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The replication licensing system

Shusuke Tada and J. Julian Blow

CRC Chromosome Replication Research Group, University of Dundee, UK

Abstract

The replication licensing system acts to ensure that no section of the genome is replicated more than once in a single cell cycle. Experiments using *Xenopus* egg extracts have revealed that the licensing system consists of two components, named RLF-M and RLF-B. Whereas the function of RLF-B is still unclear, RLF-M has been shown to consist of all six members of MCM/P1 family proteins, which appear to be the structural component of the licensing system. The origin recognition complex (ORC) and Cdc6/Cdc18 are needed on chromatin before the licensing reaction can take place, although they are not themselves components of the licensing system. Cell cycle events and cyclin-dependent protein kinases (Cdks) also seem to be involved in controlling the licensing system to ensure once per cell cycle DNA replication. The subject of this review is to detail our current understanding of the licensing system and the way that it interacts with other components of the cell cycle machinery.

Keywords

DNA replication; licensing; RLF-M; RLF-B; MCM; ORC; Cdc6; Cdc18; Cdk

Introduction

To ensure the precise duplication of chromosomal DNA during the cell division cycle, two conditions must be met. First, DNA must be duplicated only in the period specified for DNA replication in cell cycle (S phase), which is clearly separated from the period for chromosome segregation and cell division (M phase). Second, all regions of the genome must be duplicated in a single cell cycle, but no regions should be replicated more than once. The replication licensing system, originally defined in *Xenopus*, acts to ensure that no section of the genome is replicated more than once in a single S phase. As the components of the *Xenopus* licensing system have been identified, it has become clear that this system has been highly conserved in eukaryotes.

The Replication Licensing System in *Xenopus*

The replication licensing system was first proposed as a consequence of experiments using *Xenopus* egg extracts that support faithful chromosome duplication in vitro (Blow and Laskey, 1988). DNA replication in this system is dependent on the temporal DNA being assembled into functional interphase nuclei (G1 nuclei) (Blow and Laskey, 1986; Newport, 1987; Blow and Watson, 1987). Although G1 nuclei could be induced to initiate replication for extended periods of time (Blow and Watson, 1987), no DNA was re-replicated in a single cell cycle. Even if replicated G2 nuclei were added back to early interphase extract, no re-replication occurred (Blow and Laskey, 1988). However, if the G2 nuclei were allowed to progress into mitosis and undergo nuclear envelope breakdown and chromosome condensation, they could then undergo a further round of DNA replication when added back to fresh extract. Therefore some metaphase process had permitted G2 nuclei to revert to the responsive G1 state. To identify what this metaphase process is, G2 nuclei were subjected to

various treatments to see whether they could induce G2 nuclei to re-replicate in fresh extract without entering metaphase (Blow and Laskey, 1988). Treatments that caused nuclear envelope permeabilisation left the G2 nuclei capable of re-replicating in fresh extract. This nuclear envelope permeabilisation presumably mimics the effect of nuclear envelope breakdown that normally occurs during mitosis in higher eukaryotes.

To explain these results, the original model proposed the existence of a replication licensing factor (RLF), that must be bound to chromatin in order for the initiation of DNA replication to occur but is displaced as the DNA is replicated (Blow and Laskey, 1988; Fig. 1). If RLF is incapable of crossing an intact nuclear envelope, licensing is strictly periodic, only occurring when the nuclear envelope is broken down in mitosis or by artificial means in vitro (Blow and Laskey, 1988). This model was capable of explaining the results of the classic cell fusion experiments of Rao and Johnson (Rao and Johnson, 1970). Results consistent with this model were also obtained on addition of nuclei from mammalian tissue culture cells into *Xenopus* eggs (De Roeper et al., 1977) or egg extracts (Leno et al., 1992): although intact G1 nuclei replicated after transfer to *Xenopus* extract, G2 nuclei replicated only if they had been permeabilised prior to transfer.

