld2-Dpb11 complex formation, and thus loading of polymerases on unwound origins. The same conclusion was reported in *S. pombe*: SpDrc1 forms a complex with Cut5, a Dpb11 homologue(103).

7. PREVENTION OF RE-REPLICATION

After DNA replication is initiated, the MCM complex and Cdc45 move along the chromatin together with replication enzymes assembled at the replication fork to complete DNA replication, while ORC complexes remain on the origins (3, 130). To ensure genome integrity, re-replication of replicated DNA must be inhibited until chromosome separation is completed (Figure 1). Upon activation, licensed origins (pre-RC) convert to the unlicensed state (post-RC). If cells maintain the replicated chromosomes in an unlicensed state until the end of mitosis, re-replication is prevented. Thus, the temporal and special regulation of these two states in the cell cycle is crucial for once-per-cell-cycle replication. How is this achieved? Genetic experiments in *S. pombe* provided a profound insight into this important regulation by examining mutants that lead to re-replication of chromosomal DNA (Figure 3). The first key regulator to be identified turned out to be the master regulator of the cell cycle, the CDK Cdc2.

7.1. Global regulation of replication by CDKs

CDKs, associated with stage specific cyclins, drive cells into S-phase and M-phase. The M-CDKs (CDKs associated with B type mitotic cyclins) are essential for entry into mitosis. Loss of M-CDKs would therefore be expected to cause cell cycle arrest at G2. An important finding was first observed using a *S. pombe* cdc2 temperature sensitive mutant. This cdc2 mutant arrests in G2-phase at the restrictive temperature of 36°C. When the

