The ‘ORC cycle’: a novel pathway for regulating eukaryotic DNA replication

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Abstract

The function of the ‘origin recognition complex’ (ORC) in eukaryotic cells is to select genomic sites where pre-replication complexes (pre-RCs) can be assembled. Subsequent activation of these pre-RCs results in bi-directional DNA replication that originates at or close to the ORC DNA binding sites. Recent results have revealed that one or more of the six ORC subunits is modified during the G1 to S-phase transition in such a way that ORC activity is inhibited until mitosis is complete and a nuclear membrane is assembled. In yeast, Cdk1/Clb phosphorylates ORC. In frog eggs, pre-RC assembly destabilizes ORC/chromatin sites, and ORC is eventually hyperphosphorylated and released. In mammals, the affinity of Orc1 for chromatin is selectively reduced during S-phase and in some cases degraded. Thus, most, perhaps all, eukaryotes exhibit some manifestation of an ‘ORC cycle’ that restricts the ability of ORC to initiate pre-RC assembly to the early G1-phase of the cell cycle, making the ‘ORC cycle’ the premier step in determining when replication begins.

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1. DNA replication and the cell division cycle

In normal cells, the cell division cycle is highly regulated and consists of four easily recognized phases: G1-phase (preparation for DNA replication), S-phase (DNA replication), G2 phase (preparation for mitosis) and M-phase (mitosis). Following mitosis, each cell must decide whether to enter G1-phase, which is a commitment to cell division, or to enter a quiescent state (G0-phase). Once cells are committed to undergoing DNA replication, they must complete the process of cell division or die. Thus, commitment to DNA replication is the primary point at which cells regulate their division cycle. In contrast, cells in G0-phase remain capable of proliferating for long periods of time (e.g. hepatocytes, lymphocytes). Alternatively, cells can undergo terminal differentiation (e.g. neurons, germ cells), a state from which they do not reenter the cell division cycle. Unregulated cell proliferation is the primary characteristic of the pathological state known as cancer.

One critical feature of all eukaryotic cell cycles, regardless of species or developmental stage, is that initiation of DNA replication is limited to once per replication origin per cell division cycle. Not all origins are used during every cell cycle, but if they are, they cannot be used a second time until after cell division has occurred. Presumably, this mechanism evolved to avoid the genomic instability that would invariably result if some sequences were duplicated more than others and replication forks remained during mitosis. Therefore, it is not surprising that the proteins and sequence of events that comprise eukaryotic DNA replication are highly conserved (Bogan et al., 2000; Bell and Dutta, 2002).

Abbreviations: ORC, origin recognition complex; Orc1–Orc6, ORC subunits; Mcm, 2–10, minichromosome maintenance proteins; Mcm(2–7), heterohexameric complex of Mcm proteins 2–7; Cdc, cell division cycle protein; Cdt1, protein encoded by Cdc10 dependent transcript 1 in Schizosaccharomyces pombe; RLF-B, Cdt1 and replication licensing factor B are the same protein; Cdk, cyclin dependent protein kinase; Pre-RCs, pre-replication complexes consisting of ORC, Cdc6, Cdt1 and Mcm(2–7); Sc, Saccharomyces cerevisiae; Sp, Schizosaccharomyces pombe; Xl, Xenopus laevis; Dm, Drosophila melanogaster.

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Eukaryotic DNA replication begins with the binding of a six subunit ‘origin recognition complex’ (ORC) to DNA (reviewed in Bell and Dutta, 2002; outlined in Fig. 1). Proteins Cdc6 and Cdt1 (RLF-B) are then required to load Mcm proteins 2–7 onto the ORC/chromatin site to form a pre-replication complex (pre-RC). Mcm2–Mcm7 exist as a hexameric complex [Mcm(2–7)] that appears to be the helicase responsible for unwinding parental DNA strands. Presumably, there is at least one Mcm(2–7) complex per replication fork, although more are possible (Edwards et al., 2002). Pre-RCs are activated upon binding of Mcm10 protein (Wohlschlegel et al., 2002). Cdc6 is then released by the cyclin dependent protein kinase, Cdk2/Cyclin A, and replaced by Cdc45 with the help of the protein kinases Cdc7/Dbf4 and Cdk2/cyclin E. Cdc45 associates with DNA polymerase-α. DNA primase and guides it to the complex to initiate RNA-primed DNA synthesis.

Several mechanisms have been identified that limit initiation of eukaryotic DNA replication to once-per-origin-per-cell division. In yeast, Cdc6 is phosphorylated when

![Diagram](image-url)
cells enter S-phase, released from pre-RCs, ubiquitinated and degraded [(Drury et al., 1997; Weinreich et al., 2001) and references therein]. When yeast enter M-phase, the Cdk1/cyclin B phosphorylates any newly synthesized Cdc6, thereby preventing it from binding to ORC until mitosis is completed. The same mechanism exists in frogs and mammals, except that in human cells phosphorylated Cdc6 is transported out of the nucleus during S-phase and remains in the cytoplasm during G2 and M-phases [(Jiang et al., 1999; Petersen et al., 1999; Coverley et al., 2000; Mendez and Stillman, 2000; Petersen et al., 2000; Delmolino and Dutta, 2001) and references therein]. During the M to G1-transition, HsCdc6 is degraded and then resynthesized later during G1-phase (Mendez and Stillman, 2000; Petersen et al., 2000). However, the amount of chromatin bound Cdc6 in hamster cells appears to remain constant throughout the cell cycle, with no indication of any degradation during G1 (Okuno et al., 2001). This difference may reflect a larger pool of Cdc6 in human cells than hamster cells. Nevertheless, under conditions where Cdc6 cannot be phosphorylated and remains in the nucleus during S-phase, reinitiation of DNA replication still does not occur, revealing the existence of other regulatory mechanisms (Pelizon et al., 2000; Petersen et al., 2000). One of these mechanisms is inhibition of Cdt1(RLF-B) activity by geminin, a protein produced during the S to M transition (Wohlschlegel et al., 2000; Tada et al., 2001). Geminin has not been found in yeast, but in yeast, as well as in mammals, Cdt1 is degraded during S-phase. The combined inactivation of both Cdc6 and Cdt1 prevents rebinding of the Mcm(2–7) to ORC/chromatin sites. Nevertheless, Mcm proteins themselves are phosphorylated during S-phase and released from chromatin. This mechanism is presumably triggered by termination of DNA replication when two oncoming replication forks collide. Moreover, in budding yeast, the phosphorylated Mcm proteins are exported from the nucleus during the G2 to M transition, thereby providing an additional barrier to reinitiation during S-phase (Labib et al., 1999; Pasion and Forsburg, 1999; Nguyen et al., 2000). In fission yeast as well as in the metazoa, Mcm proteins remain in the nucleus, although their affinity for chromatin is markedly diminished (Lei and Tye, 2001).