In order to subject the licensing system to biochemical analysis, it was necessary to determine conditions where its activity could be manipulated. When metaphase *Xenopus* extracts are treated with certain protein kinase inhibitors, such as 6-dimethylaminopurine (6-DMAP) (Blow, 1993; Kubota and Takisawa, 1993; Vesely et al., 1994), subsequent DNA replication is blocked. These 6-DMAP-treated extracts lack an essential initiation activity that conforms to all the definitive features of RLF: the activity can bind to chromatin prior to, but not after nuclear assembly, and is removed from chromatin during replication (Blow, 1993). Metaphase-arrested *Xenopus* extracts lack this licensing activity, but it is rapidly activated on transition into anaphase (Blow, 1993; Mahbubani et al., 1997). It appears that 6-DMAP and related protein kinase inhibitors block the activation of licensing activity, rather than the licensing reaction itself, since 6-DMAP does not significantly inhibit any of the steps in DNA replication once the licensing system has become activated (Blow, 1993; Kubota and Takisawa, 1993; Mahbubani et al., 1997). The mechanism by which these protein kinase inhibitors inhibit activation of the licensing system is discussed in more detail below.

These observations led to the development of a biochemical assay for RLF components (Chong et al., 1995; Chong et al., 1997). Sperm nuclei are briefly incubated in 6-DMAP-treated extract to assemble it into chromatin that is unlicensed but is in a competent state for licensing to occur. This '6-DMAP chromatin', containing any (non-RLF) proteins required for licensing to occur, is then isolated and incubated with fractions potentially possessing licensing activity. The degree of licensing is assessed by transferring the chromatin back to 6-DMAP-treated extract and measuring the ensuing DNA replication. A series of experiments with the cell-free system revealed that the licensing of unlicensed 6-DMAP chromatin needs at least 3 distinct factors: ATP and 2 putative protein factors called RLF-M and RLF-B (Chong et al., 1995, 1997).

Replication Licensing Factor-M (RLF-M)

Using the 6-DMAP chromatin assay RLF-M activity was purified to homogeneity and shown to consist of complexes containing all six members of the MCM/P1 family (Chong et al., 1995; Thömmes et al., 1997). Consistent with their role in licensing, the *Xenopus* MCM/P1 proteins were shown to be assembled onto chromatin shortly after exit from metaphase, and removed from the chromatin as a consequence of DNA replication (Kubota et al., 1995, 1997; Chong et al., 1995; Madine et al., 1995a, 1995b; Coue et al., 1996; Hendrickson et al., 1996; Romanowski et al., 1996a; Thömmes et al., 1997). The MCM proteins had first been

identified in a screen for mutations of *S. cerevisiae* that were defective in replication of minichromosomes and which showed genetic interactions with replication origins (Maine et al., 1984; Sinha et al., 1986; Gibson et al., 1990). Six of these proteins, MCM2-7, are closely related to one another to form the MCM/P1 protein family which is highly conserved in eukaryotes (Yan et al., 1991; Chen et al., 1992; Chong et al., 1996). Some of the conserved domains show some homology to known DNA helicases (Koonin, 1993). All members of the MCM/P1 family studied in other eukaryotes appear to show periodic chromatin association, similar to that observed in *Xenopus* (for reviews see Chong et al., 1996; Kearsey et al., 1996). These results strongly suggest that the MCM/P1 family proteins form the structural component of the replication licensing system in all eukaryotes, and that the stable loading of MCM/P1 family proteins onto chromatin is the functional equivalent of the replication licensing defined in *Xenopus*.

