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Review

Cell Cycle Dependent Regulation of the Origin Recognition Complex

Melvin L. DePamphilis

Correspondence to: Melvin L. DePamphilis; National Institute of Child Health and Human Development; Building 6, Room 3A-15; 9000 Rockville Pike; National Institutes of Health; Bethesda, Maryland 20892-2753 USA; Fax: 301.570.8797; Email: depamphm@mail.nih.gov

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ABBREVIATIONS

Cg	Chinese hamster, *Cricetulus griseus*
Dm	*Drosophila melanogaster*, *D. melanogaster*
Hs	human, *Homo sapiens*
Mm	mouse, *Mus musculus*
ORC	origin recognition complex
PreRC	prereplication complex
Sc	*Saccharomyces cerevisiae*, *S. cerevisiae*
Sp	*Schizosaccharomyces pombe*, *S. pombe*
Xl	*Xenopus laevis*, *X. laevis*

KEY WORDS

ORC, Orc1, replication origins, cell division cycle, ubiquitination, Cdk, phosphorylation

ABSTRACT

The eukaryotic origin recognition complex (ORC) not only selects the sites where prereplication complexes are assembled and DNA replication begins, it is the first in a series of multiple coherent pathways that determines when prereplication complexes are assembled. Data from yeast, frogs, flies and mammals present a compelling case that one or more of the six ORC subunits undergoes cell cycle dependent modifications involving phosphorylation and ubiquitination that repress ORC activity during S, G_2 and M-phases. ORC activity is not restored until mitosis is complete and a nuclear membrane is present. In yeast, frogs and mammals, the same cyclin-dependent protein kinase [Cdk1(Cdc2)] that initiates mitosis also inhibits assembly of functional ORC/chromatin sites. In yeast, ORC remains bound to chromatin throughout cell division, but in the metazoa either ORC or the Orc1 subunit appears to cycle on and off the chromatin. Thus, this "ORC cycle" is the premier step in preventing rereplication of DNA during a single cell division cycle.

INTRODUCTION

One universal feature of eukaryotic DNA replication is that the genome is replicated once and only once each time a cell divides. This is accomplished in two ways. First, prereplication complexes (preRCs) that are assembled during the M to G_1-phase transition in the cell division cycle are inactivated during S-phase, and second, new preRCs cannot be assembled until mitosis is complete and a nuclear membrane is present. Regulation of preRC activity occurs through multiple coherent pathways that inactivate several of the proteins involved in either assembly or activation of preRCs. These include Cdc6, Cdt1, Mcm(2–7), Cdk1 and Cdk2.[1-4] This review summarizes recent results that expand this list to include the origin recognition complex (ORC). Data gathered in yeast cells, frog egg extracts, fly embryos and cultured mammalian cells reveal that ORC structure and activity are regulated by cell cycle dependent modifications of one or more of the ORC subunits. However, as with other regulatory pathways in DNA replication, different manifestations of this "ORC cycle" exist among different eukaryotes.

Prereplication Complexes.

Eukaryotic DNA replication begins with the assembly of a prereplication complex (preRC) at DNA replication origins distributed throughout the genome. This is a highly conserved process in which orthologs of the proteins responsible for DNA replication in the budding yeast *Saccharomyces cerevisiae*[1] are found in all other eukaryotes and in which preRC assembly involves the same sequence of events.[1,5,6] Replication begins with the assembly of an origin recognition complex (ORC) composed of six different subunits (Orc1 to Orc6) at DNA replication origins distributed throughout the genome. Cdc6 protein then binds to ORC/chromatin sites, allowing Cdt1(RLF-B) to load Mcm proteins 2 through 7 onto these sites thereby forming a preRC. In addition, these events will not occur in the absence of Noc3, a highly conserved DNA binding protein that is associated with *S. cerevisiae* replication origins and interacts with ORC and Mcm proteins.[7]

A hexameric Mcm(2–7) complex is believed to be the helicase responsible for unwinding parental DNA strands, a process that is facilitated by the single-stranded DNA binding, trimeric protein complex, RP-A. Since most, if not all, eukaryotic replication origins initiate DNA replication in both directions, at least two Mcm(2–7) complexes presumably are loaded at each ORC/chromatin site. In fact, under some conditions, multiple Mcm(2–7) complexes can be loaded at a single ORC/chromatin site.[8,9] DNA synthesis (S-phase) is triggered by addition of Mcm10[10] followed by the action of the protein kinase Cdc7/Dbf4, and the cyclin-dependent protein kinase Cdk2 coupled with cyclin E or

cyclin A. Both Cdk2 and Cdc7/Dbf4 can phosphorylate Mcm proteins. These events allow Cdc45, Sld, and GIN proteins to escort DNA polymerase α:DNA primase to the preRC and initiate RNA-primed DNA synthesis at or close to the ORC/chromatin sites.

DNA replication origins

Initiation of eukaryotic DNA replication occurs at specific loci in the genomes of all adult organisms studied so far. In yeast, these replication origins exist, on average, once every 40 kb.[11,12] In mammalian cells, replication bubbles are, on average, 200 to 300 kb apart, although clusters of origins can be found more closely spaced.[13,14] Some mammalian origins have been shown to be at least 500 kb from their nearest neighbor.[15]

All DNA replication origins examined so far rely upon internal DNA sequence information, although a genetically required consensus sequence has been identified only in *S. cerevisiae* replication origins.[16] These origins are ~100 bp in size and contain three to four genetically required domains. However, they contain only one genetically required consensus sequence, a 17 bp sequence of which 82% is either A or T. The most striking feature outside this consensus sequence is an A-rich region 25–105 nucleotides to the 3'-side. This A-rich region encompasses the "DNA unwinding element" where the first RNA primers are synthesized.[17] Replication origins in the fission yeast *Schizosaccharomyces pombe* consist of two or more genetically required, asymmetric A:T-rich sequences (≥70% AT residues with A on one strand and T on the other), but a genetically required consensus sequence has not been identified.[12] Replication origins in multicellular organisms such as flies, frogs and mammals are similar in size and complexity to those in *S. pombe*. They depend on internal DNA sequence information, because they can be translocated to other chromosomal loci and retain their ability to promote initiation of DNA replication, and internal sequence alterations can either enhance or repress origin activity.[18-21]

