CELL CYCLE REGULATION OF DNA REPLICATION INITIATION PROTEINS IN MAMMALIAN CELLS

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1. ABSTRACT

Genomic DNA has to be replicated completely and only once during a single cell cycle in order to maintain integrity. Eukaryotes have developed highly regulated machinery for precisely replicating genomic DNA that is fragmented into multiple chromosomes. Our knowledge of such mechanisms largely depends on findings with budding yeast, since identification of specific DNA sequences acting as replication origins, autonomously replicating sequences, has allowed extensive analyses of the initiation of DNA replication. Several factors essential for regulation of initiation have been identified, including ORC, CDC6 and MCM. Subsequent work has suggested that the fundamental machinery for DNA replication may be conserved in metazoan embryonic cells in which replication occurs sequence-independently, and also in mammalian nonembryonic cells, where replication origins are more specific. However, there are specific differences. In this review, information on function and regulation of mammalian initiation factors, ORC, CDC6 and MCM, is summarized, and yeast and embryonic systems are compared. A hypothetical model for the state of prereplication chromatin in mammalian cell nuclei and regulation during the cell cycle is also proposed.

2. INTRODUCTION

In eukaryotic cells, genomic DNA is fragmented into multiple chromosomes and replication initiates from multiple origins on these chromosomes. Therefore, the amount of DNA replicated from each origin is relatively little. Thus in metazoans, there is an efficient replication of large amounts of DNA required to expand genome size during evolution. If origin specificity is low and multiple origins fire synchronously, then genome replication is completed rapidly. This is indeed the case in early embryonic cells in metazoans (1). In contrast, in nonembryonic cells, the origins become more specific and firing is highly asynchronous, leading to an increase in S phase length (1). This firing of multiple replication origins throughout S phase gives rise to at least one problem; since each origin must fire once and only once during a single cell cycle, the already replicated DNA must be clearly differentiated from unreplicated DNA.

Our knowledge of mechanisms responsible for highly regulated eukaryotic DNA replication largely depends on findings with budding yeast, since identification of specific DNA sequences acting as replication origins, autonomously replicating sequences (ARSs), has made way for extensive analysis of the initiation step. Several factors essential for regulation of initiation have been identified, ORC, CDC6 and MCM being representative crucial proteins. An *in vitro* DNA replication system with *Xenopus* egg extracts is often used as a model system for metazoan DNA replication. This

system may mimic the condition in early embryonic cells, where DNA replication occurs sequence-nonspecifically, in contrast to the sequence-specific initiation in budding yeast (1). Nevertheless, *Xenopus* homologues of yeast initiation factors have also been identified, and shown to be necessary for DNA replication.

Conservation of essential machinery between yeast and embryonic cells suggests that similar events function in DNA replication in mammalian non-embryonic cells. Mammalian homologues of initiation proteins have been identified and appear to be essential for DNA replication. I will summarize here information on function and regulation of initiation factors ORC, CDC6 and MCM in mammalian cells, with a comparison with yeast and embryonic systems.

3. ORC (Origin Recognition Complex)

3.1. Budding yeast
ORC, consisting of six subunits ORC1 to 6, was initially identified as binding specifically to ARSs *in vitro* (2). Findings in genomic footprint analyses (3, 4) and immunoprecipitation assays of cross-linked chromatin (5, 6) suggested that ORC binds to ARSs *in vivo* throughout the cell cycle. Current models suggest that ORC acts as a flag for assembly of other replication proteins such as CDC6 and MCMs, by which the prereplication complex (pre-RC) is formed that is competent for initiation of DNA replication (7).