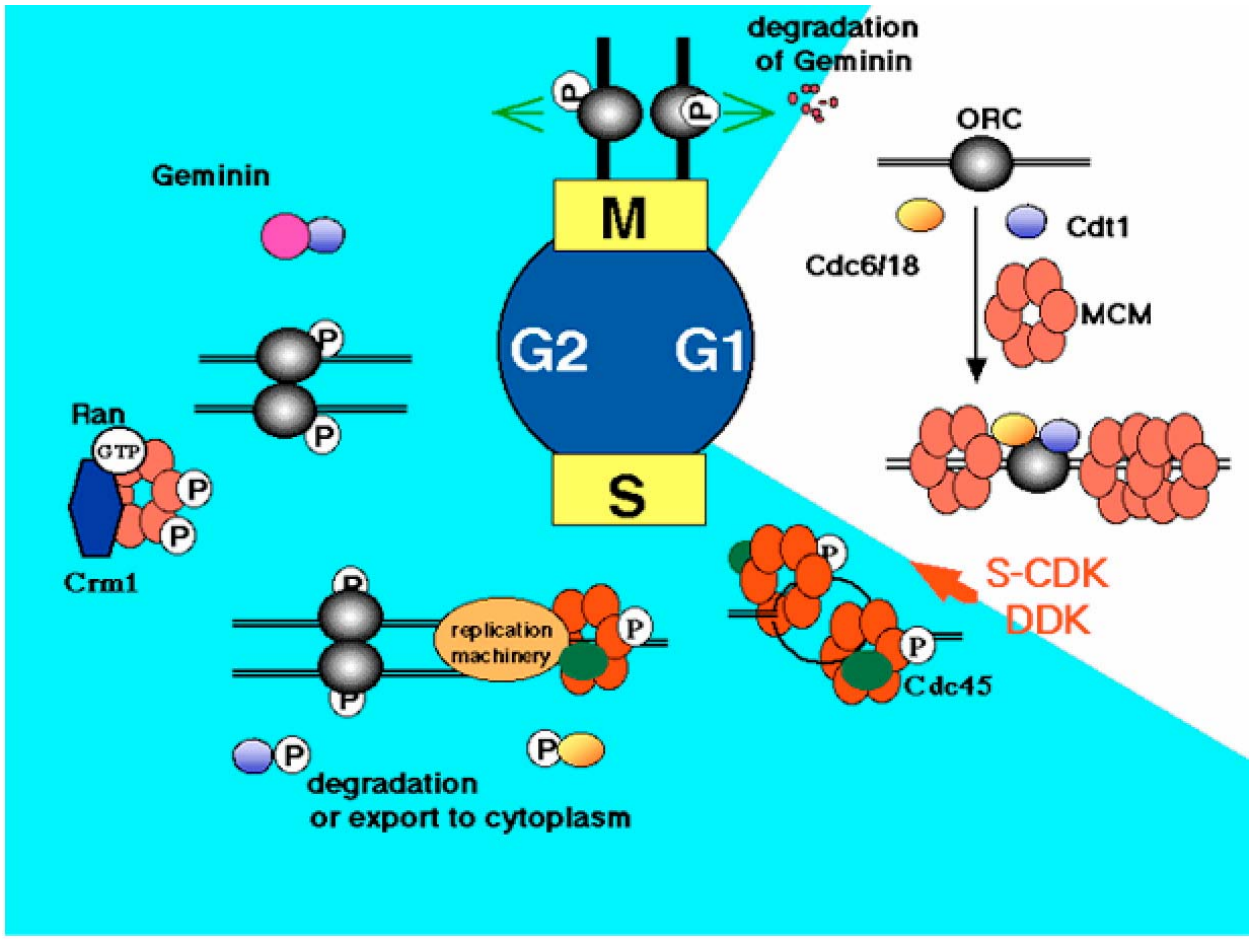

Figure 4. Licensing control during the cell cycle. Origin licensing is allowed when CDK activity is low, normally at the end of M-phase and/or G1-phase. When cells are in a permissive growth conditions, S-CDK is activated and together with DDK initiates DNA replication. Phosphorylation of MCM and its association with Cdc45 appears to be important for activation of MCM complex as a helicase. DNA is unwound, the replication enzymes are recruited and replication proceeds with MCM-Cdc45 complex. After origin is activated, it is brought to an unlicensed state. CDK inactivates licensing factors by phosphorylating them until the end of mitosis. In higher eukaryotes, Cdt1 is inactivated by Geminin, which appears at the beginning of S-phase. Phosphorylated MCM complexes in S and G2-phases are sequestered by Crm1 and RanGTP binding.

cdc2 cells were treated briefly at higher temperature, to completely destroy the Cdc2 protein, and were then returned to the permissive temperature, cells re-started S-phase instead of entering into mitosis (9). An even more dramatic phenotype was observed when the mitotic cyclin cdc13 was depleted. A strain depleted for Cdc13 underwent multiple rounds of S-phase without intervening M-phase, and its DNA content doubled from 2C to 4C, 8C… like a kind of genome wide PCR (40). Similarly, high-level expression of the Cdc2-Cdc13 inhibitor, rum1 resulted in re-replication (19, 95). The same phenomena were observed in *S. cerevisiae* by ectopically expressing the CDK inhibitor Sic1 in G2-phase (21). Also in mammalian cells, a conditional knockout of Cdc2 caused re-replication (48).

As described in Figure 1.B, re-replication may take place from the G2-phase or within S-phase. The above results demonstrated that M-CDKs are essential for preventing re-replication in G2 cells. However, in these cells, the control that prevents re-replication within the same S-phase is still intact and cells completed a round of replication before reinitiating replication. Recent results suggest that the periodic appearance of S-CDKs is essential for initiating multiple rounds of S-phase, and that this kinase activity is at the same time important for preventing re-initiation within the same S-phase (143).

On the other hand, premature activation of M-CDKs brings about untimely mitosis without DNA replication. Ectopic expression of the Cdc2 protein kinase induces mitosis, even from G1. Importantly, Cdc6/18 must be expressed and licensing established before CDK kinase activity increases (113).

Based on these observations, a concept emerged that the CDK cycle globally regulates the temporal order of S-phase and M-phase. By controlling origin licensing, the CDK cycle ensures one round of replication per cell cycle (23, 144). At the end of mitosis, CDK is inactivated and

licensing of origins is allowed. Activation of S-CDK triggers initiation of replication and at the same time brings origins to the unlicensed state. Subsequent increase of M-CDK maintains the unlicensed state until the end of mitosis. If M-CDK is absent and S-CDK activity low, cells in G2-phase become capable of establishing licensing.

How do CDKs regulate licensing to prevent re-replication? One interesting observation is that in *S. pombe* Cdc2-Cdc13 associates with replication origins through the ORC complex, and its association is mutually exclusive to that of MCM (143). Cdc2-Cdc13 may physically inhibit the recruitment of licensing factors and/or phosphorylate the ORC complex, rendering it refractory to association with licensing factors.