Metazoan replication origins also are determined by epigenetic information, because site-specific initiation in the metazoa is developmentally acquired. In the rapidly cleaving embryos of frogs and flies, initiation of DNA replication does not require either specific sequences or specific genomic loci, but later in their development, initiation events are restricted to specific genomic loci.[26,27] So far, each organism appears to contain a single copy of each ORC subunit, implying that developmental differences in site specificity do not result from different ORC gene products. In fact, they appear to result from epigenetic parameters such as nucleotide pool levels,[28] transcription factor binding sites,[29] ratio of initiation proteins to DNA,[30] gene transcription,[31] chromosome structure,[32-34] nuclear organization,[35] DNA topology[36] and DNA methylation.[9,37] These are all cellular parameters that change during animal development and therefore may restrict initiation at some sites while facilitating it at other sites ("Jesuit Model"[23]). Thus, while binding of ORC to chromatin is required to initiate preRC assembly, specific DNA sequences are not required for ORC function although they may promote ORC binding to specific loci. This is in marked contrast to animal virus genomes where specific DNA sequences are required to assemble and activate replication proteins.[38]

Origin recognition complex (ORC)

Eukaryotic ORCs share several things in common. They all bind DNA with nanomolar affinity. They all bind preferentially to asymmetric A:T-rich sequences. ORC subunits 1, 4 and 5 bind ATP. ORC exhibits ATPase activity, and the ATP binding site in Orc1 is required for ORC function. Only Orc1 and Orc2 in the metazoa, and Orc1, Orc2 and Orc6 in yeast contain multiple consensus sites for phosphorylation by cyclin-dependent protein kinases, suggesting that these subunits are targets for regulation. Nevertheless, there are also notable differences. Yeast ORCs bind to specific DNA sites both in vivo and in vitro, whereas metazoan ORCs do not. Metazoan ORCs are bound to site-specific replication origins in vivo, but in vitro they exhibit only preferential binding to asymmetric A:T-rich sequences. Yeast ORCs remain bound to chromatin throughout the cell division cycle, whereas the association of one or more of the metazoan ORC subunits with chromatin is cell cycle dependent. Conservation among eukaryotic ORCs is limited, allowing the possibility that ORC function and regulation may vary among species.

Sequence conservation. The average amino acid sequence identity among Orc1 proteins from 11 different species is 24% [range = 17 (Sc) to 100 (Cg)] and the similarity is 35% [range = 27(Sc) to 100(Cg)].[39] Even comparisons between different mammals can reveal surprising variation. For example, human, mouse and hamster Orc1 sequences are 67% to 76% identical overall, but most of the identity resides in the C-terminal half which contains a strong homology to Cdc6 and its ATP binding domain. The most notable difference among ORC proteins is the fission yeast *S. pombe* Orc4 subunit. SpOrc4 is unique among all ORC subunits in that its N-terminal half contains nine AT-hook motifs that specifically bind AT-rich sequences, while its C-terminal half is 35% identical and 63% similar to the human and *Xenopus* Orc4 proteins.[40,41]

DNA binding. ORCs purified from yeast (*S. cerevisiae*,[42] *S. pombe*[43-45]), flies (*D. melanogaster*[36]) and mammals (*H. sapiens*[46]) bind preferentially to asymmetric A:T-rich DNA of the type characteristically found in *S. pombe* replication origins, and that are found in most, if not all, eukaryotic replication origins. In *S. cerevisiae*, ORC subunits one to five, in the presence of ATP, are sufficient to bind specifically to origins of DNA replication.[42] Mutants lacking ScOrc6 are not viable,[47] confirming that Orc6 is required for some cellular function, but this function may have more to do with cytokinesis than with DNA replication.[48,49] The ScORC DNA binding site contains the 17 bp genetically required origin consensus sequence (domain A) and encompasses a 36 bp region [domains A and B1] that is ~70% AT-rich and protects a region that is ~80 to 90 bp.[39] It appears that *S. cerevisiae* double stranded origin DNA, ATP, and Cdc6 stabilize bound ScORC in a conformation that allows preRC assembly, whereas ssDNA, ATP hydrolysis, and the loss of Cdc6 converts ScORC into a form that may release the Mcm(2–7) helicase to continue unwinding DNA at replication forks.[1]

SpORC appears unique in that it relies solely on its Orc4 subunit to bind specific genomic loci.[43-45] While SpOrc4 has a general affinity for all AT-rich DNA, it has a higher affinity for specific asymmetric A:T-rich sequences found within *S. pombe* replication origins [43-45,50-52] and initiates bi-directional DNA replication primarily at a single site within the origin.[44,52,53] Some genetically required regions bind SpORC,[43-45] assemble a preRC and initiate bi-directional DNA replication, while others with the same AT-content do not.[44] Thus, while individual AT-hook motifs binds tightly to [AAA(T/A)], site specificity likely results from the arrangement of all nine motifs acting in concert.

Replication origins in flies and mammals contain homologies to the *S. cerevisiae* ORC binding site and contain regions that are 70% to 80% AT-rich[28,39,54] ORC from the fly *Drosophila melanogaster*[1] can bind selectively to AT-rich replication elements in either *D. melanogaster*[55] or *Sciara coprophila*[56] amplification origins. Furthermore, although neither XlORC nor HsORC bind to specific

DNA sequences when initiating DNA replication in a *Xenopus* egg extract, they do bind preferentially to the same AT-rich sequences selected by SpORC.[46,57] Thus, the fact that HsORC is located at specific genomic sites in vivo that are coincident with DNA replication origins[58-60] means that additional, epigenetic, parameters must determine the specificity of ORC binding in vivo (see "DNA Replication Origins").