3.2. Early embryonic system
ORC-like complexes probably containing all six ORC subunits have been isolated from *Xenopus* egg extracts and early *Drosophila* embryos (8-10). Immunodepletion experiments indicate that XORC (*Xenopus* ORC) is essential for chromatin binding of XCDC6 and subsequent DNA replication (8, 9, 11). However, contrary to the situation in the yeast, physical interactions between XORC and XCDC6 have not been evidenced (9, 11).
Because DNA replication is sequence-independent in *Xenopus* egg extracts (12), XORC may be a low sequence-specific DNA-binding protein. Immunolocalization suggests that it binds to chromatin in interphase (8, 11). Biochemical analyses have indicated that XORC2 and XCDC6 are excluded not only from chromatin but also from the nuclear matrix during mitosis (11). However, it was also reported that *Drosophila* ORC2 remains binding to chromosomes in the mitotic phase (13).

3.3. Mammalian somatic cells
Conservation of ORC between yeast and metazoan embryos suggests the existence of an ORC in nonembryonic mammalian cells. However, the properties of mammalian ORC appear somewhat different from those of the embryonic counterpart. Although several human ORC subunits have been identified and physical interactions among them do occur (14-16), complexes apparently containing all six ORC subunits have not been identified. This might simply represent instability of the putative mammalian ORC. However, it has also been

suggested that the major, if not the entire, localization in nuclei differs between human ORC1 and 2. Immunostaining of transfected ORC1 shows nuclear foci, the pattern often representing nuclear matrix localization (17). Biochemical analysis also demonstrated that most endogenous ORC1 is associated with the matrix throughout the cell cycle (Y. Tatsumi and C. Obuse *et al.*, manuscript in preparation). In contrast, ORC2 is released by nuclease treatment, suggesting chromatin binding (18, 19). These findings suggest the existence of ORC subunits that are not associated with the ORC hexamer. One possibility is that some mammalian ORC proteins function in areas other than DNA replication, when they are present in the non-hexamer form.

Why is there the difference in properties of ORC and specificity of origins between embryonic and differentiated cells? One simple possibility is that, metazoans have evolved a special embryonic ORC with low sequence-specificity. If this is not the case, the most possible scenario would be as follows. Metazoan ORC is a DNA-binding protein with relatively low sequence-specificity. In early embryos, there is such a large amount of maternal ORC that the specificity is reduced, leading to sequence-independent DNA replication. In differentiated cells, more complicated nuclear structures and functions have developed, for example for transcription, which does not function in early embryos. Although there are many potential ORC binding sites on genomic DNA, such nuclear events make most sites inaccessible to the ORC. Some are occupied by transcription factors, and others are internalized through formation of high-order chromatin structures. Intranuclear localization of ORC is also specific, for example matrix binding, influencing ORC-DNA interactions. As a result, DNA replication initiates from apparently specific sites.

4. CDC6

4.1. Budding yeast
CDC6 is essential for pre-RC footprint and MCM recruitment (4, 20). Chromatin immunoprecipitation studies suggest that CDC6 is associated with ARSs *in vivo* (5), and this may be linked to CDC6 physical interaction with ORC (21). CDC6 has conserved Walker A and B motifs which are essential for ATP binding and hydrolysis, and mutations in these motifs abrogate CDC6 functions regarding pre-RC footprint formation and/or recruitment of MCMs (22). Furthermore, CDC6 as well as ORC1 and 4 are related to RFC, a loader for PCNA, at the amino acid sequence level (22). MCMs do not appear to be tethered to chromatin via ORC and CDC6 (20). Together, ORC and CDC6 assembled on ARSs may load MCM onto prereplication chromatin, probably using ATP.

Inhibition of re-replication may occur as follows: Once pre-RC is activated, probably by S phase CDK and CDC7 kinase, it is reversed to replication incompetent post-RC, which contains ORC but not other replication proteins such as CDC6 and MCMs (7). While the mechanisms underlying replication-coupled breakdown of pre-RC are currently unknown, inappropriate reassembly is prohibited

by the S phase and mitotic CDKs (S-CDK and M-CDK) (5, 7, 23). In fission yeast, Cdc18, a homologue of CDC6, is phosphorylated by the CDK and thereby rapidly degraded (24). This regulation of Cdc18 appears critical, since overexpression drives cells into re-replication (24, 25). Although CDC6 protein levels also drop as cells enter S phase in budding yeast, restoration by the enforced expression can only induce CDC6 association with ARSs but not reloading of MCM (5), indicating that CDC6 protein levels are not an essential target of the CDKs. In metazoans, mitotic CDK phosphorylates CDC6 and MCM without altering their levels (26-28). Therefore, re-assembly of pre-RC might be inhibited due to topological incompetence of the phosphorylated CDC6 and MCM for functional interactions.