Although the function of MCM/P1 proteins in the initiation event is still unclear, interesting observations about the behaviour of MCM/P1 proteins are accumulating. *Xenopus* RLF-M can be separated into at least two sub-complexes, MCM3/5, and MCM2/4/6/7, neither of which shows RLF-M activity on its own (Thömmes et al., 1997; Kubota et al., 1997). Similar subcomplexes have been observed in mammalian cells (Burkhart et al., 1995; Musahl et al., 1995; Schulte et al., 1995). An MCM2/4/6/7 complex from human cells is capable of binding histone H3 (Ishimi et al., 1996), whilst a related MCM4/6/7 complex shows single-stranded DNA-dependent ATPase and DNA helicase activity (Ishimi, 1997). By analogy with other replication systems, two distinct types of DNA unwinding are expected to be involved in DNA replication. One necessary function is the melting of the DNA duplex in the vicinity of the replication origin to allow replication fork proteins to be loaded onto the exposed single strands; another is the unwinding of duplex DNA in front of the replication fork to allow the fork to progress. In *E. coli*, origin unwinding is performed by the *dnaA* protein, which shows some homology to the MCM/P1 family (Koonin, 1993), whereas *dnaB* protein is the replicative helicase. A specific role for MCM/P1 proteins in the initiation of DNA replication would be consistent with immunofluorescence data in metazoan cells, which shows them localised to regions containing unreplicated DNA, and not to active sites of replication (Kimura et al., 1994; Todorov et al., 1995; Krude et al., 1996; Romanowski et al., 1996a). However, the release of MCM/P1 proteins from chromatin is delayed some what from when initiation occurs (Madine et al., 1995b). Cross-linking data from *S. cerevisiae* also suggests that the MCM/P1 proteins are associated with non-origin DNA later during S phase (Aparicio et al., 1997). These results are consistent with the MCM/P1 proteins travelling some distance with the replication fork before being released.

Replication Licensing Factor-B (RLF-B)

6-DMAP chromatin incubated with RLF-M (containing a complete set of MCM/P1 family proteins) does not result in the licensing of DNA or loading of MCM/P1 proteins onto the chromatin, even in appropriate buffers containing ATP (Chong et al., 1995; Mahbubani et al., 1997). In addition, a second component of the licensing system called RLF-B is required. At present, RLF-B has not been purified, and it is still unclear how RLF-B facilitates the assembly of MCM/P1 proteins onto chromatin. Unlike RLF-M, which is apparently active throughout the *Xenopus* cell cycle, RLF-B activity is periodic (Blow, 1993; Mahbubani et al., 1997). RLF-B activity is low in metaphase, but is rapidly activated on progression into anaphase. RLF-B activity then declines again during the first half of interphase. The regulation of RLF-B activity is discussed in more detail below.

Chromatin-Associated Proteins Required for Licensing to Occur

The assay for RLF components described above involved the incubation of RLF -M and RLF-B with chromatin previously assembled in a 6-DMAP-treated extract (Chong et al., 1995, 1997). The rationale behind this was to provide chromatin with any non-RLF proteins that may be required on chromatin before licensing can occur. At least two non-RLF proteins are now known to be required for in this: the *Xenopus* Origin Recognition Complex (XORC) and XCdc6, the *Xenopus* homologue of Cdc6 in *S. cerevisiae* (or Cdc18 in *S. pombe*) (Romanowski et al., 1996b; Rowles et al., 1996; Coleman et al., 1996).

Origin Recognition Complex (ORC) ORC was first identified in *S. cerevisiae* as a protein complex, consisting of 6 polypeptides designated Orc1-6, that specifically bound to the core component of yeast origins of replication (Bell and Stillman, 1992). In vivo footprinting suggested that ORC was bound to the origin region throughout the cell cycle (Diffley and Cocker, 1992). All 6 ORC genes have been cloned from yeast, and genetic analysis suggests that ORC plays a crucial role in the initiation of replication (reviewed in Diffley, 1996; Stillman, 1996).