ATP binding. Protein sequence analysis suggests that all Orc1, Orc4 and Orc5 subunits bind ATP, regardless of species derivation,[61] and this prediction has been confirmed experimentally in *S. cerevisiae*,[62] *S. pombe*[57] and *D. melanogaster*.[63] The importance of ATP binding and hydrolysis to site-specific DNA binding by ORC has been established with ScOrc1, although the role of ATP binding and hydrolysis in Orc4 and Orc5 remains unclear.[1,64] For example, SpOrc4 is solely responsible for DNA binding specificity, but this specificity does not require ATP.[43-45] Therefore, ATP may also contribute either to stabilization of ORC at replication origins, or to assembly of a preRC, as previously suggested for *S. cerevisiae.*[65]

Cellular concentration. Cells limit the number of ORCs to one or two per replication origin, suggesting that most, if not all, ORCs are bound to chromatin. *S. cerevisiae* contains about two ORCs per replication origin [600 ORC/cell,[66] 332 origins/cell[11]], and similar data exist for *S. pombe* ([12] and J. Hurwitz, personal communication). Mammalian cells contain 10^4 to 10^5 molecules of ORC per cell,[67-71] suggesting that they initiate replication, on average, once every 60 to 600 kb,[69] consistent with the average size of replicons in mammalian cells [200 to 300 kb[13,14]].

Regulation of ORC activity in yeast cells (Fig. 1)

In both the budding yeast *S. cerevisiae*[72-74] and the fission yeast *S. pombe,*[43,75] ORC remains intact and bound to chromatin throughout the cell division cycle. Nevertheless, ORC subunits in these two yeast undergo cell cycle dependent phosphorylation that contributes to preventing reinitiation of DNA replication prior to mitosis. In *S. cerevisiae*, Orc2 and Orc6 proteins are phosphorylated by Cdk1(Cdc28)/cyclin B(Clb) during the G_1 to S transition.[76] ScORC proteins are hyperphosphorylated during the S to M transition, and then hypophosphorylated during early G_1-phase when preRC assembly occurs. Mutations that prevent Orc2 and Orc6 phosphorylation do not affect either DNA replication or cell division.[76] However, in the presence of alterations in both Cdc6 and Mcm proteins that prevent their inactivation during DNA replication, DNA replication is reinitiated prior to cell division, resulting in polyploid cells. In other words, at least three different regulatory events must be inactivated in *S. cerevisiae* in order to induce reinitiation of DNA replication within a single cell division cycle, and one of these events is phosphorylation of ORC.

A similar phenomenon exists in *S. pombe* where Orc2 undergoes dephosphorylation during the M to G_1 transition,[43,75] and is hyperphosphorylated when cells enter S-phase and Mcm proteins are released from chromatin.[77,78] Cdk1(Cdc2)/cyclin B(Cdc13) associates with replication origins during S-phase and remains there during G_2 and early M-phases.[77] This association is ORC-dependent and prevents reinitiation of DNA replication before mitosis has been completed. These data strongly suggest that the phosphorylated state of SpOrc2 determines ORC activity.

Regulation of ORC activity in *Xenopus* egg extract (Fig. 1)

As with yeast, *Xenopus laevis* (Xl) ORC also exists as a stable complex, at least in *Xenopus* egg extracts, but in contrast to yeast cells, the affinity of XlORC for DNA in egg extracts diminishes once prereplication complexes are assembled. The extent of this change depends on whether the substrate is sperm chromatin or metaphase chromatin from somatic cells. Both chromatin preparations lack functional ORCs; when either is incubated in a *Xenopus* egg extract, XlORC rapidly binds to the chromatin and initiates DNA replication. However, XlORC becomes salt-sensitive following binding of Mcm(2–7) to sperm chromatin,[79,80] but the same event causes XlORC to be released spontaneously from somatic cell chromatin.[81] Thus, the affinity of XlORC for DNA is influenced significantly either by chromatin composition or by chromatin structure.

Somatic cell chromatin brings with it a full complement of core and linker histones, whereas sperm chromatin must undergo extensive remodeling during its first 30 minutes in egg extracts.[82] Nucleoplasmin dependent decondensation of sperm chromatin is a prerequisite for XlORC binding and subsequent assembly of preRCs.[83] Remodeling involves displacement of sperm specific protamines, uptake of core histones H2A and H2B (histones H3 and H4 are already present), and linker histone B4 (histone H1 is absent from *Xenopus* sperm and eggs), as well as phosphorylation of core histones and uptake of HMG-2 protein.[84] Sperm chromatin is not converted into somatic cell chromatin until after the midblastula transition when histone H1 is expressed, the rate of cell division slows down, and a G_1-phase first appears in the cell division cycle.

In the case of sperm chromatin, ORC apparently is not released until mitosis, a step that was blocked in these experiments by the presence of cycloheximide. XlORC is displaced from chromatin during mitosis in cultured cells,[85] and XlORC in interphase egg extracts binds to chromatin while ORC in metaphase egg extracts does not.[79,80,86,87] Moreover, XlORC in metaphase egg extract is hyperphosphorylated,[88,89] reminiscent of ORC in yeast cells. This hyperphosphorylation appears to result from Cdk/cyclin A activity, because addition of cyclin A to interphase egg extracts can release XlORC from chromatin[79,87] and prevent binding of XlORC to chromatin,[90] whereas equivalent amounts of either cyclin B or E do not have these affects.

Surprisingly, ORC is associated with Cdk1/cyclin A in interphase egg extracts,[91] where, based on the studies described above, it would be expected to inhibit ORC binding to DNA, but where it clearly does not. One explanation is that the ORC-associated Cdk1/cyclin A activity in these extracts is inhibited by Xic1, a Cdk-specific inhibitor that is degraded when interphase egg extract enters S-phase.[92] Degradation of Xic1 would then allow release of XlORC from chromatin. However, inhibition of Cdk activity in interphase egg extracts has no effect on either the binding or release of ORC from somatic cell chromatin.[81,90] Therefore, additional events must be required to trigger release of ORC. For example, Mcm10 binds to chromatin after preRC assembly and before Cdk2 activity where it is required to initiate DNA replication.[10] Cdk1/cyclin A also inhibits loading Mcm proteins onto ORC/chromatin sites, because inhibition of Cdk activity in geminin-depleted metaphase eggs strongly stimulates Mcm binding to chromatin.[93] Thus, while it is clear that the affinity of XlORC for chromatin is cell cycle dependent, the precise mechanisms that regulate this affinity are not yet clear.