4.2. Early embryonic system

Immunodepletion experiments indicate that XCDC6 is essential for DNA replication in egg extracts (11). Also in this system, S phase and mitotic CDKs appear to inhibit re-loading of the once dissociated XMCM onto postreplication chromatin (29, 30). However, levels of XCDC6 are relatively constant during the cell cycle (11). Phosphorylation of XCDC6 has not been reported. Based on results of immunostaining, XCDC6 appears to bind to chromatin in early interphase, but binds to the nuclear envelope in postreplication nuclei (11).

4.3. Mammalian somatic cells

Microinjection of antibodies against mammalian CDC6 inhibits S phase entry (31), suggesting an essential role in DNA replication. CDC6 may be an MCM-loader even in mammalian cells.

Data on mammalian CDC6 can be confusing. Based on findings with immunostaining, nuclear localization seems the case in the G1 phase (19, 26, 32, 33). In NIH3T3 and FT210 cells, almost all CDC6 is partitioned into a detergent insoluble fraction and is not released even by nuclease digestion, suggesting nuclear matrix binding (19, 26). Levels of bound CDC6 appear unchanged throughout the cell cycle (19, 26). On the other hand, in other cell lines including HeLa, CDC6 is detectable in cytoplasm as cells enter S phase (26, 32, 33). At least with transfected CDC6, this translocation is controlled by CDK2/Cyclin A (32). Biochemical analyses indicate that in HeLa cells, part of CDC6 is soluble while the remainder binds to the matrix, and that the latter level is constant during the cell cycle (26). Therefore, it may be the soluble form that is translocated in S phase. The matrix-bound, but not soluble, CDC6 is hyperphosphorylated in G2/M phase by CDC2 kinase (26), which suppresses reloading of MCMs (28). At least in certain cell types, mitotic CDC2-phosphorylation of CDC6 does not lead to degradation (26). Selective CDC2 phosphorylation of the bound CDC6 and absence of a soluble form in certain cells imply that it may be the bound form that functions in DNA replication.

5. MCM (<u>M</u>INI<u>C</u>HROMOSOME <u>MA</u>INTENANCE)

5.1. Budding yeast

Six MCM proteins, MCM2 to 7 with a related homology, comprise the MCM family. Their highly conserved central region contains an element similar to the Walker A motif. All six are essential for genome replication. Current concepts suggest that MCM loaded onto pre-replication chromatin plays an essential role in DNA replication, when displaced from chromatin and thereafter CDK activity prevents rebinding (7, 34).

5.1.1. MCM in pre-RC formation

It has been speculated that MCMs are loaded around ARSs to form pre-RC. The abundance of chromatin-bound MCM appears to be several times that of bound ORC or CDC6, which means the possible existence of multiple MCMs per one ARS (20). Chromatin immunoprecipitation assays have shown that chromatin around, but not distant from, ARSs can be cross-linked to MCM by formaldehyde in the G1 phase, and that the cross-linkable chromatin region moves away from ARSs as the S phase proceeds (5, 6). This could be interpreted as indicating that MCMs associate with ARSs and move with replication forks. Another possible interpretation is that, in G1, the chromatin-bound MCMs are spread over the relatively wide region of chromatin while those near ARSs are in a cross-linkable form. MCMs not associated with ARSs may change to such a cross-linkable form as S phase proceeds.

It has been suggested that MCMs may be activated and function through phosphorylation by CDC7 kinase, which is essential for DNA replication. A subset of MCMs is phosphorylated by CDC7 *in vitro* and probably *in vivo*, and certain MCM mutants can overcome CDC7 dysfunction (35, 36).