Experiments in the *Xenopus* egg system suggest an elegant way to activate CDKs bound to origins. Activation of CDKs depends on the inactivation of CDK inhibitors such as p27 and p57 in mammalian cells, sic1 in *S. cerevisiae*, and Xic1 in *Xenopus*. In a *Xenopus in vitro* system, Xic1, Cdk2/cyclinE and the SCF ubiquitin ligase are recruited to chromatin through association with licensing factors. This recruitment promotes Xic1 degradation and activates Cdk2 (31, 148). It remains to be seen whether a similar regulation exists in other organisms.

Several licensing factors have CDK phosphorylation sites, and their phosphorylation results in their inactivation as described in the following sections.

7.2. Inactivation of licensing factors by CDKs and proteolysis

S. pombe genetics uncovered another important control acting over re-replication. High-level expression of Cdc18 overrode the re-replication control, driving cells to a continuous replication (96, 101). In addition, Cdt1 was shown to promote Cdc18 induced re-replication (100, 147). In contrast to the re-replication induced by loss of Cdc2-cdc13, the DNA content increased continuously in cells over expressing these licensing factors, and this continuous replication was depended on checkpoint control (36), suggesting that re-licensing of origins was taking place repeatedly in a single S-phase. In mammalian cells, a partial re-replication was observed when Cdt1 was over-expressed in p53 deficient cell lines (135). Regulating the levels of Cdc18 and Cdt1 is therefore crucial for once per cell cycle replication.

Proteolysis turned out to be important for regulating the levels of licensing factors during the cell cycle: inactivation of *S. pombe* Pop1, a component of the E3 ubiquitin ligase SCF (62), resulted in polyploidy. Pop1 was shown to target Cdc18 for proteolysis following its phosphorylation by Cdc2 (5, 50). Similarly, *S.cerevisiae* Cdc6 is recognized and targeted for proteolysis by the F box protein Cdc4 (26). Similar controls appear to function in higher eukaryotes: knockout of Skp2, a mammalian F-box protein in mouse results in increase of ploidy in some tissues (97). ts41 cells have a defect in Nedd 8 conjugation of SCF complex, which is essential for its activity. At restrictive temperature, ts41 cells undergo re-replication (11, 38), when both Cdt1 and Cdc6/18 `proteins accumulate (our unpublished observation). Interestingly, it is Cdt1, rather than Cdc6/18, which appears to be the main target of the proteolysis-dependent rereplication control in metazoa. Human Cdt1 is degraded by ubiquitin dependent proteolysis so that the Cdt1 protein is only detected in G1 (102). In contrast, mammalian Cdc6/18 remains in S-phase and G2 and it is only degraded at the end of mitosis through the APC E3 ligase (89, 112). In addition, human Orc1 looses affinity for chromatin in S-phase and is degraded, in an SCF-dependent manner (73, 90). In *C. elegans*, inactivation of CUL-4, a member of the cullin ubiquitin ligase family, causes massive rereplication, producing cells with up to 100C DNA content. Removal of one genomic copy of Cdt1 suppresses the cul-4 rereplication phenotype, suggesting that Cdt1 is the main target of this control (150).

Phosphorylation may also affect the ability of licensing factors to assemble the pre-replicative complex. Both ORC and MCM proteins are phosphorylated by CDKs. Orc2, in *S. pombe* as well as *S.cerevisiae*, is phosphorylated during S-phase and mitosis (76, 99, 133). A phosphorylation site mutant of *S. pombe* Orc2 was able to enhance cdc18 mediated re-replication. Consistenlty, ScCdc6 can associate with ORC purified from G1 cells, but not from G2 cells, suggesting that a modification takes place on ORC after S-phase, which inhibits Cdc6 association (120). In mammalian cells, phosphorylation of ORC, Cdc6/18, and MCM proteins appear to decrease their affinity for chromatin (29).

7.3. Re-replication control and the Ran cycle

The initial licensing model of Blow and Laskey (8) postulated that the nuclear envelope excludes an essential licensing factor from the nucleus during G2, which only gains access to DNA after nuclear envelope break-down in mitosis. Indeed, *S. cerevisiae* MCM proteins where soon discovered and shown to be excluded from the nucleus in G2 and to accumulate in the nucleus in G1, lending support to this model. Since in yeast the nuclear envelope does not break down in mitosis, cell cycle specific import and export of MCM proteins into and out of the nucleus ensure that MCM proteins accumulate in the nucleus only in G1. MCM proteins in other species however appear constitutively nuclear, which questioned the importance of regulated nucleo-cytoplasmic transport for timely licensing in all eukaryotes. As more data accumulate however, it becomes evident that the nucleocytoplasmic transport machinery has an important role in regulating licensing in all eukaryotes. This regulation differs in its details among species, and appears to be one of several means which the cell employs to ensure re-replication control under all circumstances.