Since egg extracts contain a huge excess of ORC, some mechanism, as yet undefined, must prevent new ORC from binding to the somatic cell chromatin as the XlORC is released after preRC assembly. Otherwise, one could not observe a net loss of ORC from the chromatin. This mechanism may involve nuclear structure, since XlORC does not bind to chromatin in nuclei isolated from cells that

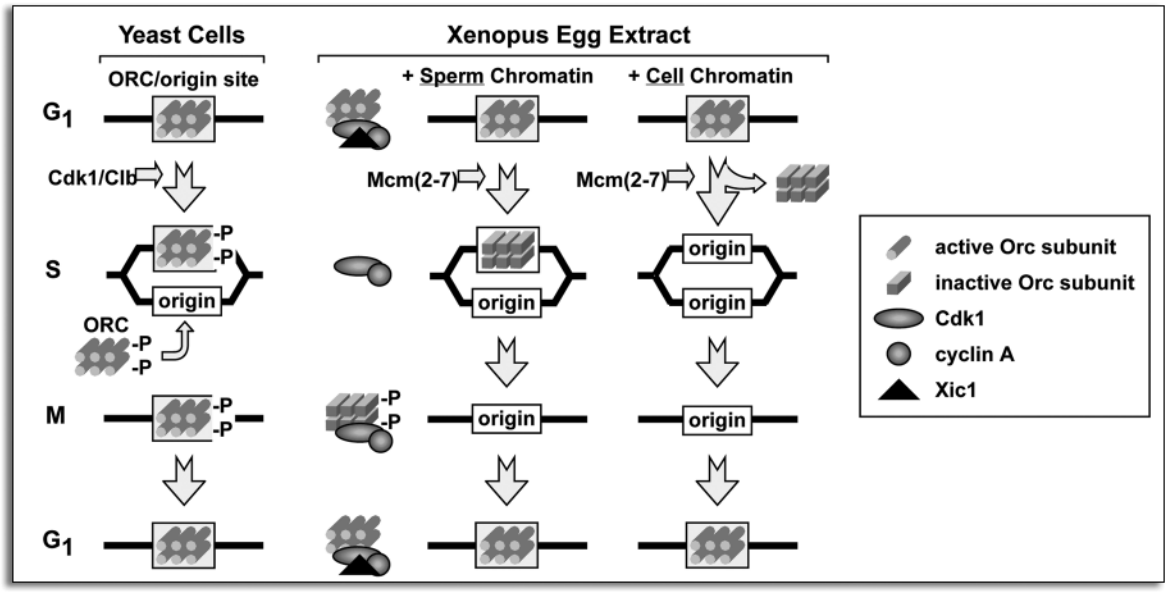

Figure 1. The "ORC cycle" in yeast cells and in frog eggs. Yeast Cells: ORC (six gray cylinders) remains bound to replication origins throughout the cell cycle, but at least Orc2 and Orc6 are phosphorylated (-P) by Cdk1/cyclin B during the S to M periods, and this phosphorylation inhibits ORC's ability to assemble a preRC. Xenopus Eggs: In extract from activated frog eggs, ORC rapidly binds to sperm chromatin and initiates DNA replication, but the affinity of ORC for chromatin is reduced (red boxes) following preRC assembly. XlORC will also rapidly bind to metaphase chromatin from somatic cells and initiate DNA replication. Under these conditions, ORC is then spontaneously released following preRC assembly. XlORC in activated egg extract is associated with Cdk1/cyclin A, but the activity of this protein kinase is inhibited by the presence of Xic1. Xic1 is degraded during S-phase. Orc1 and Orc2 are hyperphosphorylated in extracts of meiotic eggs, presumably by Cdk1/cyclin A, and this phosphorylated form of ORC does not bind chromatin.

have already passed their "origin decision point" (the time when functional preRCs first appear at specific genomic sites).[81] This observation together with the fact that XlORC cannot bind to chromatin in metaphase egg extracts suggests that once XlORC disengages from chromatin, it cannot rebind until the next G_1-phase.

Regulation Of ORC activity in *Drosophila* embryos (Fig. 2)

Several observations are consistent with a cell cycle dependent, differential association of *Drosophila melanogaster* ORC with chromatin. The cellular levels of DmOrc1 change dramatically throughout development, and its accumulation is regulated by E2F-dependent transcription.[94] In contrast, DmOrc2 levels remained constant. In embryos, Orc1 accumulates preferentially in proliferating cells, and in the eye imaginal disc. Orc1 accumulates to high levels only in late G_1 and S phase. Moreover, overexpression of Orc1 alters the pattern of DNA synthesis, implicating Orc1 in regulating initiation of DNA replication. In fact, DmOrc1 is selectively ubiquitinated by the APC/Fzr system during mitosis, degraded as cells exit mitosis and then synthesized and bound to chromatin during late G_1-phase.[95] Assuming that the DmORC, like the ScORC, cannot function without its Orc1 subunit, these data suggest that DmOrc1 association with chromatin during G_1-phase is a critical step in producing functional preRCs during *Drosophila* development.

Regulation Of ORC activity in mammalian cells (Fig. 2)

With the exception of Orc1, the levels of the other ORC subunits in mammalian cells and the amount of each subunit bound to chromatin remain essentially the same throughout cell division [Orc2,[68,69,96-99] Orc3,[68,97,100] Orc4,[68,71,100] Orc5,[68,100] and Orc6[97,101]]. Orc1 also is stably bound to chromatin, and possibly to nuclear structure,[70,100] during G_1-phase. However, in some cells such as Chinese hamster ovary (CHO) cells, the cellular level of Orc1 remains constant throughout cell division,[69,71,102] whereas in other cells, such as human tumor cells, the cellular level of Orc1 oscillates, because Orc1 is selectively degraded during S-phase.[68,70,97,103] This surprising difference among mammalian cell lines has been confirmed by direct comparison of CHO and HeLa cells synchronized by centrifugal elutriation in the absence of metabolic inhibitors (Ghosh S, Vassilev A, DePamphilis ML, unpublished data). However, Orc1 in CHO cell can be selectively eluted from during the S to M-phases of cell division under conditions in which the other ORC subunits remain stably bound (nonionic detergent, 0.1 to 0.15 M NaCl, MgATP),[69,102] revealing that its physical interaction with ORC/chromatin sites is altered, although it is not degraded. Restoration of stable Orc1 binding to chromatin occurs during the M to G_1-transition and follows the same time course as degradation of cyclin B,[97,104] suggesting that exiting mitosis inaugurates stabilization of newly synthesized Orc1 protein and its binding to chromatin. Furthermore, stable binding of Orc1 to chromatin precedes the appearance of functional preRCs at specific origins of bi-directional replication,[30,69,102] suggesting that assembly of functional ORC/chromatin sites is the rate limiting step in the assembly of preRCs at specific genomic sites.