The question remains as to whether or not ORC activity is also regulated.

2. Selection of initiation sites

Since ORC binding to DNA is the first step in pre-RC assembly, ORC determines where DNA replication can begin by binding to specific DNA sites in the genomes of budding yeast (S. cerevisiae), fission yeast (S. pombe), flies (Drosophila melanogaster, Sciara coprophila) and mammals [(Abdurashidova et al., 2003; Bell, 2002; Keller et al., 2002b; Kong and DePamphilis, 2002; Ladenburger et al., 2002) and references therein]. The budding yeast S. cerevisiae contains about two ORCs per replication origin [600 ORC/cell (Rowley et al., 1995); 323 origins/cell (Raghuraman et al., 2001)]. Since both ORC and Mcm proteins are bound to 429 sites in the S. cerevisiae genome (Wyrick et al., 2001), about 25% of these ORC/chromatin sites do not initiate DNA replication. These pre-RCs are silenced when DNA replication forks from neighboring, earlier firing origins, pass through them (Santocanale et al., 1999). Mammalian cells contain $10^4$–$10^5$ molecules of ORC per cell (Natale et al., 2000; Kreitz et al., 2001; Okuno et al., 2001; Ohta et al., 2003), suggesting that they initiate replication, on average, once every 60–600 kb (Natale et al., 2000), consistent with the average size of replicons in mammalian cells [200–300 kb; (Ockey and Saffhill, 1976; Yurov and Liapunova, 1977)]. These data suggest that most, if not all, ORCs are bound to replication origins.

Initiation sites for DNA replication are distributed throughout all eukaryotic genomes. This insures that genomes can be duplicated in a few minutes (e.g. rapidly cleaving embryos of flies, frogs, and fish) to a few hours (e.g. mammalian cells) without having to change the basic replication fork mechanism by which DNA is replicated. In yeast, flies and mammals, cis-acting sequences (replication origins) determine where DNA replication will begin [(DePamphilis, 1999; Altman and Fanning, 2001; Lu et al., 2001; Bell and Dutta, 2002; Kong and DePamphilis, 2002) and references therein], but with the exception of the small (~0.1 kb) replication origins in S. cerevisiae, no consensus sequence has been identified that is required for replication. Replication origins in fission yeast, flies and mammals are five to 20 times larger than those in budding yeast. They frequently contain large AT-rich regions and genetically identifiable sequences that are required for origin function. The activity of these sequences is often orientation or distance dependent. Furthermore, ORC not only binds to replication origins in fission yeast, flies, and mammals (Bell and Dutta, 2002), but recent studies have revealed that ORC binds to specific sites within replication origins in S. pombe (Kong and DePamphilis, 2001; Lee et al., 2001; Kong and DePamphilis, 2002) and human cells (Abdurashidova et al., 2003), and the ORC binding sites in S. pombe ARS3001 are required for origin function (Kim and Huberman, 1998; Kong and DePamphilis, 2002).

One remarkable feature of eukaryotic DNA replication is that selection of initiation sites varies from non-specific in the rapidly cleaving embryos of frogs, flies, and fish, to site-specific in yeasts, flies and mammals (DePamphilis, 1999; Bell and Dutta, 2002). Moreover, as development of frogs (Hyrien et al., 1995) and flies (Sasaki et al., 1999) changes from rapid cell cleavage events prior to the onset of zygotic gene expression to the slower cell division cycles in later
embryonic stages and adult animals, initiation events changes from non-specific to site-specific. Since only one set of Orc genes has been discovered in frogs and flies, site-specific initiation must be developmentally acquired epigenetically. Epigenetic changes that can affect site specificity include the ratio of initiation factors to DNA, chromatin or nuclear composition and structure, DNA methylation, and the acquisition of ORC accessory proteins that facilitate binding of ORC to specific sequences (DePamphilis, 1999). Recent studies have suggested that transcription factors may also facilitate binding of ORC to specific DNA sites (Bosco et al., 2001; Beall et al., 2002; Saitoh et al., 2002).

3. The “ORC cycle” in summary

Reinitiation of DNA replication at the same replication origins during a single cell division cycle is prevented in eukaryotic cells by regulating at least three steps in the assembly of pre-RCs – association of Cdc6, Cdt1 and Mcm(2–7) proteins with chromatin (see Section 1). Recent results have revealed a fourth regulatory step in which ORC activity is inhibited during the G1 to S-phase transition and not reestablished until mitosis is complete and a nuclear membrane has reassembled. Therefore, regulation of ORC activity becomes the premier step in determining when DNA replication begins. Cell cycle dependent changes in ORC activity, and in some cases the affinity of one or more ORC subunits for chromatin, is hereafter referred to as ‘the ORC cycle’. However, the manifestation of the ORC cycle can vary among species and during development (summarized in Fig. 2).

The ORC cycle was first recognized in mammalian cells (Natale et al., 2000; Kreitz et al., 2001) where the Orc1 subunit is selectively destabilized during S-phase, ubiquitinated and in some cases degraded, and then rebinds stably to chromatin during the M to G1 transition to establish pre-RCs at specific genomic sites (see Section 4). Since all six ORC subunits are required for ORC activity in yeast (Bell, 2002), release of Orc1 should prevent ORC function, and in fact, mammalian metaphase chromatin lacks functional ORCs. Cell cycle dependent loss of ORC function is even more obvious when mammalian somatic cell chromatin is incubated in a Xenopus laevis (XI) egg extract (see Section 7). In this case, XIORC binds to the cell chromatin, initiates pre-RC assembly, and the entire XIORC is then released upon completion of pre-RC assembly. The timing of this release, however, appears to be dependent on chromatin structure, because when Xenopus sperm chromatin is incubated in the same extract, the affinity of XIORC for sperm chromatin is reduced, but ORC remains bound to the chromatin throughout DNA replication. In this case, XIORC appears to be released during G2/M phase as a result of hyperphosphorylation by Cdk1/cyclin A. In fact, phosphorylation has been shown to play a role in regulating ORC activity in yeast even though all six ORC subunits remain stably bound to chromatin throughout the cell cycle (see Section 8). Whether or not an ORC cycle also exists in other eukaryotes, such as flies, remains to be demonstrated, although some evidence suggests that it does (see Section 9). Release of ORC subunits from chromatin after pre-RC assembly, either as a programmed event during DNA replication (Sun et al., 2002), or by selective elution using salt or cyclin-dependent protein kinase activity (Hua and Newport, 1998; Jares and Blow, 2000), does not interfere with assembly of active replication forks. Therefore, regulating ORC association with chromatin is a feasible mechanism for restricting reinitiation events during S-phase without interfering with DNA replication. Whether or not ORC or an ORC subcomplex binds to newly synthesized replication origins during S-phase is unknown, but in Xenopus egg extracts (discussed below) ORC cannot rebind to somatic cell chromatin until the next cell cycle begins.