In *S. cerevisiae*, not only the MCM proteins but also ScCdt1 is subjected to cell cycle specific nucleocytoplasmic shuttling. Cdt1 is present in the nucleus in G1 and appears to enter the nucleus in a manner interdependent with the nuclear accumulation of the MCM complex. ScCdt1 is then exported from the nucleus during

S-phase and G2, dependent on its phosphorylation by CDKs.

In higher eukaryotes, MCM proteins appear constitutively nuclear. However a recent report suggests that the nucleocytoplasmic transport machinery may be important for preventing re-replication also in higher eukaryotes, by binding to and inactivating licensing factors, even in the absence of net export from the nucleus. *Xenopus* MCM proteins form a complex with the exportin-1/Crm1 during S-phase, and formation of this complex is dependent on both Ran-GTP and phosphorylation by S-phase CDKs (145). Lowering Ran-GTP within nuclei allows MCM proteins to re-associate with chromatin and induces rereplication. Re-replication control is therefore added to the long list of cellular processes regulated by Ran. It is unclear whether MCM proteins are the only pre-RC components which are targeted by Ran-GTP/Crm1. In fact, previous reports have shown that a soluble pool of Cdc6 is exported from the nucleus during S-phase, dependent on its phosphorylation by CDKs (30, 52, 109, 111, 117). In addition, activation of the licensing inhibitor Geminin (see next session) requires that it is imported into the nucleus.

7.4. Geminin, an inhibitor of Cdt1

In contrast to single cell organisms, multi cellular organisms may require a more strict regulation over re-replication, since a failure in genome integrity would be detrimental for the organism. Geminin, first cloned in *Xenopus* as a substrate of the mitosis specific ubiquitin ligase APC (88), may be such a metazoa specific licensing regulator. It has a so called “destruction box” and mutating this box renders Geminin a potent inhibitor of licensing. In the presence of an undegradable form of Geminin, loading of the MCM complex was prevented, though ORC and Cdc6/18 were found on the chromatin. A Geminin interacting protein, isolated from human cells and *Xenopus* extracts, turned out to be Cdt1. Using the *Xenopus in vitro* assay, Geminin binding to Cdt1 was shown to block MCM loading (124, 141). High-level expression of Geminin in mammalian cells arrests cells in G1-phase (123, 142). It is possible that Geminin binding will block Cdt1 chromatin association, or will prevent MCM loading on chromatin bound Cdt1 (34, 146). *In vitro* binding assays show that Geminin binds to the middle of Cdt1, while Mcm6 binds to the C-terminus of Cdt1 (146).

It is shown that Geminin has a substantial role in preventing premature licensing in *Xenopus* eggs (arrested at the metaphase of meiosis II), but re-replication is not induced in Geminin depleted extracts (88). Inactivation of Geminin by anti-sense treatment caused a Chk1 dependent G2 arrest at early stages in the *Xenopus* embryo (87). The knockout of Geminin in *Drosophila* results in pleiotropic effects: increase in DNA content, anaphase defects, and increase in S-phase cells (92, 115). Further experiments are required to establish that the observed high amount of DNA content is due to re-replication. There may be a cell type specific or a stage specific effect in Geminin depleted cells. Inactivation of the Cdc2 protein kinase brings about re-replication in mammalian cells (48). Since degradation of Geminin is dependent on APC, which is in its turn activated by the Cdc2 protein kinase, Geminin may not be degraded when Cdc2 is absent. It would be interesting to study Geminin in such cells.

It is curious that Geminin and Cdt1 mostly do not co-exist during the mammalian cell cycle (102, 141). Cdt1 is present in G1-phase and disappears after initiation of replication, while Geminin is not present in G1 and appears around the beginning of S-phase. Cdt1 and Geminin may only co-exist during a small window of the cell cycle, around the G1/S transition. Geminin may have a failsafe role to inhibit any Cdt1 escaping degradation under adverse conditions, such as heat shock or DNA damage, when CDKs might be inactivated. It is possible however that Geminin may have an unidentified role in addition to inhibition of Cdt1 in the cell cycle. A careful knockout experiment of Geminin is required to address Geminin’s *in vivo* role.