These changes reflect changes in the binding of ORC to DNA in vivo. A DNA footprint at the human lamin B2 origin that encompasses the start sites for leading strand DNA replication[105] changes from a large footprint in G_1-phase, to a smaller footprint in S and G_2-phases, to the absence of a footprint during M-phase.[106] During G_1-phase, Orc1, Orc2, Cdc6 and Mcm3 proteins can be cross-linked to this DNA sequence, but during S-phase, only the Orc2 protein can be cross-linked, consistent with release of Orc1 and disassembly of a preRC.[60] By M-phase, neither Orc1 nor Orc2 can

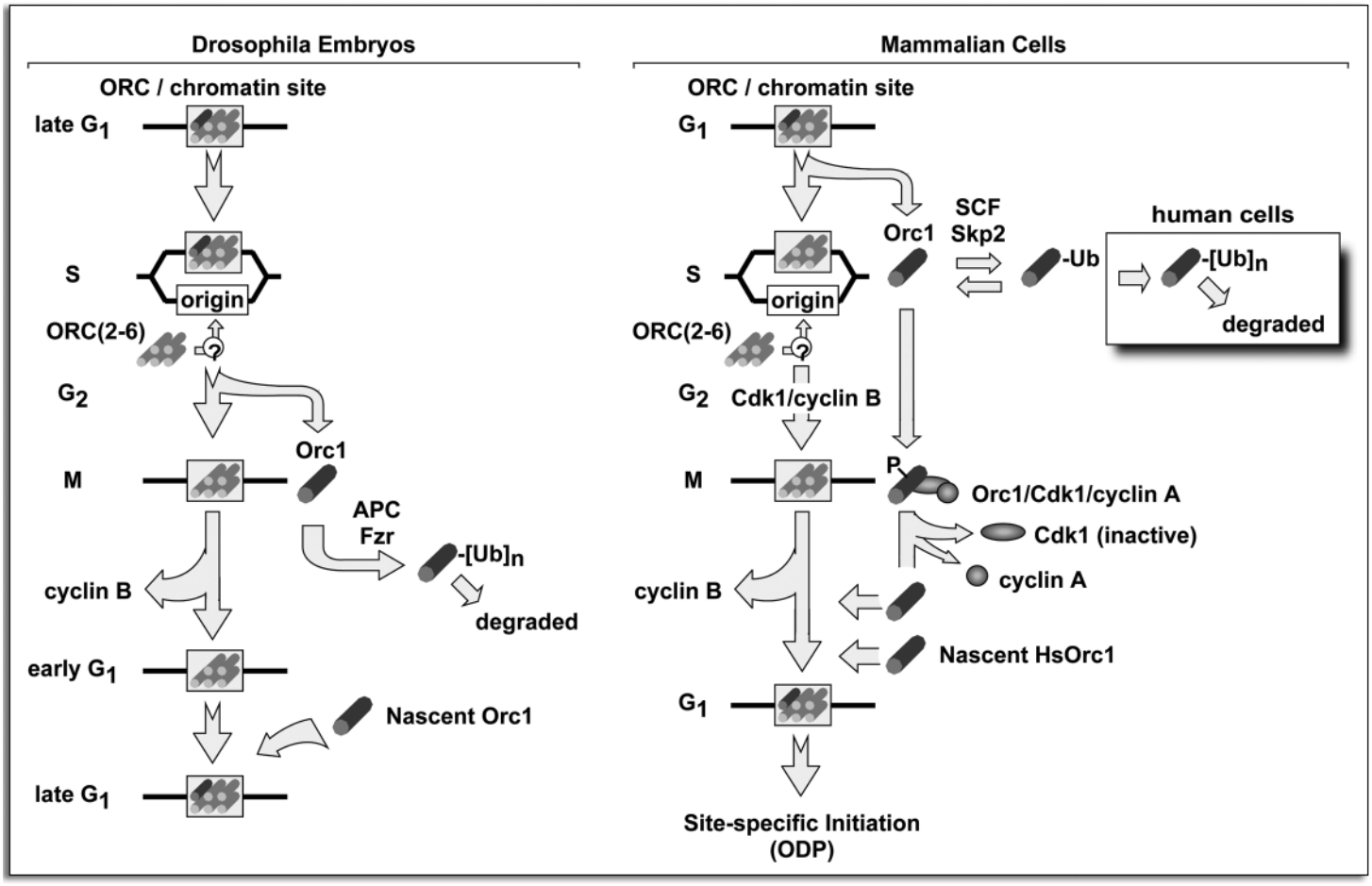

Figure 2. The "ORC cycle" in fly embryos and in mammalian cells. Fly Embryos: ORC subunits 2 to 6 (grey cylinders) remain bound to chromatin throughout the cell cycle, but Orc1 (red cylinder) is selectively ubiquitinated during mitosis by the APC/Fzr system and degraded. Orc1 reappears in late G_1-phase cells where it is bound to chromatin. Mammalian Cells: Orc1 can be selectively eluted from chromatin in S-phase cells while the remaining ORC subunits remain chromatin bound. In S-phase hamster cells, Orc1 can be mono-ubiquitinated, but it is not selectively degraded in vivo. In contrast, Orc1 in human cells can be polyubiquitinated, and it is selectively degraded. Presumably, new ORC core complexes bind to newly created origins during S-phase, although this has not been demonstrated. During the S to M transition in hamster cells, the mono-ubiquitinated form of Orc1 disappears and a hyperphosphorylated form of Orc1 appears that is associated with Cdk1/cyclin A. As cells exit mitosis and enter G_1-phase, cyclin B is degraded, Orc1-associated protein kinase activity disappears (presumably through dissociation of Cdk1 and cyclin A), Orc1 becomes dephosphorylated and bound to chromatin.

be cross-linked to DNA. This selective loss in the ability to cross-link Orc1 to DNA in S-phase cells has been reported at other mammalian replication origins, as well.[59]

These changes are also consistent with the properties of purified mammalian ORC subunits (Fig. 3). Human ORC consists of a stable core complex of Orc2, Orc3, Orc4 and Orc5, and two weakly bound subunits, Orc1 and Orc6.[107,108] This organization is reflected in the subunit interactions detected by yeast 2-hybrid analysis of the mouse subunits.[109]

Since Orc1 is required for ORC activity in yeast, release of Orc1 should prevent ORC function during the S to M transition. In fact, mammalian metaphase chromatin lacks functional ORCs, because chromatin from metaphase CHO cells will not initiate DNA replication in a *Xenopus* egg extract that has been depleted of XlORC proteins.[30,69,110] Under the same conditions, DNA in nuclei from G_1-phase CHO cells does replicate efficiently. In a complete *Xenopus* egg extract, metaphase chromatin from CHO cells also replicates efficiently.