In spite of uncertainty about what constitutes a replication origin in higher eukaryotes, homologues of ORC subunits have been identified in a variety of eukaryotes including *Xenopus*. Two ORC genes have been identified from *Xenopus*, XORC1 (Rowles et al., 1996) and XORC2 (Carpenter et al., 1996). A complex of 7 polypeptides has been purified from *Xenopus* (XORC), containing both XOrcl and XOrcl2, with an overall similarity to yeast ORC (Rowles et al., 1996). Immunodepletion of XOrcl or 2 from *Xenopus* egg extracts abolished the initiation of DNA replication (Carpenter et al., 1996; Romanowski et al., 1996b; Rowles et al., 1996), and activity can be restored by the addition of purified XORC (Rowles et al., 1996). XOrcl is found associated with chromatin through interphase, and it saturates chromatin with an average of one XOrcl molecule per 10 - 15 kb, close to previous estimates for average replicon size (Rowles et al., 1996). These data are consistent with XORC being an important determinant of replication origins in *Xenopus*.

Chromatin assembled in extracts immunodepleted of XOrcl or 2 are unable to assemble the MCM/P1 polypeptides (or XCdc6, as discussed below) onto chromatin, and are not functionally licensed (Coleman et al., 1996; Rowles et al., 1996; Romanowski et al., 1996b). There is a sequential assembly reaction, whereby first XORC is assembled onto chromatin, followed by the loading of MCM/P1 proteins and functional licensing (Rowles et al., 1996). However, it seems unlikely that RLF-M is assembled directly onto XORC on the chromatin. First, in *Xenopus*, chromatin can bind up to 10 - 20 copies of the RLF-M per replication origin (Mahbubani et al., 1997), whilst chromatin becomes saturated with 1 copy of XORC per replication origin (Rowles et al., 1996). A similar quantitative excess of MCM/P1 proteins over replication origins is seen in other cell types (Burkhart et al., 1995; Lei et al., 1996). Second, XOrcl can be removed from chromatin under conditions where MCM/P1 remain chromatin-bound and functional (Hua and Newport, 1998; S. Tada, A. Rowles and J. Blow, manuscript in preparation). A similar effect is seen in yeast (Donovan et al., 1997). These results suggest that ORC serves to assemble other replication proteins onto sites adjacent to it on chromatin.

Cdc6/Cdc18 In *S. cerevisiae*, genomic footprinting around replication origins reveals a change in the chromatin structure during the cell cycle (Diffley et al., 1994). In S, G2 and M phase, the footprint pattern is similar to that generated by ORC binding in vitro, whilst in G1 phase a more extensive footprint, called the “pre-replicative complex” (pre-RC), is seen. The pre-RC plausibly correlates to the origin being in a ‘licensed’ state. The production and maintenance of the pre-RC is dependent on the function of the yeast *CDC6* gene (Cocker et

al., 1996; Donovan et al., 1997). Significantly, association of MCM/P1 proteins with chromatin in *S. cerevisiae* requires functional CDC6 (Donovan et al., 1997; Liang and Stillman, 1997). CDC6 is essential for the initiation of DNA replication, and is a 58-kDa protein with similarity to the largest subunit of ORC, Orc1. Cdc6 protein levels are also periodic in the cell cycle, as the gene is only transcribed in late mitosis and G1 (Zwerschke et al., 1994), and the protein is degraded in early S phase (Piatti et al., 1995; Drury et al., 1997). Consistent with it playing a key role in preventing re-replication of DNA in single cell cycle, one *cdc6* mutant allele (*cdc6-3*) causes persistent initiation of DNA replication in G2 or M-phase, and displays persistent association of MCM/P1 proteins with chromatin (Liang and Stillman, 1997).

Homologues of Cdc6 have been identified in a variety of eukaryotes (Kelly et al., 1993; Coleman et al., 1996; Williams et al., 1997). The *S. pombe* homologue of CDC6, called *cdc18*, displays an even more dramatic effect on re-replication of DNA: overexpression of the gene results in the continuing initiation of replication, and leads to cells with giant nuclei containing many times the normal content of DNA (Nishitani and Nurse, 1995; Muzi-Falconi et al., 1996). Immunodepletion of the *Xenopus* Cdc6 homologue (XCdc6) from the *Xenopus* cell-free system showed that it is required for the initiation of replication and for the assembly of MCM/P1 proteins onto chromatin (Coleman et al., 1996). XCdc6 was shown to associate with chromatin, and the assembly of XCdc6 onto chromatin is in its turn dependent on the presence of chromatin-bound XORC (Coleman et al., 1996).