The data presented in the previous sections showed that the basic licensing control is conserved from yeast to mammalian cells. Though the details of regulation somewhat differ between organisms and are highly redundant, CDKs always act as the principal regulator to control licensing in a cell cycle. Only when CDK activity is low at G1-phase, licensing of origins is allowed, and following initiation, CDKs maintain the replicated DNA in an unlicensed state until chromosome separation is completed. It is therefore somewhat surprising that no Geminin homologue has yet been identified in lower eukaryotes. This could either reflect a very low level of conservation of this licensing regulator across evolution or could indicate a metazoa-specific control which has evolved to accommodate the complexity of life as a multicellular eukaryote.

8. PERSPECTIVE

ORC, Cdc6/18, Cdt1 and the MCM complex are essential for establishing licensing. *In vitro* reconstitution experiments indicate that in the presence of these four factors, substantial levels of sperm DNA was licensed for replication, in a Geminin sensitive manner (34). At least in such a simplified situation, these four factors appear therefore sufficient for licensing. However, each origin in the cell is subject to a specific surrounding chromatin structure, including nucleosomes, heterochromatin regions, adjacent transcriptional complexes etc. To establish licensing in such circumstances, additional factors may be required to promote and maintain licensing. Noc3, histone acetyl-transferases and chromatin remodelling factors may be some of the additional factors involved.

Examination of licensing in re-replicating cdc13-depleted *S. pombe* cells shows that the control of temporal licensing for each round of S-phase is maintained independent of M-CDK (143). On the other hand, oscillation of mitotic CDK activity can be driven in the absence of DNA replication in early *Xenopus* development. These observations indicate that intrinsic oscillations of DNA replication and M-CDK activity are mutually

independent events. To combine these events in the cell cycle for the production of two identical cells, cells have developed checkpoint controls to ensure the interdependency of each cell cycle event. Licensing factors are essential for the checkpoint signal which prevents activation of M-CDK, through forming replication forks, while CDKs inactivate licensing factors more directly as described. Do licensing factors also directly regulate CDKs? In *S. cerevisiae*, a role for Cdc6 to inactivate M-CDK has been proposed (10) while in mammalian cells, Cdc6/18 is present until the end of mitosis. There might be an unknown function of Cdc6/18 to regulate CDK activity. It is also important that activation of CDK and Cdc7 at the initiation of replication must depend on the completion of licensing. Is there a control to ensure that licensing is finished before accumulation of S-CDKs? The presence of a licensing checkpoint has been proposed for mammalian cells (119). Is it also possible that free forms of licensing factors act inhibitory to CDKs, and their assembly on chromatin may free CDKs for activation?

During the cell cycle, licensing takes when CDK activity is low at the end of mitosis, while increase in CDK activity after S-phase onset inhibits further licensing. CDKs are therefore unlikely to act positively on licensing. Unexpectedly, a positive role for CDKs on licensing is emerging at the transition from quiescence to proliferation. Licensing is lost when cells exit the cell cycle to G0 and it needs to be re-established when cells resume growth. Using an *in vitro* licensing system, Coverley *et al*. showed that cyclin E stimulates MCM loading onto chromatin during the transition from G0 to S-phase (20). Similarly, knocking out both E-type cyclins in mice had surprisingly little effect on cycling mouse embryonic fibroblasts, but inhibited licensing during the G0 to S-phase transition (32). It would be interesting to investigate why cyclin E becomes essential for licensing in this transition.

Do licensing factors have roles in addition to ensuring timely licensing? Orc proteins were shown early on to be involved in heterochromatin formation in both yeast and *Drosophila* and to be required for mitosis, while human Orc6 was also recently shown to be involved in mitosis (114). Geminin is involved in neurogenesis as well as cell cycle control. More experiments are required to address the multiple roles of licensing factors *in vivo*.

Licensing of replication must also be coupled to cell growth. Cells enter the cell cycle only when adequate conditions are ensured. Production and inactivation of licensing factors should depend on such growth conditions. In this sense, anchorage dependent expression of Cdc6/18 (53), and the involvement of factors such as Noc3 and Yph1, may provide a new insight to connect growth to licensing.

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