What prevents Orc1 from binding tightly to chromatin during the S to M transition? One mechanism is ubiquitination. In human (Hs) cells, HsOrc1 is selectively polyubiquitinated by the SCF/Skp2 ubiquitination systems and then degraded by 26S proteasomes during S-phase. HSOrc1 reappears during the M to G_1 transition.[68,97,103,111] However, this mechanism alone does not appear sufficient to regulate ORC function. Significant amounts of HsOrc1 remain during the S to M-phase transition in HeLa cells, and this residual Orc1 would account for the observation that nuclei from G_2-phase HeLa cells can still replicate their DNA when incubated in an ORC-depleted *Xenopus* egg extract.[85] In fact, human cells containing only 10% their normal level of Orc2 can still replicate their genome.[112] Furthermore, Orc1 in S-phase Chinese hamster (Cg) cells is selectively mono-ubiquitinated, but CgOrc1 is not selectively degraded unless the cells are lysed.[102] Mono-ubiquitination of CgOrc1 may be sufficient to inhibit assembly of functional ORCs during S-phase, but very little, if any, of the CgOrc1 in G_2/M phase cells is ubiquitinated, despite the fact that Orc1 in these cells is easily eluted from chromatin under conditions where Orc2 is not. Whether ubiquitination is responsible for releasing chromatin bound Orc1 or is simply a mechanism for preventing Orc1 from rebinding to chromatin during S-phase remains to be determined.

What is clear is that by the time cells have entered mitosis, the Orc1 that is present is not ubiquitinated. This can occur in three ways: (1) degradation of polyubiquitinated Orc1 by the 26S proteasome and resynthesis of Orc1, (2) removal of mono or di-ubiquitin moieties by ubiquitin hydrolases[113] and (3) degradation and resynthesis of Orc1 by other pathways. The half-life for both hamster Orc1 and Orc2 in vivo is ~3 hours,[71,102] which allows sufficient time for at least 75% of the Ub-Orc1 pool to be replaced by Orc1.

A second mechanism for regulating Orc1 activity has recently been reported.[104] Hamster Orc1 was found to be selectively bound to a protein kinase activity predominantly, if not exclusively, during the G_2/M-phase of the cell division cycle. This G_2/M-phase Orc1-associated protein kinase was identified as Cdk1/cyclin A. It was able to phosphorylate Orc1 in vivo as well as in vitro, and inhibition of its activity resulted in rapid and stable binding of Orc1 to chromatin. Thus, the same cyclin-dependent protein kinase (Cdk1) that regulates the onset of mitosis also inhibits ORC activity during mitosis.

Recently, an analogous observation was made with Cdc6 in *S. cerevisiae*.[114] Here Cdk1(Cdc28)/cyclin B(Clb2) binds tightly to Cdc6, rendering Cdc6 in this complex unable to assemble preRCs. Thus, mammals appear to regulate Orc1 activity in much the same way that yeast regulate Cdc6 activity. In fact, as with Cdc6 in human cells, Cdk2/cyclin A can also associate with human Orc1 and phosphorylate it in vitro.[97] However, the amount of protein kinase activity associated with Orc1 during G_1 and S-phases (the period of greatest Cdk2 activity) is < 1% of the amount associated with Orc1 during G_2/M-phase.[104] Therefore, the significance of Cdk2/cyclin A phosphorylation of ORC subunits remains to be determined.

Three points of confusion need to be addressed. First, the amount of chromatin bound Orc1 in M-phase hamster cells compared with G_1-phase hamster cells can sometimes appear greatly reduced.[71] The problem is that mammalian Orc1 is sensitive to ubiquitination and degradation in these extracts even when samples are on ice whereas Orc1 in the chromatin bound fraction is not.[97,102] This problem can be avoided by including MG-132, a specific inhibitor of the 26S proteasome, in the cell lysis buffer along with a cocktail of other protease inhibitors.[104]

The second point is whether Orc1 actually dissociates from chromatin during the S to M-phases of the cell cycle, or remains associated with chromatin, but with an altered affinity that inhibits its ability to form a functional ORC/chromatin site. The available data support the second conclusion. During the S to M-phase period of cell division, Orc1 can be selectively released from chromatin under conditions in which the other ORC subunits remain bound. The same conditions that selectively elute Orc1 from chromatin also selectively elute histone H1.[102,104] In fact, Orc1, like histone H1, remains with the chromatin fraction when metaphase cells are permeabilized with digitonin under low salt conditions that support initiation of DNA replication,[69] and ectopically expressed Orc1 remains associated with chromatin even in metaphase cells.[71] However, the fact that metaphase chromatin cannot replicate in an ORC-depleted *Xenopus* egg extract reveals that although mammalian ORC proteins are present, they are not functional.[30,69,110] When metaphase chromatin is incubated in complete egg extract, XlORC proteins rapidly bind to the chromatin, initiate DNA replication, and are then released spontaneously.[81] Thus, both the intact frog ORC and the mammalian Orc1 subunit exhibit a cell cycle dependent change in their affinity for mammalian chromatin. This change in affinity of Orc1 for chromatin is reflected in the binding of Orc1 to DNA. During G_1-phase, Orc1 can be cross-linked to DNA either with formaldehyde[59] or with UV-irradiation,[60] but not during the S to M transition.