Sequential Assembly of Origin-associated proteins

These results can be used to draw a slightly more complex picture of events that lead up to the licensing of replication origins in *Xenopus* (Fig. 2, slice a); a similar sequence of steps is also likely to occur in other eukaryotes. XORC is assembled onto chromatin at future origins of replication spaced approximately 10 - 15 kb apart. Specification of the sites used as replication origins in *Xenopus* is unlikely to depend solely on DNA sequence (Harland and Laskey, 1980; Hyrien and Mechali, 1992; Hyrien and Mechali, 1993; Mahbubani et al., 1992), but may instead involve some feature of chromosome structure (Gilbert et al., 1995). Once XORC is assembled onto the chromatin, XCdc6 is then also able to become chromatin-bound, possibly by directly binding to XORC. Chromatin containing bound XORC and XCdc6 can then serve as substrate for the licensing reaction, which requires the activity of both RLF-B and RLF-M. This results in the assembly onto chromatin of an average of 10-20 copies of the RLF-M complex per replication origin, presumably at sites adjacent to where XORC is bound. The sole requirement of XORC and XCdc6 in DNA replication may simply be to load RLF-M onto chromatin.

Subsequent to RLF-M loading, replication origins then interact with further proteins essential for the initiation of DNA replication such as Cdc45 and Cdc7. In *S. cerevisiae*, Cdc45 has been shown to interact with ORC and MCM/P1 proteins genetically (Zou et al., 1997; Hardy, 1997). The cell-cycle specific association of Cdc45 with chromatin appears to depend on Cdc6 and MCM/P1 proteins as well as active Cdk kinase, although the dissociation of Cdc45 takes place at later stage of the cell-cycle than the release of MCM/P1 proteins from chromatin (Zou and Stillman, 1998). Cdc7, a protein kinase regulated by Dbf4 in a cell-cycle specific manner, has been shown to phosphorylate Mcm2, 3, 4 and 6 in vitro and Mcm2 in vivo at the G1/S transition (Lei et al., 1997). Significantly, a gain of function mutation in the Mcm5 gene (*bob1* mutation) allows the initiation of DNA replication in the absence of the Cdc7 kinase (Hardy et al., 1997). These results strongly suggest MCM/P1 proteins are activated through phosphorylation by the Cdc7-Dbf4 kinase after they have been loaded on the chromatin.

Regulation of Replication Licensing

The key feature of replication licensing is to explain why DNA is not re-replicated in a single cell cycle. How well can it do this? Although we do not understand the precise biochemical role that the MCM/P1 proteins play in the initiation of replication, their association with chromatin appears to adequately determine whether the chromatin is functionally licensed. However, it is less clear what restricts the binding of these proteins to origins in late mitosis and early G1. Although the nuclear envelope plays an important part in this process in *Xenopus*, it appears that the cell cycle regulation of licensing is more complex than was originally envisaged.

Regulation by subcellular localisation

The original licensing factor model proposed that in *Xenopus*, RLF was incapable of crossing an intact nuclear envelope. Indeed, before re-licensing of replicated chromatin can occur and the MCM/P1 polypeptides re-associate with replication chromatin, the nuclear envelope must be permeabilised and the chromatin exposed to both RLF-B and RLF-M (Chong et al., 1995). However, it appears that in *Xenopus*, and probably most other eukaryotes as well, MCM/P1 polypeptides are capable of crossing the nuclear envelope, and are accumulated in the nucleus throughout interphase (Thömmes et al., 1992; Todorov et al., 1994; Madine et al., 1995a). Two different explanations have been made to explain the absence of licensing in G2 despite the presence of MCM/P1 polypeptides within the nucleus.

