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Cell Cycle Control of DNA Replication

Bruce Stillman

The initiation of DNA replication in eukaryotic cells is a highly regulated process that leads to the duplication of the genetic information for the next cell generation. This requires the ordered assembly of many proteins at the origins of DNA replication to form a competent, pre-replicative chromosomal state. In addition to this competent complex, at least two cell cycle regulated protein kinase pathways are required to affect a transition to a post-replicative chromosomal state. Protein kinases required to establish mitosis prevent re-replication of the DNA. As cells exit mitosis, the cell cycle is reset, allowing the establishment of a new, competent replication state.

The transmission of genetic information from one cell generation to the next requires the accurate duplication of the DNA during the S phase of the cell cycle and the faithful segregation of the resultant sister chromatids during mitosis. In most eukaryotic cells, these two events are normally dependent on each other and thus the replication of the genome and mitosis occur in alternative, oscillating cycles. The molecular mechanisms that determine how DNA replication is initiated, how it is restricted to S phase, and how replication occurs only once per cell cycle in most eukaryotic cells have become major areas of attention. In this review, recent progress in these exciting areas is discussed. More detailed reviews on these issues can be found elsewhere (*1*, *2*).

The groundwork for understanding the control of DNA replication came from cell fusion experiments (*2*, *3*). Cells were synchronized at various stages of the cell cycle, then fused, and the marked nuclei were maintained to direct DNA replication and mitosis. For example, when a cell in the G1 phase of the cell cycle was fused to a cell in S phase, the G1-derived nucleus immediately initiated DNA replication, much earlier than if the cell had not been fused (Fig. 1). Other cell fusions demonstrated that G2 cells could not activate G1 nuclei, nor could G2 nuclei initiate DNA replication when fused to S-phase cells.

The cell fusion experiments revealed three important phenomena. First, only chromosomes from G1 cells are competent to initiate DNA replication. Second, S-phase cells, but not cells in G1 or G2, contain an activator of initiation of DNA replication that can work on the competent (G1) chromosomal state. Third, G2 nuclei do not re-replicate DNA until they pass through mitosis. The key goals of current research are to understand the molecular nature of the competent state and how is it established; the nature of the activator or activators present in S-phase cells; what prevents G2 nuclei from re-replicating, and how the competent state is erased during mitosis. This review focuses on these issues, primarily through discoveries in yeast that have general relevance to control of DNA replication in all cells.

Initiation: Replicators and Initiators

A key starting point to understanding the cell cycle controls that are imposed on the process of DNA replication is the origin of DNA replication. In eukaryotes, just as in bacteria, the location of the origin of DNA replication is determined by cis-acting DNA sequences (the replicator element in the DNA) and a trans-acting protein (the initiator protein) that binds to the replicator (*4*–*6*). Eukaryotic chromosomes are too large to replicate from a single origin and so contain multiple origins, more than are actually needed to replicate each chromosome (*7*). Although best understood at the present time in the yeast *Saccharomyces cerevisiae*, replicators and potential initiator proteins are beginning to be characterized in a wide variety of eukaryotes (*4*, *7*, *8*–*18*). In *S. cerevisiae*, replicators consist of multiple functional DNA elements, only one of which is essential (A) (*19*–*22*). Adjacent to the essential element are two or three functionally conserved DNA elements (B1, B2, and B3) that, although not individually essential, are necessary for initiation and influence the frequency with which an origin is used (*19*–*22*). The A, B1, and B2 elements form the core of the replicator and bind essential DNA replication proteins, whereas the B3 element functions as a replicator enhancer by binding a protein called autonomously replicating sequence (ARS)–binding Factor 1 [Abf1p, (*23*)].