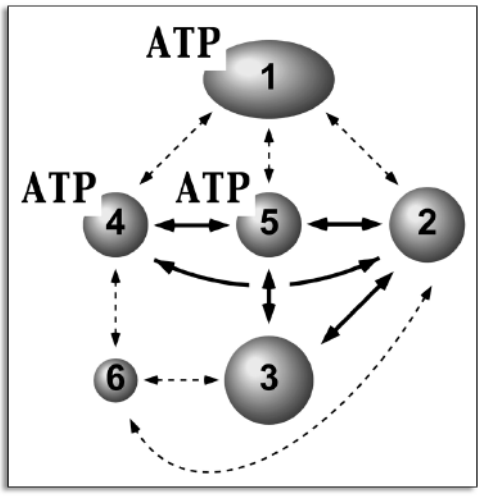

Figure 3. Organization of the mammalian origin recognition complex. Mammalian ORC consists of a stable core of subunits 2, 3, 4 and 5 that interacts weakly with subunits 1 and 6. Strong (solid arrows) and weak (broken arrows) interactions are indicated. HsORC subunits are 97 (Orc1), 66 (Orc2), 82 Orc3, 50 (Orc4), 50 (Orc5) and 28 (Orc6) kilodaltons. MmORC and CgORC subunits are similar, but not identical, to those in HsORC in size and amino acid sequence. This interactive map is based on immuno-precipitation analysis of purified HsORC subunits expressed in baculovirus infected insect cells,[107,108] and 2-hybrid analysis of MmORC subunits expressed in yeast.[109] ORC subunits 1, 4 and 5 bind ATP.

The third point concerns antibody specificity. An earlier report that human Orc1 remained stably bound to chromatin throughout the cell cycle and could be cross-linked to metaphase chromatin[115] was later called into question when the same group discovered that the antibody (3A2A) they had used to detect HsOrc1 in these experiments cross-reacted with another protein whose size closely matched that of HsOrc1.[68]

Principle features of eukaryotic "ORC cycles"

The results summarized in this review reveal various manifestations of cell cycle dependent regulation of ORC throughout the eukaryotic kingdom. Nevertheless, several features of these pathways are shared.

(1) In yeast (and perhaps other unicellular eukaryotes), ORC remains stably bound throughout the cell cycle to specific sites (replication origins) along the genome. In multicellular eukaryotes such as frogs, flies and mammals, one or more ORC subunits disengages from ORC/chromatin soon after preRC assembly is complete, resulting in its increased sensitivity to salt and its accessibility to ubiquitination and perhaps other modifications.

(2) In frogs, the affinity of ORC for chromatin is either reduced or lost under DNA replication conditions, depending on whether sperm chromatin or somatic cell chromatin is the substrate. In flies and mammals, changes in the affinity of ORC for chromatin also occur, but they appear to be restricted primarily to the Orc1 subunit. Interestingly, yeast chromatin, like *Xenopus* sperm chromatin, lacks a classical histone H1 linker,[116] suggesting that reduced chromatin condensation may facilitate binding of ORC during S-phase.

(3) Yeast, frogs and mammals phosphorylate one or more ORC subunits sometime during the S to M-phase transition using Cdk1/cyclin A or cyclin B. This mechanism has the advantage of blocking assembly of new preRCs until mitosis is complete and a

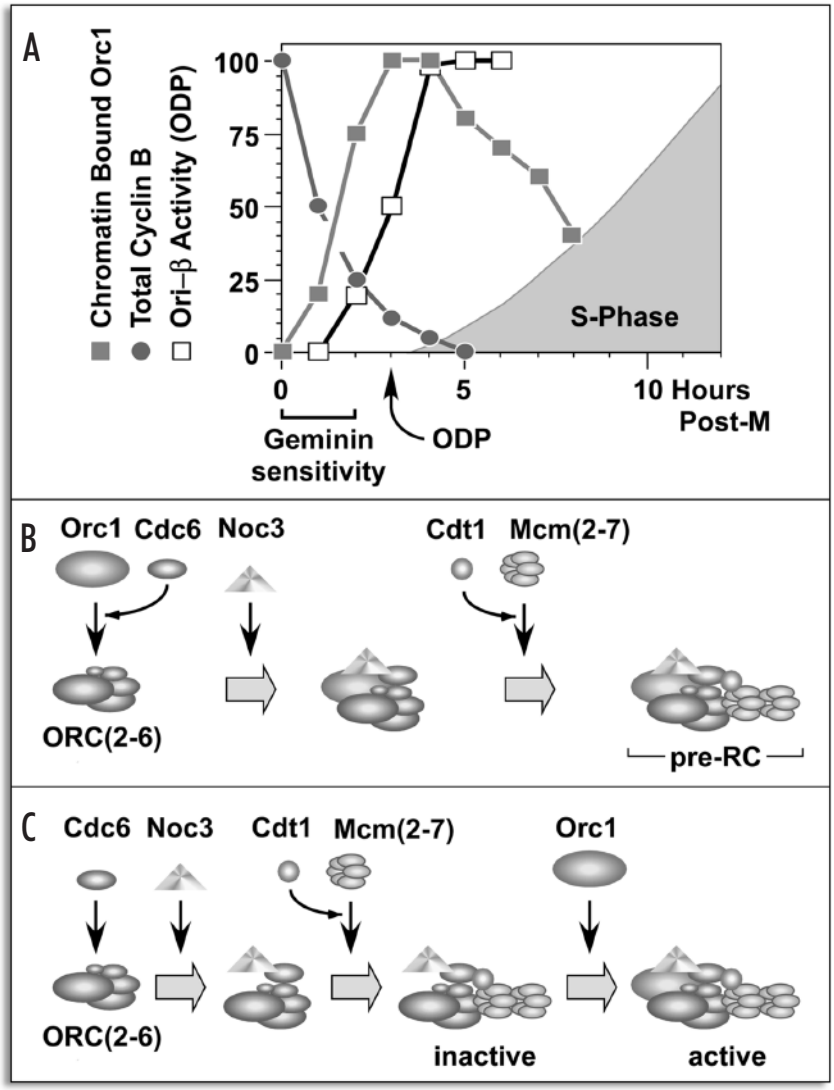

Figure 4. Binding of mammalian Orc1 to chromatin may occur after Mcm(2-7) binding, instead of before it. (A) Time courses in hamster CHO C400 cells for stable binding of CgOrc1 to chromatin, total cyclin B present, preferential initiation at ori-β [which marks the "origin decision point" (ODP)][69,102] and sensitivity to geminin.[71,119] The time period for geminin sensitivity has been extended 1 hour in an attempt to normalize the data to similar rates of cyclin B loss. Similar time courses for Orc1 binding to chromatin and for cyclin B degradation under similar experimental conditions have been reported for human cells. [68,97] Orc1 could trigger assembly of functional preRCs by either binding to ORC core/chromatin sites before (B) or after (C) Mcm(2–7) has bound.