It should not be surprising that species-specific variations exist in the regulation of ORC activity, because although ORC proteins are highly conserved within a single taxonomic family, conservation among all species is modest. For example, human, hamster and mouse Orc1 proteins are ~81% identical and 84% similar in their total amino acid sequence, and 93% identical and 95% similar in their C-terminal portion (Bogan et al., 2000). The C-terminal portion contains a homology with Cdc6 protein and an ATP binding domain that appears to be required for site-specific DNA binding (Bell, 2002). Among all species, however, total amino acid sequence conservation drops to 24% identity and 35% similarity.

4. The ORC cycle in mammals

4.1. Selective release Of Orc1

In mammals, both the cellular concentrations of Orc proteins 2–6 and the amount of each protein bound to chromatin appear constant throughout the cell division cycle [Orc2 (Rizzi et al., 1998; Saha et al., 1998; Mendez and Stillman, 2000; Natale et al., 2000; Mendez et al., 2002; Ohta et al., 2003), Orc3 (Mendez et al., 2002; Ohta et al., 2003), Orc4 (Okuno et al., 2001; Ohta et al., 2002), Orc5 (Ohta et al., 2003), Orc6 (Dhar and Dutta, 2000; Mendez et al., 2002). Nevertheless, there are changes in the distribution of ORC subunits that suggest they have activities independent of their role in DNA replication. For example, both the cellular concentration (Dhar and Dutta, 2000) and the amount of chromatin bound HsOrc6 (Mendez et al., 2002) remain constant throughout the cell cycle, but some Orc6 is found at non-chromosomal sites in mitotic cells (Prasanth et al., 2002). This appears to be due to a fraction of Orc6 that is not associated with ORC. Furthermore, the abundance of individual ORC subunits relative to each other can vary among tissues, and some
subunits are expressed in non-proliferating tissues (Thome et al., 2000). Orc1, however, is unique in that it appears to regulate ORC activity in a cell cycle dependent manner (Fig. 1, ‘Mammals’; Fig. 2, ‘Mammalian cells’).

In hamster cells, the cellular concentration of Orc1 is constant throughout the cell cycle (Natale et al., 2000; Okuno et al., 2001; Li and DePamphilis, 2002), but Orc1 is selectively released from chromatin during the S and M-phases while the remaining Orc proteins remain chromatin bound when cells are lysed with a non-ionic detergent in the presence of 0.1–0.15 M salt and ATP (Natale et al., 2000; Li and DePamphilis, 2002). The same is true in human cells, except that the cellular concentration of Orc1 oscillates during the cell cycle, because Orc1 is degraded during S-phase (Kreitz et al., 2001; Fujita et al., 2002; Mendez et al., 2002; Ohta et al., 2003); J. Teer and A. Dutta, unpublished data]. Previous reports that HsOrc1 levels did not decrease during S-phase (Saha et al., 1998; Tatsumi et al., 2000) appear to have resulted from differences in the specificity of the antibodies used to detect HsOrc1 in various experiments (A. Dutta; C. Obuse, personal communications).

In contrast to the reports described above, release of chromatin bound Orc1 during S-phase in hamster cells was not detected by Okuno et al. (2001) who concluded that both the amount of chromatin-bound Orc1 and Orc4 as well as their cellular concentrations remained constant throughout the cell cycle. However, although it is not clear why they did not observe a release of Orc1 during S-phase, it does appear that the amount of Orc1 bound to chromatin in their experiments was markedly diminished during M-phase, suggesting that Orc1 is selectively destabilized sometime during the cell cycle. The absence of Orc1 in their chromatin-unbound fraction from M-phase cells (Okuno et al., 2001) probably resulted from the fact that unbound Orc1 is easily degraded, even when cell extracts are on ice, whereas chromatin bound Orc1 is stable (Li and DePamphilis, 2002). In addition, the amount of chromatin bound Orc1 in M-phase arrested cells can vary depending on the length of time cells are held in nocodazole. For example, chromatin bound Orc1 was not detected in M-phase cells that were not collected in nocodazole (Natale et al., 2000; Li and DePamphilis, 2002), whereas cells arrested in nocodazole could contain up to 20% of the amount of chromatin bound Orc1 observed in G1-phase cells, depending on how long the cells were held in nocodazole (Li and DePamphilis, 2002). Nocodazole blocks anaphase by preventing microtubule assembly, which does not necessarily prevent other cell cycle related events.

The selective release of Orc1 during S-phase accounts for the fact that one or more ORC subunits are both functionally and physically absent from metaphase chromatin. The absence of ORC activity is revealed by the fact that hamster M-phase chromatin will replicate in a complete Xenopus egg extract, but not in one that has been depleted of Xenopus Orc proteins (Yu et al., 1998; Li et al., 2000; Natale et al., 2000). In contrast, nuclei isolated in the same manner

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**Fig. 2.** Four manifestations of the ORC cycle in eukaryotes. Yeast cells: ORC (six gray cylinders) remains bound to replication origins throughout the cell cycle, but ORC is phosphorylated (-P) during the S to M periods, and this phosphorylation inhibits its ability to assemble a pre-RC. Xenopus egg extract: ORC binds to sperm chromatin, but the stability of ORC/chromatin sites is reduced (red boxes) following pre-RC assembly. ORC is phosphorylated by Cdk1/cyclin A (yellow ball) during G2/M and released from chromatin. If somatic cell chromatin instead of sperm chromatin is incubated in the extract, then ORC is released from chromatin following pre-RC assembly. Mammalian cells: The Orc1 subunit is selectively destabilized (red box) and released from chromatin when cells enter S-phase. Orc1 is then mono-ubiquitinated (Ub) and in some cases polyubiquitinated ([Ub] n) and degraded. Orc1 in mitotic cells appears hyperphosphorylated. Thus, in yeast, frogs and mammals, stable ORC/chromatin sites that can initiate assembly of a pre-RC are not present until mitosis is complete and a nuclear membrane is present.
from G1-phase cells will initiate DNA replication de novo under both conditions, revealing that hamster pre-RCs are assembled during the M to G1 transition. Loss of ORC activity can be accounted for by the fact that Orc1 protein is not associated with metaphase chromatin under conditions where other Orc proteins remain stably bound (Natale et al., 2000; Tatsumi et al., 2000; Okuno et al., 2001; Mendez et al., 2002) and mammalian Cdc6 protein is abundant [(Mendez and Stillman, 2000; Petersen et al., 2000). This observation is consistent with the cell cycle-dependent changes observed in a footprint at the human lam B2 origin (Abdurashidova et al., 1998) that encompasses the start sites for leading strand DNA replication (Abdurashidova et al., 2000). A large G1-phase footprint changed into a smaller S and G2 phase footprint that disappeared during M-phase. Recent UV-induced cross-linking experiments reveal that the large G1-phase footprint could be accounted for by the presence of a pre-RC containing at least Orc1, Orc2, Cdc6, and Mcm3 proteins (Abdurashidova et al., 2003). During S-phase, only the Orc2 protein can be cross-linked to chromatin, consistent with the disappearance of the pre-RC and the release of Orc1. By M-phase, not even Orc2 could be cross-linked to DNA, suggesting additional changes occur in the affinity of ORC for chromatin. Similarly, both Orc1 and Orc2 can be cross-linked to specific replication origins in vivo by treating human G1-phase cells with formaldehyde, but only Orc2 can be cross-linked during S-phase (Ladenburger et al., 2002). These observations in mammalian cells are consistent with earlier reports that both Orc1 and Orc2 are present on chromatin in Xenopus interphase cells but not in metaphase cells (Romanowski et al., 1996). Moreover, Orc proteins in Xenopus eggs competent for DNA replication will bind to sperm chromatin, whereas Orc proteins in Xenopus eggs in metaphase II will not (Coleman et al., 1996; Hua and Newport, 1998; Findeisen et al., 1999; Rowles et al., 1999).