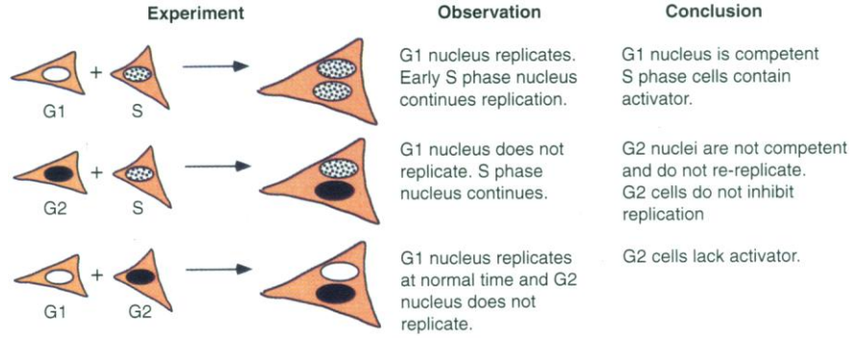

Fig. 1. Cell fusion experiments. Human HeLa cells that had been synchronized at different stages of the cell cycle were fused and the fate of the marked nuclei was followed. Data from (*3*).

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An important advance was the discovery of the eukaryotic cell initiator protein, a multi-subunit protein called the origin recognition complex [ORC (*24*)] that binds to the A and B1 elements in *S. cerevisiae* replicators (24–26). ORC contains six polypeptides that are all essential for cell division and for the initiation of DNA replication (*24–29*). ORC serves as a landing pad for protein-protein interactions that are regulated during the cell cycle and is thus one of the focal points for replication control.

The structure of replicators and the mechanisms that determine origin location in eukaryotes other than *S. cerevisiae* appear to be more complicated, but some general trends are emerging. It is clear that initiation is sequence specific because in diverse organisms such as fission yeast, Physarum, insects, and mammals, origins are localized to discrete regions of chromosomes (*4, 9, 10, 12, 14, 16, 18, 30, 31*). Although there is still some uncertainty about how localized the start sites of DNA replication are in mammalian cells, initiation generally occurs in intragenic regions. In contrast to the small, discrete (100 to 200 bp) origins in *S. cerevisiae*, initiation zones, the regions where initiation takes place, have been reported that range from 0.5 to 50 kilobase pairs of DNA in mammalian chromosomes (*4, 5, 14*).

Studies of DNA replication in *Xenopus* embryos revealed that the zone where replication initiates changes during development (*32*). In the very early embryonic cell divisions, initiation occurs over a broad region in the ribosomal DNA (rDNA) locus, but during the mid-blastula transition, this zone becomes restricted. This switch may reflect a reduction in maternal stores of ORC and other initiation proteins and global changes in chromosome structure. A similar phenomenon may occur during every cell cycle in mammalian cells. When very early G1-phase nuclei from human cells were incubated in a *Xenopus* egg extract, initiation near the dihydrofolate reductase (DHFR) gene was dispersed, however, when nuclei from cells in late G1 were similarly incubated, initiation was more localized (*33*).

Initiation can also occur on reconstituted chromatin in extracts from activated *Xenopus* eggs, but the initiation is not localized to a specific DNA region (*34*). But paradoxically, initiation of DNA replication requires the *Xenopus* ORC (*35–37*). One possibility is that in the early embryo, high concentrations of maternally inherited ORC can interact with DNA sequences that occur quite frequently in the genome because the chromatin structure that is established in the early embryo does not restrict ORC binding to many potential binding sites. Later during development, ORC may be restricted to specific chromosomal regions by higher order chromatin structure, necessitating a dependence of origins on other chromosomal elements such as enhancers. Even within a region that is activated by these chromosomal elements, ORC may still have multiple sites to select and this might explain why in a population of mammalian cells, initiation can occur in a broad initiation zone (*4, 5*). Because an active origin can suppress the activity of a nearby potential origin (*38*), in a single cell the site of initiation within any given zone is probably unique and determined by the location of ORC on the DNA.

Apart from *S. cerevisiae*, in only a few organisms have the DNA sequences that are required for origin function been characterized in detail by genetic analysis. In the fission yeast *Schizosaccharomyces pombe*, the replicators are longer, of the order of 500 to 1000 bp, and as in the budding yeast, are located in inter-genic regions (*9, 10*). In mammals, the human β-globin locus has been the focus of intense study because of the existence of chromosomal deletions in thalassemia patients (*31, 39*). Two non-overlapping deletions that remove sequences either near the promoter for the β-globin gene or sequences that cover the locus controlling region (LCR) eliminate the origin located within the β-globin locus (*31, 39*). The latter observation is interesting because the LCR controls the developmentally regulated activation of the β-globin locus that is required for gene expression and possibly DNA replication. This suggests that quite diverse DNA sequence elements can control the positioning of origins of DNA replication to specific sites within chromosomes, even elements that affect large chromosomal domains.