nuclear membrane is present. In mammals, the Cdk1/cyclin A association with Orc1 is lost during the M to G_1-phase transition, whereas in frogs it is retained. Perhaps the compressed cell cycle in rapidly cleaving frog eggs (30 minutes) and the lack of a G_1-phase precludes dissociation of XlORC from Cdk1/cyclin A. Instead, XlORC-associated Cdk1 activity may be repressed by association with Xic1, a Cdk specific peptide inhibitor that is degraded only after assembly of preRCs on chromatin.[92]

(4) Flies and mammals employ ubiquitination to selectively target and in some cases destroy Orc1. Mammals use the SCF(Skp2) ubiquitination system during S-phase to mark the Orc1 subunit,[97] although subsequent degradation of Orc1 is not mandatory. Flies use the APC(Fzr) ubiquitination system during G_2/M-phase.[95] Both flies and mammals then synthesize Orc1 and bind it to chromatin during the M to G_1-phase transition.

Functions of eukaryotic "ORC cycles"

Restricting initiation events. The obvious function of the cell cycle dependent changes in ORC subunits described above would be to act as the premier step in restricting initiation events to once per origin per cell division. Three lines of evidence support this conclusion. First, in yeast, the ability to initiate DNA replication is dependent on the phosphorylated state of ORC subunits, as well as the status of Cdc6 and Mcm proteins.[76-78] Second, chromatin from M-phase hamster cells cannot initiate DNA replication in *Xenopus* egg extracts depleted of XlORC proteins whereas chromatin from G_1-phase cells can.[30,69,110] Third, the cell cycle dependent destruction of Orc1 in fly embryos[95] and in cultured human cells[68,97,111] implies that functional preRCs cannot be assembled in these organisms during S, G_2 and M-phases. However, loss of Orc1 protein was not complete in these studies. Reports that ORC levels as low as 10% are capable of initiating DNA replication,[112] and that sufficient ORCs are present in G_2-phase nuclei to initiate DNA replication[85] suggest that cell cycle dependent changes in Orc1 may have other functions as well.

Initiation site selection. In an effort to account for the "random" initiation events observed in the rapidly cleaving embryos of frogs, flies and fish, the broad initiation zones detected by 2D gel electrophoresis in mammalian cells, and the site-specific initiation events detected by labeling nascent DNA in mammalian cells, a model was proposed based on the Jesuit dictum that "many are called, but few are chosen".[117,118] As additional studies accumulated, this model appeared event more apropos.[23,38] Simply stated, it was proposed that DNA contains many potential initiation sites, but that as cells undergo differentiation and animal development proceeds, the epigenetic changes described under "replication origins" may repress some sites while activating others. The cell cycle dependent behavior of Orc1 suggests that it may participate in this process by choosing which ORC core/chromatin sites can produce functional preRCs. Moreover, overexpression of DmOrc1 altered the pattern of DNA synthesis in developing *Drosophila* embryos, implicating Orc1 in regulating initiation of DNA replication.[94]

Genetic evidence in yeast,[1,64] biochemical evidence in *Xenopus* egg extracts,[46] and the periodic oscillation of Orc1 levels in mammalian cells and fly embryos[68,95,97] support the conclusion that Orc1 is required to initiate DNA synthesis, but not necessarily for binding of ORC to DNA. In *S. pombe*, only Orc4 is required for site-specific DNA binding.[43-45] In *S. cerevisiae*, Orc1 and ATP are required to bind specifically to DNA replication origins, but they do not appear to be required for nonspecific DNA binding.[64] Therefore, when mammalian Orc1 rebinds to chromatin during the M to G_1-phase transition, it may bind either before (Fig. 4B) or after (Fig. 4C) the binding of Mcm(2–7).

Some results favor Orc1 binding to chromatin after Mcm(2–7) is bound. The bulk of CgOrc1 is stably bound to chromatin[69,102] after initiation of DNA replication is no longer sensitive to geminin,[71,119] a specific inhibitor of Cdt1-dependent loading of Mcm(2–7) (Fig. 4A). Similarly, DmOrc1 binds to chromatin during late G_1-phase.[95] Other results favor Orc1 binding before Mcm(2–7). Reducing the level of Orc1 in human cells also reduces association of MCM proteins with chromatin.[100] Therefore, assuming that this treatment di dnot affect any other protein required for preRC assembly, HsOrc1 is required for binding MCM proteins to chromatin.

Interestingly, the affinity of Orc1 for chromatin in mammalian cells is not determined simply by its affinity for other ORC subunits, because Cdk activity in metaphase cells was solely responsible for preventing stable binding of Orc1 to chromatin, but it was not responsible for preventing binding of Orc1 to Orc2 during mitosis.[104] Since the affinity between Orc1 and Orc2 did increase during the M to G_1-phase transition, a second event must occur during the M to G_1-phase transition that assembles a functional ORC complex on the chromatin. This event may correspond to the "origin decision point",[120] that time during G_1-phase when functional preRCs appear at specific sites on mammalian genomes. If the ORC(2–5) core complex remains bound to specific genome sites throughout the cell cycle, then Orc1 could determine which of these sites is selected for preRC assembly based on the interaction of Orc1 with chromatin bound proteins other than ORC subunits. Moreover, Orc1 may chaperon Cdc6 to specific ORC/chromatin sites. Cdc6 can bind to Orc1,[99] and Cdc6 can facilitate binding of XlORC to somatic cell chromatin[81,124] and ScORC to yeast replication origins.[121] Thus, the "ORC cycle" provides a mechanistic basis for the "Jesuit Model" of initiation site selection in mammalian cells.

Cell growth and proliferation. Orc1, like Orc2 and Orc6, may interact with other proteins to coordinate DNA replication with other events in cell growth proliferation such as gene expression and cell division.[122,123] In addition, the status of Orc1 may signal the cell when DNA replication is proceeding normally and when it is aberrant. Thus, while the results described in this review clearly reveal the existence of an "ORC cycle" in eukaryotes, the biological significance of the various cell cycle dependent changes in ORC structure and activity is only beginning to be understood.

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