Whether Orc1 is associated with chromatin in vivo in an unstable state or is actually released from chromatin in vivo as well as in cell extracts is difficult to assess. What is clear is that Orc1 is not stably bound to chromatin in comparison with other Orc proteins, and that metaphase cells do not contain functional ORCs. M-phase cells permeabilized with digitonin retain Orc1 in the chromatin pellet (Natale et al., 2000), but this M-phase chromatin does not initiate DNA replication in an ORC-depleted Xenopus egg extract (Yu et al., 1998; Li et al., 2000; Natale et al., 2000). Expression of a GFP-tagged Orc1 protein in hamster cells resulted in GFP-labeled metaphase chromosomes (Okuno et al., 2001), but there is no evidence that the GFP-Orc1 protein is assembled into ORCs and if so, that the GFP-ORCs are active.

4.2. Ubiquitination Of Orc1

Orc1 in S-phase mammalian cells is selectively ubiquitinated and, under some conditions, degraded. In hamster cells, two forms of Orc1 are released concurrently during S-phase, one that is not ubiquitinated and one that is mono-ubiquitinated (Li and DePamphilis, 2002). Orc1 and mono-ubiquitinated Orc1 also were detected in the non-chromatin bound fraction from human S-phase cells (Mendez et al., 2002). However, in human cells, Orc1 became polyubiquitinated and was degraded via the 26S proteasome pathway (Fujita et al., 2002; Mendez et al., 2002), a reaction mediated by the SCF(Skp2) ubiquitin-ligase complex (Mendez et al., 2002). In contrast, only trace amounts of polyubiquitinated Orc1 were detected in hamster cells. Hamster Orc1 was polyubiquitinated and degraded only when it was released from nuclei into cellular extracts. Thus, the chromatin unbound fraction of Orc1 in non-ionic detergent extracts of hamster mitotic cells can be lost, even when cell lysates are held in ice (Okuno et al., 2001; Li and DePamphilis, 2002).

In contrast to Orc1, Orc2 not only remains bound to chromatin throughout the cell cycle, but Orc2 is not a substrate for ubiquitination, even as a soluble protein (Li and DePamphilis, 2002). Interestingly, the half-life of Orc1 in both hamster and human exponentially proliferating cells is ~3 h, the same as observed for Orc2 [(Li and DePamphilis, 2002); J. Mendez, personal communication]. Therefore, it may not be necessary to deubiquitinate Ub-Orc1 in order to reestablish functional ORC/chromatin sites in early G1-phase, because in both hamster and human cells, most of the Orc1 in M-phase cells will have been synthesized during the previous 6 h.

5. Orc1 and the assembly of pre-replication complexes

Several observations suggest that the stable binding of Orc1 to chromatin is the premier regulatory step in the assembly of pre-RCs and thereby the commitment to cell proliferation. First, both HsOrc1 (but not HsOrc2) and DmOrc1 expression are regulated by E2F (Ohtani et al., 1996; Asano and Wharton, 1999), a transcription factor that plays an important role in driving cells from G1 into S-phase. E2F also regulates expression of Cdc6 and Mcm genes (Leone et al., 1998; Ohtani et al., 1998; Yan et al., 1998), suggesting that assembly of pre-RCs depends on the E2F/Rb pathway with ORC activity regulated by expression of Orc1. Second, the Orc1 gene is the only ORC gene whose expression is correlated with cell proliferation: Orc1 is expressed only in proliferating mammalian cells whereas Orc2–Orc5 are expressed to varying extents in many non-proliferating cells (Thome et al., 2000). Third, stable binding of Orc1 to chromatin immediately precedes assembly of pre-RCs at specific chromosomal loci.

During the M to G1 transition, Orc1 rebinds stably to chromatin with the concomitant appearance of functional ORCs. M-phase hamster cells contain as much Orc1 as G1-phase cells, but most, if not all, of the Orc1 in M-phase hamster cells is not bound to chromatin and only a minor
fraction is ubiquitinated (Li and DePamphilis, 2002). M-phase human cells generally contain much less Orc1 than G1-phase human cells, as a result of Orc1 degradation during S-phase (Mendez et al., 2002). Therefore, human Orc1 must be resynthesized during the M to G1 transition, whereas hamster Orc1 does not (Okuno et al., 2001). ORC activity is restored during the first 2 h after either hamster or human M-phase cells (arrested in nocodazole) are released into G1-phase (Yu et al., 1998; Natale et al., 2000). This corresponds to the time period (1–3 h post-M) when Orc1 (Natale et al., 2000; Li and DePamphilis, 2002; Mendez et al., 2002; Ohta et al., 2003), Mcm2 and Mcm3 reassociate stably with chromatin (Dimitrova et al., 1999; Natale et al., 2000; Dimitrova et al., 2002; Ohta et al., 2003). Nuclei isolated from these early G1-cells can initiate de novo DNA replication in a Xenopus egg extract depleted either of ORC or of Mcm proteins. The appearance of chromatin bound Orc1 is inversely related to the lost of cyclin B (Mendez and Stillman, 2000), which is required to drive cells into mitosis, and directly linked to formation of an intact nuclear envelope (Dimitrova and Gilbert, 1998; Natale et al., 2000), which is required for initiation of DNA replication. Thus, the bulk of Orc1 and Mcm binding occurs after mitosis is complete and a nuclear membrane has reformed around the genome, a time referred to either as late telophase or early G1-phase. Consistent with this view is the fact that HsCdc6 protein, like cyclin B, is degraded during the M to G1 transition and then resynthesized during G1 (Petersen et al., 2000; Mendez et al., 2002), just in time to be incorporated into the newly reconstituted active ORC/chromatin sites. Therefore, stable rebinding of Orc1 to chromatin appears to be the rate-limiting step for assembly of pre-RCs.