Two States of the Replication Complex

Early studies of the budding yeast demonstrated that a non-nucleosomal chromatin structure existed in origins of DNA replication (*40*). Furthermore, the chromatin structure at two chromosomal origins changes during the cell cycle (*41*). Extension of these observations by high resolution analysis of the chromatin structure at origins of DNA replication has greatly contributed to our understanding of cell cycle control of DNA replication (*42, 43*). Genomic footprinting at the nucleotide level reveals a pattern that is remarkably similar to the footprint obtained by ORC on naked DNA, suggesting that ORC is bound to replicators throughout the cell cycle (*24, 42*). The pattern of the footprint, however, changes throughout the cell cycle, with a nuclease hypersensitive site in the B1 region changing most obviously (*43*). The hypersensitive site is present in the S, G2, and early M (before anaphase) stages of the cell cycle, but disappears as cells exit mitosis or in early G1. Moreover, the appearance of the site requires the Cdc7p protein kinase.

These studies support the idea that there are at least two states of the chromosome during DNA replication. One state is determined by binding of a pre-replication complex (pre-RC) at the origin and probably corresponds to the competent state of G1 chromosomes. In *S. cerevisiae*, the pre-RC is established after cells pass through anaphase and the sister chromatids separate (*43*). The pre-RC must be inherited with the chromosomes into the two daughter cells, but because G1 cells lack the S-phase activator, initiation of DNA replication cannot occur. Once initiation does occur, however, it is probable that the pre-RC is disrupted, perhaps by the act of initiation itself, and a second state, the post-RC, which also contains ORC, is established.

It appears that ORC is a landing pad for the assembly of a pre-RC that can be formed only at certain stages of the cell cycle. Thus understanding cell cycle control of initiation boils down to identifying the proteins that form the pre-RC and determining how the pre-RC is activated by cell-cycle regulated activators. Progress in this area has been quite rapid.

Cdc6, cdc18$^+$, and the Replication Complex

The *S. cerevisiae* Cdc6 protein (Cdc6p) is essential for DNA replication and has sequence similarity to the large subunit of ORC (*29, 44*). If cells enter the cell cycle in the absence of the Cdc6p, DNA replication does not occur but the cell cycle continues and cells enter into an abortive, pseudo-mitotic state without duplicating their DNA (*45*). This abnormal situation leads to a reductional anaphase during which the unreplicated chromosomes segregate to the daughter cells. Consequently, each daughter cell ends up with less than a full complement of chromosomes, resulting in cell death. A similar situation occurs in the fission yeast *S. pombe* where the lack of synthesis of the Cdc6p-related cdc18$^+$ protein in late G1 causes cells to enter mitosis in the absence of DNA replication (*46*). These and other studies (*47, 48*) suggest that the Cdc6p and the cdc18$^+$ protein are required for DNA replication, but also that they play a role in ensuring that DNA replication occurs before mitosis.

Both the Cdc6p and the cdc18$^+$ protein can be synthesized in late G1 (*45–47, 49*). The Cdc6p (and perhaps cdc18$^+$) is also

synthesized in late G2, allowing it to assemble on to the pre-RC as cells exit mitosis and enter G1 (*45*). This cell cycle–controlled transcription of the genes is apparently not essential for regulation of DNA replication because constitutive expression of either the Cdc6p or the cdc18$^+$ protein in normal amounts does not result in unrestricted DNA replication (*45*, *46*). Gross over-expression (10- to 20-fold) of the cdc18$^+$ protein in *S. pombe*, however, inhibits mitosis and allows a number of cycles of DNA replication, resulting in polyploidy without nuclear division (*49*, *50*). The over-replication, however, requires very large amounts of the cdc18$^+$ protein.