The first explanation invokes the requirement for RLF-B in the licensing reaction. If RLF-B were excluded from the nucleus, then the presence of RLF-M within the nucleus would not result in licensing until passage through mitosis (Chong et al., 1996). Until RLF-B is purified and identified, this explanation remains plausible, but hard to test. However, experiments involving transfer of mammalian nuclei to the *Xenopus* extract are consistent with this idea. Nuclei prepared from cells in G2 ('G2 nuclei') could only replicate in *Xenopus* extract if the nuclear envelope had been permeabilised prior to transfer (Leno et al., 1992). When permeabilised G2 nuclei were resealed using nuclear envelope precursors prior to transfer to *Xenopus* extract, they remained incapable of re-replicating in *Xenopus* extract (Coverley et al., 1993). This suggests that a factor required for licensing (most plausibly RLF-B) could not gain access to chromatin surrounded by an intact nuclear envelope.

The second explanation invokes the presence of an intra-nuclear inhibitor of the licensing reaction (Mahbubani et al., 1997; Hua et al., 1997; Walter et al., 1998). Walter et al (1998) have recently shown that chromatin previously assembled in interphase *Xenopus* extract (and so has associated XORC, XCdc6 and RLF-M) can undergo complete replication in a highly concentrated nuclear extract made from the *Xenopus* cell-free system. However, DNA templates introduced directly into the nuclear extract are not replicated, and this appears to correlate with an activity present in the nuclear extract that inhibits the binding of XMcm3 onto chromatin (Walter et al., 1998). Therefore nuclear permeabilisation may be required for re-licensing of replicated nuclei in order to release the inhibitor from the nuclei. Since cyclin-dependent kinases are known to directly inhibit licensing (Blow, 1993; Mahbubani et al., 1997; Hua et al., 1997) and are accumulated within nuclei, they could plausibly supply the inhibitor (see below).

It should be noted that these two explanations are not mutually exclusive, and given the importance of preventing re-replication of DNA, both processes may be operative together.

Regulation by the Cell Cycle Engine

In most eukaryotic cells, cyclin-dependent kinases (Cdks), are activated sequentially to promote progression through the cell cycle, providing the so-called 'Cell Cycle Engine'. In G1 phase, Cdk activity is typically low, and this period correlates with the time when 'licensed' or 'pre-replicative' chromatin is seen. A range of results suggest that this relationship is causal, and that licensing is actively inhibited by Cdks. The most striking observations have been made in *S. pombe*, where re-replication of DNA is seen in a range of treatments that lower Cdc2 activity in G2, either as a result of a the cell possessing a temperature-sensitive *cdc2* gene (Broek et al., 1991), deletion of the *cdc13* gene (the cyclin B associated with mitotic entry in *S. pombe*; Hayles et al., 1994), or overexpression of Rum1, a Cdc2 inhibitor (Moreno and Nurse, 1994; Correa-Bordes and Nurse, 1995). The same sort of function is also suggested in *S. cerevisiae*, since establishment of the pre-replicative footprint over replication origin s can only take place in G1 before Cdks are activated (Piatti et al., 1996).

In *Xenopus*, direct inhibition of licensing by Cdks is observed. The licensing of replication origins can be blocked by cyclin A (Blow, 1993), cyclin E-Cdk2 (Hua et al., 1997), and by cyclin B-cdc2 (Mahbubani et al., 1997). The inhibition of licensing by Cdks appears to be a direct effect, since reactions involving chromatin, purified RLF-M and partially-purified RLF-B could be blocked with recombinant Cyclin B-Cdc2 (Mahbubani et al., 1997).