One experiment, however, suggests that pre-RC assembly is completed about 2 h before the stable binding of hamster Orc1, Mcm2 and Mcm3 is completed. Geminin, a specific inhibitor of Cdt1, prevents stable binding of Mcm(2–7) to chromatin. Prior to pre-RCs are assembled, DNA replication is no longer sensitive to geminin. DNA replication in hamster metaphase chromatin incubated in Xenopus egg extract is sensitive to geminin, but DNA replication in chromatin isolated 1 h after metaphase is not (Okuno et al., 2001; Dimitrova et al., 2002). In these experiments, Orc1 appears to bind to chromatin within the 1st h after release of cells from the nucodazole block, consistent with the execution point for geminin. Interestingly, Mcm proteins continued to bind to chromatin until mid to late G1-phase (Dimitrova et al., 1999; Natale et al., 2000; Dimitrova et al., 2002), suggesting that more Mcm proteins bind to chromatin than are actually assembled into pre-RCs. Apparent differences in the published time courses for protein binding and the geminin execution point likely reflect differences in experimental protocols that affect the amount of protein recovered in different fractions and the time required for cells to progress from M to G1 when the synchronizing agent, nocodazole, is removed.

6. ORC and the ‘origin decision point’

Xenopus egg extract can initiate DNA replication de novo in virtually any DNA, chromatin or nuclei provided, but these initiation events are generally distributed non-specifically throughout the DNA substrate. The only exception is when nuclei are isolated from mammalian cells under conditions that preserve their impermeability to macromolecules (Gilbert et al., 1995). Under these conditions, initiation events are distributed non-specifically throughout the genome when ‘nuclei’ are isolated from M-phase or early G1-phase cells, but at specific sites when nuclei are taken from late G1-phase cells (Wu and Gilbert, 1996; Wu and Gilbert, 1997; Li et al., 2000; Okuno et al., 2001). DNA replication in late G1-nuclei is independent of XIO RC proteins, begins in vitro at the same site-specific origins of bi-directional DNA replication used by the cells in vivo, and is sensitive to protein kinase inhibitors. Therefore, it appears that pre-RCs in late G1-phase nuclei are present at specific genomic sites waiting to be activated by Cdk2/cyclin A, E and Cdc7/Dbf4 (Fig. 1). This cell cycle dependent transition that occurs in mammalian chromatin, from a substrate for non-specific initiation events to a substrate for site-specific initiation events, is referred to as the ‘origin decision point’ (ODP).

Two views of the ODP have emerged. In one view, the ODP marks the transition from Xenopus ORC directed initiation events to mammalian ORC directed initiation events. Prior to the ODP in hamster cells (midpoint ~ 2 h post-M), initiation of DNA replication in hamster chromatin requires the presence of Xenopus ORC in the egg extract (Natale et al., 2000). XIO RC present in these experiments rapidly binds to hamster metaphase chromatin within the first few minutes of incubation (Sun et al., 2002) and is responsible for most of the DNA replication that occurs during the first 1–2 h post-M (Yu et al., 1998; Natale et al., 2000) where it initiates replication non-specifically throughout the genome (Yu et al., 1998; Dimitrova and Gilbert, 1999; Li et al., 2000; Natale et al., 2000). After the ODP in hamster cells, Xenopus egg extract simply activates hamster pre-RCs that were assembled in vivo. Initiation sites are established in hamster cells upon the stable binding of Orc1 (midpoint ~ 1.5 h post-M) to Orc2–6/chromatin complexes located at specific sites along the genome (Natale et al., 2000; Li and DePamphilis, 2002). This view is supported by the studies in human cells where Orc1 binding to chromatin follows a similar time course to that in hamster cells (Mendez et al., 2002) and where cross-linking studies have revealed that Orc2 is bound to specific genomic sites, some of which correspond to origins of bi-directional DNA replication (Abdurashidova et al., 2003; Keller et al., 2002b; Ladenburger et al., 2002). This view also accounts for the observation that the normal temporal order for initiation is absent in chromatin isolated from cells in M-phase to 1 h post-M phase but present in chromatin isolated from late G1-phase cells (Dimitrova and Gilbert, 1999; Li et al., 2000).
XlORC initiates replication non-specifically in chromatin from M-phase and telophase cells.

An alternative view of the ODP is that pre-RCs are initially assembled ‘randomly’ along the genome during the M to G1-transition in mammalian cells, and then reorganized, by some as yet unidentified mechanism, into specific sites [(Okuno et al., 2001) and references therein]. One suggested mechanism is that some pre-RCs are inactivated at the ODP, leaving those pre-RCs at specific genome sites to initiate replication at the start of S-phase. However, this implies the existence of a larger number of ORC/chromatin sites in mammalian cells that the cellular concentration of ORC would permit. This view of the ODP is based on the conclusion that all six mammalian ORC subunits are stably bound to chromatin throughout the cell cycle, that pre-RCs are assembled in hamster cells within 1 h after metaphase, and that pre-RC assembly precedes the ODP in hamster cells (midpoint ~3 h post-M) by 2–2.5 h. Various experimental artifacts that are innumerable in the papers cited above may well explain some of the differences between these two views. Nevertheless, the possibility remains that there exists a novel, as yet undiscovered, mechanism for determining where initiation events occur in mammalian genomes.

6.1. Association of Orc1 with nuclear structure

The fact that assembly of pre-RCs is delayed until mitosis is complete and a nuclear membrane is assembled implies that nuclear structure plays a role in the initiation of DNA replication. The nucleus appears to regulate the mechanism for determining where initiation events occur in the genome. Nevertheless, the possibility implies the existence of a larger number of ORC/chromatin sites in mammalian cells that the cellular concentration of ORC would permit. This view of the ODP is based on the conclusion that all six mammalian ORC subunits are stably bound to chromatin throughout the cell cycle, that pre-RCs are assembled in hamster cells within 1 h after metaphase, and that pre-RC assembly precedes the ODP in hamster cells (midpoint ~3 h post-M) by 2–2.5 h. Various experimental artifacts that are innumerable in the papers cited above may well explain some of the differences between these two views. Nevertheless, the possibility remains that there exists a novel, as yet undiscovered, mechanism for determining where initiation events occur in mammalian genomes.