5.1.2. Complex formation by MCM

In metazoans, while hexameric complexes consisting of all six MCMs may be their predominant form, some authors also suggest the existence of more heterogeneous complexes with different compositions (34; also see below). However, it is possible that such heterogeneous complexes are generated *in vitro* since the heterohexamer tends to be destabilized during experimental processes. Although the existence of the heterohexamer has not been noted in budding yeast, it has been purified from fission yeast (37).

Functions of MCMs remain largely unknown. The observation that a certain subcomplex of human MCMs has weak but ATP-dependent helicase activity is of interest (38). However, such activity has not been identified in other systems. In fission yeast, the purified MCM heterohexamer has no detectable ATPase or helicase activity (37).

5.2. Early embryonic system

Immunodepletion experiments suggest that all six XMCMs are essential for DNA replication and that their chromatin binding depends on the XORC and XCDC6 (9, 11, 39, 40). Chromatin-bound XMCMs dissociate as replication proceeds (39, 40). In the original licensing model, the nuclear membrane was considered to block rebinding of an essential replication factor, *i.e.* licensing factor, to chromatin, and MCMs were thought to be

components of the factor. However, constitutive nuclear localization of mammalian MCM (see below) suggests the non-generality of this model, although it could be important in the embryonic situation (34).

5.2.1. Complex formation

In egg extracts, anti-XMCM3 antibodies can precipitate all six XMCMs in approximately equal amounts (39, 40), and no change in net molecular mass (~600kDa) of the XMCM complexes was detected when comparing samples from different cell cycle stages (27). The kinetics of chromatin binding and displacement is synchronous among the six XMCMs (40). Together, these data suggest that the heterohexameric XMCM complexes may be the predominant form. However, in purified XMCM fractions, more heterogeneous XMCM complexes, rather than the heterohexamer, were detected (40).

5.2.2. Regulation by protein kinases

Chromatin-bound XMCM4 is phosphorylated in early S phase (41). In yeast and mammals, MCM4 appears to be a substrate of CDC7 kinase (36, 42). Therefore, it may be that phosphorylation is achieved by CDC7 which activates XMCM, although direct evidence is lacking. S-CDK is also essential for inducing DNA replication in extracts (7), and as described above, it may also negatively regulate XMCM-chromatin interaction. However, XMCM itself might not be a good substrate for the S-CDK (27). M-CDK phosphorylates XMCM4, and treatment of S phase chromatin with exogenously added M-CDK dissociates XMCMs from chromatin (27). However, *in vivo*, M-CDK may function to suppress re-loading of already dissociated XMCMs.

5.3. Mammalian somatic cells

All six MCMs have been identified in mammals. Microinjection of antibodies against human MCM2 or murine MCM3, or treatment with antisense oligonucleotide against human MCM7 inhibit S phase entry (43-45), suggesting their requirement for DNA replication.

5.3.1 Chromatin association

When assessed by immunostaining, mammalian MCMs exclusively show nuclear localization throughout the interphase (44, 46-48). These MCMs are biochemically separable into two forms, one extractable by nonionic detergent and the other associated with the nuclei and thus resistant to such extraction (43, 46-48). Most of the bound form is released by nuclease treatment, indicating chromatin binding (46, 49, 50). This is in striking contrast to matrix-associated CDC6 and ORC1. Immunostaining of MCM in detergent-extracted G1 nuclei resulted in homogeneous nuclear staining (44, 46, 48), which is in contrast to the focal staining observed for ORC1. Taken together, the data suggest that MCMs are bound to chromatin over relatively wide areas rather than in specific locations. While the total amount is constant during the cell cycle, the bound MCM gradually dissociates as cells pass through the S phase (44, 46-48).