Potential Targets of Cdk Inhibition

By what mechanism do Cdks inhibit licensing? Any of the four proteins currently implicated in the licensing reaction (ORC, Cdc6, RLF-B and RLF-M) are potential targets of the kinase, in addition to whatever other unknown factors may also be involved. Below, the potential for each of these four activities to be inhibited by Cdks is briefly considered.

Regulation of ORC function—A physical interaction between Orp2p, the *S. pombe* Orc2 homologue and Cdks has been shown by two-hybrid interaction (Leatherwood et al., 1996), suggesting the regulation of ORC function by Cdks in *S. pombe*. Although genomic footprinting in *S. cerevisiae* shows that ORC is present on the chromatin throughout the cell cycle, it is still possible that it could be inactivated for licensing by Cdks without being removed from the chromatin. In *Xenopus*, XORC can be removed from licensed chromatin by incubation with Cyclin A-Cdc2, although the MCM/P1 proteins, however, remained on the chromatin (Hua and Newport, 1998). Dissociation of XORC following addition of Cyclin A-Cdc2 is plausibly mediated by the direct phosphorylation of XORC though this has not been demonstrated. Although XORC remains on chromatin throughout interphase (Rowles et al., 1996), there is some evidence that it is removed during metaphase (Romanowski et al., 1996b). The dissociation of ORC during mitosis may be more important in higher eukaryotes, as origins of DNA replication may be different in each cell cycle and are clearly changed during the shift from embryonic to somatic cell cycles.

Regulation of Cdc6/Cdc18 function—Like Cdc6 in *S. cerevisiae*, Cdc18 in *S. pombe* is a highly unstable protein present only during G1 of the cell cycle. Deletion of the *rum1* gene, encoding a Cdc2 inhibitor, increased the severity of the *cdc18* mutant phenotype, whereas overexpression of Rum1 induced the accumulation of Cdc18 and rescued the *cdc18* mutant (Jallepalli and Kelly, 1996). GST-Cdc18 formed a complex with Orp2, Cdc2, and the B-type cyclins Cdc13 and Cig2 (Jallepalli et al., 1997; Lopez-Girona et al., 1998). Cdc18 phosphorylation is dependent on Cdc2 and results in rapid degradation of the protein. Mutation at one of the phosphorylation sites was sufficient to cause re-replication of DNA (Jallepalli et al., 1997; Lopez-Girona et al., 1998). These results suggest that in *S. pombe*, Cdc18 is regulated by its abundance during the cell cycle, and its destruction is mediated

directly by Cdk phosphorylation. Similarly, in *S. cerevisiae*, degradation of Cdc6 appears to correlate with Cdk activation (Piatti et al., 1996; Drury et al., 1997). However, if the protein is expressed during G2, re-replication of DNA is not observed. In this case, association of Cdk-Clb activity with Cdc6 during S, G2 and M phase was reported (Piatti et al., 1996).

Regulation of RLF-M function—MCM/P1 proteins are observed to be phosphorylated or dephosphorylated in a cell cycle-specific manner. *Xenopus* XMcm4 becomes hyperphosphorylated in mitosis, showing a pattern of phosphorylation similar to that induced by Cdks (Coue et al., 1996; Hendrickson et al., 1996). However, the potential effect of phosphorylation on RLF-M activity is less clear. Although Hendrickson et al. (1996) reported the removal of MCM/P1 proteins from chromatin on incubation with Cdks, this effect has not been observed by others (Hua and Newport 1998; S. Tada, unpublished observations). The activity of RLF-M does not change dramatically in the cell cycle in *Xenopus* egg extract, and it appears fully active, even in metaphase when Cdk levels are at their highest (Mahbubani et al., 1997). Thus the phosphorylation of MCM/P1 proteins has yet to be shown to have a clear effect on their capacity to license chromatin.