7. The ORC cycle in frogs

Metaphase chromatin lacks functional ORC complexes, and therefore when metaphase chromatin from hamster cells is incubated in a Xenopus egg extract, XlORC rapidly binds to the chromatin and initiates DNA replication. However, in contrast to mammalian ORC, where only the Orc1 subunit is destabilized upon DNA replication, the entire XlORC is released from the somatic cell chromatin into the replication extract (50 mM KCl) as soon as pre-RCs have been assembled [(Fig. 1, ‘Xenopus’; Fig. 2, ‘somatic cell chromatin’; (Sun et al., 2002)]. This programmed release of XlORC was insensitive to aphidicolin (specific inhibitor of replicative DNA polymerases) and olomoucine (specific inhibitor of cyclin dependent protein kinases), but sensitive to geminin (specific inhibitor of Cdt1), and prevented by immuno-depletion of XlMcm proteins from the egg extract. Therefore, assembly of pre-RCs triggers release of ORC from cell chromatin. Alternatively, ORC release may be triggered by some event that occurs between completion of pre-RC assembly and the action of Cdk2. For example, Mcm10 binds to chromatin after pre-RC assembly and before Cdk2 activity where it is required to initiate DNA replication (Wohlschlegel et al., 2002).

Once pre-RCs were assembled in egg extract, XlORC was no longer present on somatic cell chromatin, despite the fact that egg extract contained a great excess of soluble XlORC. Furthermore, XlORC could not bind to chromatin in nuclei isolated from hamster cells that had progressed past their ‘origin decision point’ (Sun et al., 2002). Cdk/cyclins were not involved in these events, since neither binding nor release of XlORC to somatic cell chromatin was affected by olomoucine. Moreover, XlORC in meiotic eggs (metaphase II) does not bind to sperm chromatin (Coleman et al., 1996; Hua and Newport, 1998; Findeisen et al., 1999; Rowles et al., 1999), and XlORC is not bound to metaphase chromatin in Xenopus somatic cells (Romanowski et al., 1996). This inability of XlORC in cells undergoing meiosis or mitosis to bind to chromatin likely reflects the fact that XlORC is hyperphosphorylated in these cells where it associates with Cdk1/cyclin A (Romanowski et al., 2000). These data strongly suggest that XlORC cannot rebind to either somatic cell chromatin or sperm chromatin until cell division is completed.

7.1. Chromatin structure and the affinity of ORC for DNA

Xenopus sperm chromatin, like mammalian metaphase chromatin, lacks ORC proteins, and XlORC rapidly binds to chromatin from M-phase and telophase cells.
sperm chromatin and initiates DNA replication. However, in this case, XIORC is not released from chromatin, although it does undergo a demonstrable change in its affinity for chromatin [Fig. 2, ‘sperm chromatin’; (Hua and Newport, 1998; Rowles et al., 1999)]. Prior to pre-RC assembly, ORC remains bound to sperm chromatin in 0.25 M salt, but after pre-RC assembly, ORC can be eluted from sperm chromatin by exposure to high levels of Cdk2, or to mitotic extract, or 0.1–0.15 M KCl, leaving the Mcm proteins bound to the chromatin and capable of initiating DNA replication. This destabilization of ORC’s association with sperm chromatin does not require DNA replication, protein synthesis, cyclin-dependent kinases or Cdc7. It does require prior action of Cdc6, Mcm(2–7), and Cdt1, suggesting that binding of the Mcm(2–7) to chromatin weakens the association of ORC with sperm chromatin and thus increases its sensitivity both to salt and to phosphorylation. Thus, XIORC undergoes a programmed change in its affinity for chromatin immediately following pre-RC assembly. However, 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.

The fact that XIORC remains bound to sperm chromatin under conditions where it is released from somatic cell chromatin reveals that the affinity of ORC for DNA depends on chromatin structure. Since both chromatin substrates are rapidly assembled into nuclei by Xenopus egg extract, these differences likely reflect differences in their composition. For example, somatic cell chromatin brings with it a full complement of core and linker histones, but sperm chromatin must undergo extensive remodeling during its first 30 min in egg extract (Philpott and Leno, 1992). Nucleoplasmin dependent decondensation of sperm chromatin is a prerequisite for XORC binding and subsequent assembly of pre-RCs (Gillespie et al., 2001). Remodeling involves displacement of sperm specific proaminates, 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 (Dimitrov et al., 1994). 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 G1-phase first appears in the cell division cycle.

### 7.2. Comparison of frogs and mammals

The difference in behavior between mammalian and Xenopus ORCs is in keeping with the properties of the purified proteins. XIORC proteins exist as a stable complex of XIorc(1–6) that can be immunoprecipitated from Xenopus egg extract (Rowles et al., 1996; Tugal et al., 1998), although the identity of XIorc6 has not been confirmed. In contrast, human ORC proteins exist as a stable complex of HsOrc(2–5) to which HsOrc1 and HsOrc6 bind only weakly (Dhar et al., 2001; Vashee et al., 2001). Therefore, it is not surprising that mammalian Orc1 is selectively released from chromatin when cells enter S-phase and then rebinds when cells enter G1-phase, while Xenopus Orc1 remains associated with the other ORC subunits, regardless of whether they are bound or released from chromatin. Thus, XIORC is well suited to initiating DNA replication at many sites as cells undergo rapid cleavage events during early frog development. Xenopus ORC appears to bind weakly to chromatin and then release intact in order to reinitiate quickly at other sites. Mammalian ORC, on the other hand, is better suited to binding tightly to specific genomic sites where it remains throughout cell proliferation and differentiation. In mammals, site specificity is maintained by releasing only one of the six subunits during S-phase, leaving the core ORC tightly bound to a replication origin.

### 8. The ORC cycle in yeast

In contrast to mammalian cells, yeast ORC remains stably bound to chromatin throughout the yeast cell cycle. In *S. cerevisiae*, both DNA footprinting (Diffley et al., 1994; Fujita et al., 1998) and immuno-precipitation (Liang and Stillman, 1997) analyses reveal that a complete ORC binds to yeast replication origins immediately after initiation of replication occurs and remains there throughout the cell division cycle (Fig. 2, ‘yeast’). Similarly, in *S. pombe* all six ORC subunits remain bound to chromatin throughout the cell cycle (Lygerou and Nurse, 1999; Kong and DePamphilis, 2001). Nevertheless, re-initiation within a single S-phase is prevented by a combination of ORC phosphorylation, down regulation of Cdc6 activity, and nuclear exclusion of Mcm proteins (Nguyen et al., 2001).