5.3.2 Complex formation

In the soluble fraction, heterohexameric complexes consisting of six MCMs are detected throughout the cell cycle (28, 49, 51, 52). The complexes are unstable but there is strong binding of MCM3 to 5, and MCM4, 6 and 7 form another tight subcomplex (44, 49-53). The hexameric complexes tend to break down into such subcomplexes during experimental procedures. Density gradient centrifugation analyses have also suggested the existence of more heterogeneous MCM complexes (50, 53), some may exist *in vivo* but others might be generated *in vitro*.

A very important issue is composition of the bound form. Immunoprecipitation of chromatin-bound MCMs solubilized by DNase I digestion under relatively physiological salt concentrations suggests that the predominant form is the heterohexamer (28, 49). In immunoprecipitated chromatin-bound MCM complexes, no other protein(s) have to date been detected which have a comparable stoichiometry to MCMs (49), suggesting that there is binding to DNA without mediation of other protein(s) (20, 54). However, purified MCMs do not show significant DNA binding activity (37 and our unpublished data). Therefore, it could be that MCM hexamers like PCNA trimers clamp DNA. ATP stabilizes MCM complex-chromatin binding *in vitro*, without hydrolysis (49), and might therefore function as a co-factor for MCM-DNA interactions *in vivo*.

5.3.3. Regulation by phosphorylation

The MCM complexes undergo complicated phosphorylation during the cell cycle. Here, I would like to focus on two points.

One is the phosphorylation of chromatin-bound MCM2 and 4 in the S phase (28). This is reminiscent of S phase XMCM4 phosphorylation (41), and likewise it is conceivable that it is achieved by S-CDK and/or CDC7 kinases. Indeed, MCM2 and 4 appear to be substrates of these kinases *in vitro* (42). However, it is not clear whether this is the case *in vivo*. Ishimi showed that a MCM complex with a specific composition, namely a hexamer consisting of two molecules each of MCM4, 6 and 7, has weak but ATP-dependent helicase activity which is inhibited by MCM2 binding (38, 55). On the other hand, as described above, the predominant chromatin-bound MCM form appears to be the hexamer consisting of all six MCMs. An intriguing possibility is that S phase phosphorylation of the latter leads to rearrangement to generate the former active hexamers, which function in DNA replication. However, such has not been demonstrated either *in vivo* or *in vitro*.