Regulation of RLF-B function—In the *Xenopus* cell-free system, RLF-B activity is periodic in the cell cycle, peaking in late mitosis. Further analysis of this cell cycle variation indicated the existence of at least two control systems for RLF-B activity: the presence of an RLF inhibitor in metaphase and a decline of RLF-B activity during the progression of interphase (Mahbubani et al., 1997). Cdks play an important role in the metaphase inhibition, as affinity depletion of Cdks from metaphase extract removed the inhibitory activity. The inhibitory effect of 6-DMAP is likely to be mediated by a stabilisation of the Cdc2-cyclin B kinase leading to a stabilisation of the metaphase inhibitor. Since purified MPF shows an inhibitory effect on the licensing reaction, it is possible that the metaphase inhibition may be result from a direct phosphorylation of RLF-B by Cdks. On the other hand, the decay of RLF-B activity in interphase did not appear to depend on Cdk activity since the cell cycle profile of RLF-B activity was unaffected by treating extracts with the Cdk inhibitor p21Cip1 (Mahbubani et al., 1997). However, until the identity of RLF-B is known, its ability to serve as a direct Cdk substrate cannot be tested.

Conclusions

As described above, a consistent picture is emerging about how replication origins are controlled in eukaryotes and how precise genome duplication is achieved. However, there appear to be some discrepancies in the regulation systems used in different organisms. Thus, it is difficult to summarise the general regulation system of the licensing reaction at present, and hence Fig. 2 shows a model for the control of the replication licensing system proposed mainly from the biochemical research in *Xenopus*. The abrupt disappearance of MPF activity at the metaphase-anaphase transition allows the licensing reaction to occur. This involves the sequential binding to chromatin of O RC, Cdc6, and MCM/P1 proteins in RLF-M. The MCM/P1 protein-binding depends on RLF-B activity which only becomes active after exit from metaphase (Fig. 2, a). After activation of S-phase promoting Cdks (SPF) within nuclei in late G1 phase, the further loading of MCM/P1 proteins does not occur possibly because of the inhibition of RLF-B or ORC/Cdc6, or by the exclusion of RLF-B from the nucleus (Fig. 2, b). S-phase promoting Cdks then cause the initiation of replication on the chromatin where MCM/P1 proteins are loaded. MCM/P1 protein is removed from chromatin as it is replicated, so that further initiation does not occur on the replicated region (Fig. 2, c). At G2 phase, DNA replication is complete and the genome has been faithfully duplicated. However, another round of the licensing reaction involving MCM/P1 loading is still prohibited possibly because of inactivation of RLF-B and/or absence of Cdc6 on the chromatin (Fig. 2, d). MPF inhibits the replication licensing reaction, presumably through t

the inhibition of RLF-B function and/or the chromatin bound component until the disappearance of MPF activity at the metaphase-anaphase transition (Fig. 2, e).

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Figure 1.

Original model for the licensing system in the *Xenopus* early embryo. a, Licensing factor (+) binds to DNA. b, DNA is assembled into nucleus. c, Initiation at licensed sites occurs co-ordinately throughout individual nuclei. d, Licensing factor is inactivated by initiation or the passage of a replication fork. e, Fully replicated DNA cannot re-replicate due to exclusion of licensing factor from DNA by nuclear envelope. Breakdown of nuclear envelope during mitosis allows access of the licensing factor to the DNA, to prepare it for DNA synthesis in the next cell cycle.

Figure 2.

Model for the control of the licensing reaction during cell cycle progression in *Xenopus* egg extract. The behaviour of XORC, XCdc6, RLF-M and RLF-B on a single origin of DNA replication is shown. The activities of S phase-promoting Cdk (SPF) and M phase-promoting Cdk (MPF) are indicated in the middle wheel. The cell cycle is divided into 5 stages in terms of licensing activity as follows: stage a is from metaphase to late G1 where there is no significant Cdk activity; stage b is from late G1 to G1/S transition where there is SPF activity but prior to initiation taking place; stage c is S phase; stage d is G2 phase; and stage e is from the onset of M phase to the end of anaphase where MPF is activated. See text for more details.