In *S. cerevisiae*, ScOrc1, ScOrc2 and ScOrc6 contain consensus phosphorylation sites, and these proteins are phosphorylated by the cyclin dependent protein kinase Cdk1(Cdc28)/Clb1 during the G1 to S transition (Nguyen et al., 2001). ScORC proteins are hyperphosphorylated during the S to M transition, and then hypophosphorylated during early G1-phase when pre-RC assembly occurs. Genetic alterations of ScOrc proteins that prevent their phosphorylation do not affect either DNA replication or cell division. However, when this alteration is accompanied by alterations in both Cdc6 and Mcm proteins that prevent their export from the nucleus, then DNA replication is reinitiated prior to cell division, resulting in polyplloid cells. In other words, all three regulatory pathways must be inactivated in order to induce reinitiation of DNA replication within a single cell division cycle.

A similar phenomenon exists in *S. pombe*. SpOrc2 undergoes dephosphorylation during the M to G1 transition (Lygerou and Nurse, 1999; Kong and DePamphilis, 2001), and is hyperphosphorylated when cells enter S-phase and Mcm proteins are released from chromatin (Vas et al., 2001; Wuarin et al., 2002). Furthermore, the mitotic B type cyclin
Cdc13 complexed with the Cdk1(Cdc2) protein kinase associates with replication origins during S-phase and remains there during G2 and early M-phases (Wuarin et al., 2002). This association is ORC-dependent, apparently by association of Cdk1(Cdc2) with the Orc2 subunit, and prevents reinitiation of DNA replication before mitosis has been completed. These data strongly suggest that the phosphorylated state of SpOrc2 determines ORC activity.

8.1. Comparison of yeast and frogs

The ORC cycle in yeast resembles that in Xenopus egg extract when sperm chromatin in the substrate (Fig. 2). ORC proteins in both species are hyperphosphorylated during metaphase by Cdk1, and phosphorylation inhibits its ability to initiate pre-RC assembly. Phosphorylated XlORC binds poorly to chromatin (Romanowski et al., 2000), while pre-RC assembly is inhibited by Cdk1 dependent phosphorylation of ScORC (Nguyen et al., 2001) or by association of SpORC with Cdk1 (Wuarin et al., 2002). Whether or not phosphorylation of XlORC subunits, like those in yeast, also occurs during DNA replication is not clear. Whether or not yeast ORC, like XlORC, can be selectively eluted at lower salt concentrations following pre-RC assembly or DNA replication also is not clear. Dephosphorylation of the Orc2 subunit occurs during the M to G1 transition in Xenopus (Sun et al., 2002) and in yeast (Nguyen et al., 2001; Vas et al., 2001), presumably as an activation step in pre-RC assembly. Phosphorylation of ORC may alter its affinity for chromatin or for other pre-RC proteins during the G1 to S transition, while chromatin composition may determine whether the modified ORC subunits are remain bound or are released. Interestingly, yeast chromatin, like Xenopus sperm chromatin, lacks a classical histone H1 linker (Landsman, 1996), suggesting that reduced chromatin condensation may facilitate binding of ORC during S-phase.

9. The ORC cycle in flies

Direct analyses of cell cycle dependent changes in the activity, post-translational modifications or chromatin affinity of DmORC are not yet available. However, several observations are consistent with a cell cycle dependent, differential association of DmORC proteins with chromatin. In Drosophila, Orc2 is distributed fairly homogeneously in interphase nuclei, while it only remains bound to the heterochromatin region of chromosomes through mitosis in embryos and larval neuroblasts (Pak et al., 1997; Loupart et al., 2000). Mutations in Orc2 exhibited delayed entry into S-phase as well as delayed exit from metaphase with some euchromatic regions replicating even later than heterochromatin (Loupart et al., 2000). In addition, mitotic chromosomes were irregularly condensed, suggesting a novel role for ORC in chromosome architecture as well as DNA replication. In contrast to Orc2, Orc1 levels change dramatically throughout Drosophila development, and its accumulation is regulated by E2F-dependent transcription (Asano and Wharton, 1999). In embryos, Orc1 accumulates preferentially in proliferating cells, and in the eye imaginal disc, Orc1 accumulation is cell cycle regulated, with high levels in late G1 and S phase. Moreover, overexpression of Orc1 altered the pattern of DNA synthesis, implicating Orc1 in regulating initiation of DNA replication.

The DNA binding activity of purified DmORC is largely non-specific and ATP-independent, although DmORC, like ScORC, requires only the ORC1 component of the complex to bind ATP for tight DNA interactions (Chesnokov et al., 2001). In vivo, DmORC does bind to specific genomic sites (Austin et al., 1999; Bielinsky et al., 2001). Such site-specific DNA binding may require association with other proteins (Bosco et al., 2001; Beall et al., 2002), and these associations may regulate ORC activity as well.

10. Mechanisms that regulate ORC activity

The results described above reveal at least five mechanisms by which ORC activity is regulated during cell proliferation.

10.1. Ubiquitination

Since both Orc1 and Ub-Orc1 are rapidly released from chromatin when hamster cells enter S-phase and Ub-Orc1 is largely absent from mitotic cells, ubiquitination per se is not likely the cause of Orc1 release, but a mechanism to prevent its reassociation with ORC/chromatin sites during S-phase. Two phenomena suggest that Ub-Orc1 may become sequestered in the nuclear membrane. First, several examples have been reported where monoubiquitination is required for endosomal sorting of proteins into membrane vesicles (Hicke, 2001; Haglund et al., 2002). Second, a protein called SUMO that is very similar in structure to ubiquitin conjugates proteins with a single adduct and this modification generally results in the protein’s relocation to the nucleus (Wilson and Rangasamy, 2001). Some cells, such as HeLa and other cancer cells, may contain larger pools of Orc1 that cause it to be exported to the cytoplasm where it is polyubiquitinated and degraded. In fact, differences in pool sizes could account for the observation that Cdc6 is exported from the nucleus and degraded in human cells but not in hamster cells (Okuno et al., 2001).

10.2. Phosphorylation

Phosphorylation of Orc proteins can affect both their affinity for chromatin and their activity. In yeast, Cdk1/cyclin B prevents pre-RC assembly until mitosis has been completed by phosphorylating one or more of the ORC subunits and Cdc6. In frogs, both Orc1 and Orc2 are hyperphosphorylated in metaphase-arrested eggs relative to
activated eggs (Carpenter and Dunphy, 1998; Tugal et al., 1998). Moreover, XIORC can be selectively released from chromatin by incubating chromatin either in a metaphase extract (Rowles et al., 1999) or with Cdk1(Cdc2)/cyclin A (Hua and Newport, 1998; Findeisen et al., 1999). In mammals, hyperphosphorylation of Orc1 has been detected in metaphase cells (Tatsumi et al., 2000; Thome et al., 2000), and over-expression of cyclin A results in export of Orc1 to the cytoplasm, a process that is dependent on the phosphorylation status of Cdk target sites in Orc1 (Laman et al., 2001). Given the fact that Cdk2/cyclin A interacts with HsOrc1 (Mendez et al., 2002) and Cdk1/cyclin A interacts with XIORC (Romanowski et al., 2000), cyclin dependent phosphorylation may be the mechanism that releases ORC proteins from chromatin during the G1 to S transition, and the released protein then may be exported to the cytoplasm where it undergoes polyubiquitination and degradation.