The other point is hyperphosphorylation of MCM2 and 4 in the G2/M phase, when almost all MCMs are dissociated from chromatin (28). CDC2/cyclin B kinase, a regulator of G2/M progression, phosphorylates MCM2 and 4 in complexes *in vitro*, and CDC2 inactivation leads to disappearance of the hyperphosphorylated forms in CDC2 temperature-sensitive mutant murine FT210 cells, which means that the hyperphosphorylation is directed by CDC2/cyclin B *in vivo* (28). As described above, the matrix-bound CDC6 is also hyperphosphorylated by CDC2/cyclin B (26). Furthermore, CDC2 inactivation in FT210 cells increases the level of chromatin-bound MCMs
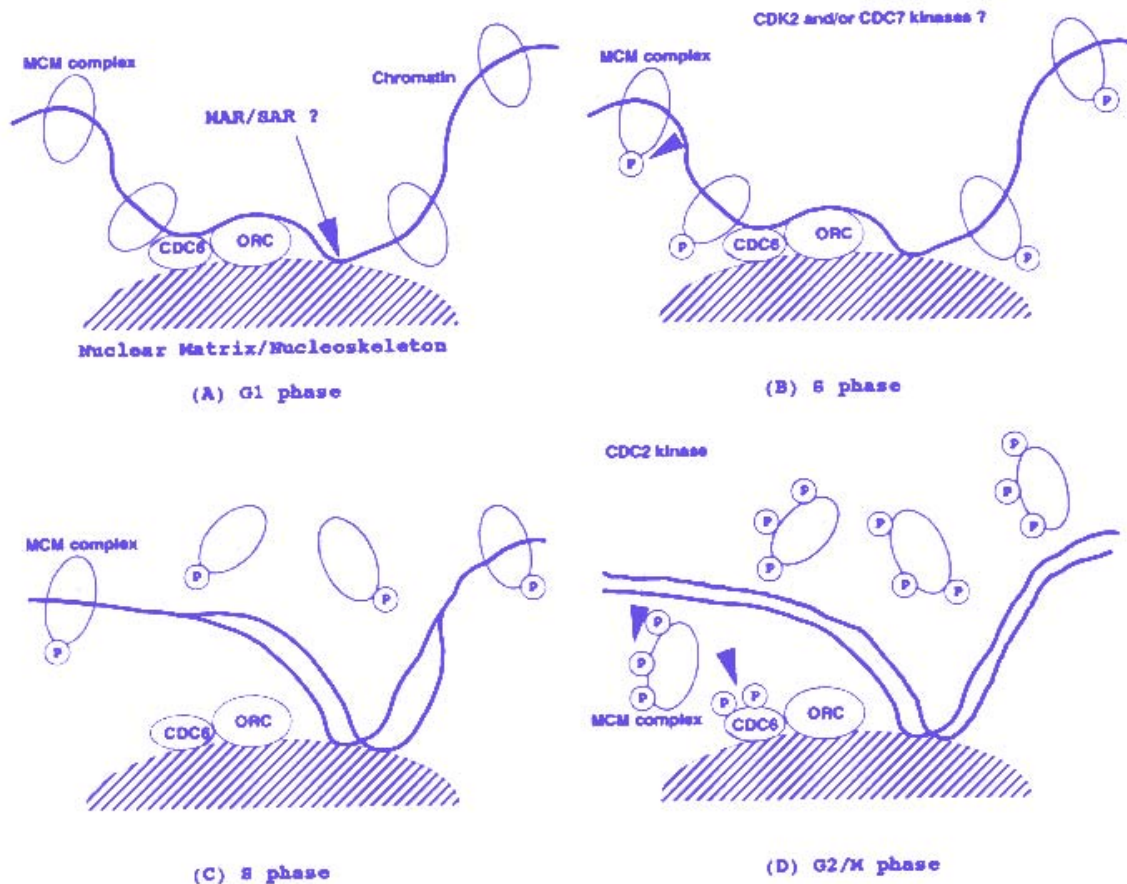
Figure 1. A hypothetical model for the state of pre-replication chromatin and cell cycle regulation in mammalian cell. In this model, the soluble CDC6 and MCM present in G1 phase are omitted. Also note that exact phosphorylation sites and numbers in CDC6 and MCM have not been determined, so that the phosphorylation in the model is figurative. (A) In G1 phase, ORC and CDC6 are assumed to be associated with the matrix, MCM complexes being loaded onto chromatin regions not associated with the matrix, including those near ORC/CDC6 binding sites. (B) In S phase, the bound MCM might be activated through phosphorylation, possibly by CDC7 and/or CDK2 kinases, but this phosphorylation does not induce its dissociation from chromatin. (C) Activated MCM plays an essential role in DNA replication, and is simultaneously displaced from chromatin through an unknown mechanism. In S phase, reloading of once dissociated MCM may be suppressed by CDK2 kinase, possibly through phosphorylation. (D) In G2/M phase, CDC2 kinase hyperphosphorylates CDC6 and MCM complexes, thereby prohibiting reloading of MCM onto postreplication chromatin. During exit from mitosis, CDC2 kinase activity is restrained and the prereplication chromatin structure is again constructed.

in G2 phase (28), indicating that mitotic CDK prohibits inappropriate reloading of MCMs onto chromatin and thereby contributes suppression of re-replication, even in mammalian cells. This might occur through the phosphorylation of MCMs and CDC6.