10.3. DNA replication

DNA replication appears to be required to destabilize Orc1 in mammalian cells, because significant amounts Orc1 are not released from chromatin in cells arrested at their G1/S interphase (Li and DePamphilis, 2002). Therefore, Orc1 release in mammals may be triggered by DNA synthesis through the origin region. This hypothesis is supported by data from yeast and flies. Orc1, Orc4, and Orc5 from yeast and flies can each bind ATP, and the same ORC subunits in all other eukaryotes contain potential ATP binding sites [reviewed in (Bell, 2002)]. Moreover, the affinity of both DmORC (Chesnokov et al., 2001) and ScORC (Klemm et al., 1997) for DNA increases by binding of ATP to the Orc1 subunit. ATP binding to ScOrc1 is required for site-specific binding of ScORC to origin DNA. The rate of ATP hydrolysis is then reduced in response to origin binding, suggesting that ATP is not hydrolyzed until a subsequent step in replication. That step appears to be the generation of single stranded DNA at replication origins. ScORC binds to ssDNA, but its affinity for ssDNA is dependent only on ssDNA length, not on either DNA sequence or ATP binding. In fact, association of ScORC with ssDNA stimulates ATP hydrolysis, and stabilizes an altered ORC structure (Lee et al., 2000). In yeast, this altered ORC structure may be locked in place by phosphorylation of ORC subunits and thereby prevent subsequent pre-RC assembly. In mammals, this altered ORC structure may cause selective release of Orc1.

10.4. Pre-replication complex assembly

In Xenopus egg extract, assembly of pre-RCs triggers a change in the affinity of ORC for chromatin that is manifested differently, depending on the chromatin substrate. In the case of somatic cell chromatin, the entire ORC is released under DNA replication conditions. In the case of sperm chromatin, the entire ORC becomes salt-sensitive.

10.5. Auxiliary protein interactions

Interactions between ORC and other proteins can facilitate pre-RC assembly (Fig. 1). For example, HsOrc1 binds Cdc6 (Saha et al., 1998), and Cdc6 facilitates binding of XIORC to somatic cell chromatin (Sun et al., 2002) and ScORC to its cognate replication origin (Mizushima et al., 2000). Moreover, ScCdc6 specifically recognizes the ATP-bound state of ScOrc1 (Klemm and Bell, 2001). Therefore, Orc1 may recruit Cdc6, which is present during M-phase, and this Orc1/Cdc6 complex may then target specifically one of the many Orc2–6 complexes that are bound to various sites along the genome (the 'Jesuit Model' for site selection, (DePamphilis, 1993, 1996, 1999).

Interactions between ORC and other proteins can also prevent pre-RC assembly. For example, Skp2, a component of the mammalian ubiquitination system, binds to Orc1 and is required for Orc1 ubiquitination during S-phase (Mendez et al., 2002). Association of ORC/chromatin sites with Cdk1/cyclin B during mitosis in S. pombe prevents assembly of pre-RCs (Wuarin et al., 2002). Sic1, a specific inhibitor of Cdk5, prevents the S-phase-specific Cdk1/Cln5 protein kinase in S. cerevisiae from interacting with ORC, but does not prevent the G1–phase specific Cdk1/Cln2 kinase from binding to ORC (Weinreich et al., 2001). Therefore, Sic1 can prevent Cdk1 activation of pre-RCs during the G1 to S-phase transition, without also preventing subsequent inactivation of pre-RC assembly by the same protein kinase during the S to M-phase transition.

11. Regulating initiation of DNA replication by regulating ORC activity

One can envisage at least four advantages to regulating ORC activity. The first is to prevent assembly of pre-RCs during S-phase by inactivating the premier step in their assembly. By applying this strategy to newly assembled ORC/chromatin sites as well as to those sites where initiation has already occurred, initiation of DNA replication is restricted to once-per-origin-per-cell division cycle. Moreover, the assembly of pre-RCs cannot begin again until mitosis is complete and a nuclear membrane has been reassembled. To this end, mammals have taken the more sophisticated approach. Since Cdc6 binds specifically to Orc1, selective inactivation of Orc1 would directly prevent recruitment of Cdc6 to ORC/chromatin sites without disturbing the genomic locations of the remaining chromatin bound Orc proteins.

A second advantage is to provide the metazoa with a mechanism for selecting which of the many potential initiation sites along the genome will be activated ['Jesuit Model', (DePamphilis, 1993, 1996)]. A core complex
consisting of Orc(2–5) or Orc(2–6) could be assembled at many potential initiation sites, but which of these sites is activated during each round of cell proliferation would be determined by the binding either of Orc1 or of an Orc1/Cdc6 complex. This would help to resolve the problem of preventing DNA replication from interfering with DNA transcription as the pattern of gene expression and chromatin organization changes during animal development. The ORC cycle would allow some replication origins to be inactivated while activating others. A dramatic example of this is the transition from non-specific selection of initiation sites in embryos undergoing rapid cell cleavages to site-specific initiation in cells of the same animal undergoing a normal mitotic cell cycle (Hyrien et al., 1995; Sasaki et al., 1999).

Third, the ORC cycle offers an excellent opportunity for checkpoint control mechanisms. For example, cells that have sustained DNA damage, or that have entered a quiescent or terminally differentiated state could prevent activation of the pathway leading to S-phase by inactivating one or more ORC subunits. Human cells treated with adriamycin, a DNA damage-inducing agent, do, in fact, selectively degrade Orc1 (Mendez et al., 2002).

Finally, the existence of multiple mechanisms for regulating initiation of DNA replication serves to prevent terminally differentiated cells from reentering their cell proliferation cycle. Cells entering a quiescent state have been shown to lose their ability to establish pre-replication complexes at specific genomic sites (Wu and Gilbert, 1997) and their ability to bind Cdc6 and Mcm proteins to their chromatin (Madine et al., 2000; Sun et al., 2001). Moreover, terminally differentiated cells lack critical proteins required for assembly of pre-replication complexes (Lu et al., 1999). While the mechanisms involved have not yet been elucidated, destabilization of one or more ORC subunits, followed by the sequestration of Ub-Orc or the destruction of [Ub]n-Orc1 may well trigger a cascade of events that shuts down cell proliferation pathways.

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