6. A SUGGESTED HYPOTHETICAL MODEL FOR PREREPLICATION CHROMATIN AND CELL CYCLE REGULATION IN MAMMALIAN CELL

A hypothetical model for the state of prereplication chromatin and its cell cycle regulation in mammalian cells is shown in figure 1. A putative hexameric mammalian ORC has not been identified, however, given the conservation of ORC in eukaryotes, a mammalian ORC may exist. If so, what is its intranuclear localization? While ORC1 appears to be associated with the matrix, ORC2 binds mainly to chromatin. Expression of MCMs and CDC6, as well as other replication proteins such as PCNA, is cell growth dependent (31, 33, 34, 47, 56). Among known mammalian ORC subunits, ORC1, but not ORC2, 4 and 5, appears cell growth dependent (15, 16, 33). This does not preclude a role for ORCs other than ORC1 in DNA replication, but does imply other function(s). On the other hand, it also suggests that ORC1 may be of prime importance for DNA replication. ORC1 can bind to CDC6 *in vitro*, and these co-fractionate through multiple column steps from cell lysates (33). In addition, CDC6 is also associated with the matrix (26). Together, in this model, ORC and CDC6 are assumed to be associated

with the matrix throughout the cell cycle. There are no available data indicating that mammalian ORC/CDC6 binds to chromatin. Again given the conservation of ORC/CDC6 functions, ORC/CDC6 may bind to prereplication chromatin in G1. However, a possible dissociation from chromatin may occur after the S phase, as suggested for the *Xenopus* egg system (54).

MCM complexes are loaded, presumably by ORC/CDC6, mainly onto chromatin regions not associated with the matrix. The abundance of chromatin-bound MCM is several times that of bound CDC6 (26), and the relatively large extent of MCM-bound chromatin could explain the pattern of initiation seen in metazoan nonembryonic cells, namely the so-called "initiation zone". Given the analogy with the yeast system, however, it is also possible that at least a small proportion of the complexes is loaded on chromatin near ORC/CDC6 binding sites. The data obtained with immunoprecipitation for lysates prepared from the nuclei chemically cross-linked with Dithiobis-succinimidylpropionate suggest that this is indeed the case (26). The bound MCMs might be activated through phosphorylation possibly by CDC7 and/or CDK2 kinase, but this phosphorylation does not induce dissociation from chromatin (28, 29, 54). Regarding the role of S-CDK, recently reported findings with yeast and *Xenopus* eggs that CDC45 protein is loaded onto chromatin, probably via MCM, under the control of S phase CDK (57, 58) are of much interest. The activated MCM plays an essential role in DNA replication, and is simultaneously displaced from chromatin through unknown mechanism(s), coupled to DNA replication. In the S phase, reloading of once dissociated MCMs may be suppressed by CDK2 kinase (29), possibly through phosphorylation. In the G2/M phase, CDC2 kinase hyperphosphorylates CDC6 and MCM complexes. Reloading the MCM onto postreplication chromatin may thereby be prohibited (26, 28). During exit from mitosis, CDC2 kinase activity is restrained and the prereplication chromatin structure is again constructed.

Little is known of the chromatin region associated with these initiation proteins. However, the observation that candidate replication origins in mammals frequently contain matrix attachment region (MAR/SAR) seems indicative (59-61). Chromatin regions associated with ORC1/CDC6 on the matrix might act as centers of initiation zones. Some of the problems with this model could be addressed by chromatin immunoprecipitation assays in mammalian cells.

7. PERSPECTIVES

It is important to investigate in detail *in vitro* biochemical properties of mammalian initiation proteins. Questions of whether or not a mammalian ORC hexamer exists, and if so, what is its binding specificity for DNA, need to be considered. The issue of helicase activity associated with a human MCM complex also warrants further studies. It will be of much importance if an *in vitro* reconstitution system for ORC-CDC6 binding to DNA and subsequent MCM loading could be established. At the same time, it is also important to ascertain the *in vivo*

situation. For example, actual binding sites of ORC, CDC6 or MCMs in mammalian replicators such as that in the beta-globin locus have not been clarified, and research in this area is expected to provide pertinent information. Functions of the nuclear matrix in DNA replication have long been considered. Understanding the high-order nuclear and chromatin structures is also required. Elucidation of actual processes responsible for DNA replication in mammalian cells will be greatly facilitated by resolution of these issues.

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