Both the Cdc6 and the cdc18$^+$ proteins have a very short half-life, on the order of less then five min (*45*, *47*, *49*). When the gene encoding Cdc6p is placed under control of a promoter that can be repressed, the amount of Cdc6p drops rapidly when the gene is turned off (*45*). Using this technology, it has been demonstrated that the Cdc6p is essential for formation of the pre-RC (*52*). Furthermore, Cdc6p can only function to establish the pre-RC during a specific time window, from exit from mitosis until late G1 (*51*). Most interestingly, the window is bracketed by the destruction of the mitotic cyclins at anaphase and the activation of S-phase cyclin (Clb5 and Clb6)–CDK activity after START [the commitment point in the cell cycle to cell division (*51*)]. Thereafter, the pre-RC cannot be formed (Fig. 2).

The most probable scenario is that Cdc6p loads onto the ORC that is bound to the replicator. Indeed evidence for both genetic and physical interactions between ORC and Cdc6p in *S. cerevisiae* or cdc18$^+$ protein in *S. pombe* have been obtained (*15*, *53*, *54*). Moreover, recent studies on the replication of chromatin in a *Xenopus* egg extract demonstrate that the loading of the essential *Xenopus* Cdc6p onto chromatin requires the *Xenopus* ORC (*35*). Thus, an interaction between ORC and Cdc6 determines the formation of the pre-RC. Consistent with this, when the functional amounts of ORC or Cdc6p are lowered compared to the amount present in wild-type cells, as occurs in mutants at the permissive or semi-permissive temperatures, the frequency of firing origins of DNA replication along the chromosomes is lower than normal (*54*).

The MCM Family and Licensing Replication

Studies on the replication of DNA in extracts from *Xenopus* eggs have been valuable for understanding the biochemistry of the initiation of DNA replication because this system reproduces many aspects of the cell cycle control observed in cultured cells. A "licensing factor" model has been proposed on the basis of the above-mentioned cell fusion studies and more recent studies using the *Xenopus* egg extract replication system (*55*). For example, G1 nuclei from human HeLa cells are competent to replicate in a *Xenopus* egg extract, but G2 nuclei are not (*56*). When, however, the G2 nuclei were permeabilized and then repaired, they replicated again to yield tetraploid nuclei. The model posited that replication licensing factors (RLFs) would bind to chromatin when the nuclear envelope was disassembled during mitosis (or in an experimental situation when chromatin was exposed to the egg extract). During interphase, however, when the nuclear envelope was intact, the RLFs would not be able to assemble onto chromatin. The binding to the chromatin of the licensing factor or factors causes the chromatin to become competent for DNA replication, but once initiation of DNA replication has occurred, the licensing factor would be destroyed, thereby preventing re-replication of the DNA. Although the original model may not be entirely correct, many studies have reported results that are consistent with the licensing factor model in general and it remains an important guide (*34*). For example, recent studies designed to test the model have demonstrated links to genetic studies in the yeasts that have pointed to a role for the MCM family of proteins in contributing to the competent state of replication in G1 (*57*–*60*).

The MCM proteins were first identified by genetic studies in *S. cerevisiae* but are now known to be present in all eukaryotes (*58*, *61*). The phenotype of yeast cells that have a mis-sense mutation in any one of these essential genes is a high rate of loss of plasmids (mini-chromosome), or an arrest of the cell cycle consistent with a proposed role in DNA replication. There are six MCM proteins in the yeast *S. cerevisiae* [(*58*, *62*, *63*); MCM2; MCM3; CDC46/MCM5; CDC54/MCM4; CDC47/MCM7; and the yeast genome project has identified a protein that is related to the *S. pombe* mis5$^+$ protein].

The MCM proteins, such as MCM3 and MCM7, are essential for DNA replication in *Xenopus* egg extracts (*35*, *36*, *57*, *59*, *60*, *64*). Furthermore, two activities called RLF-M and RLF-B have been identified that are required to modify or "license" chromatin (*57*). This modified chromatin is then capable of replicating in extracts where

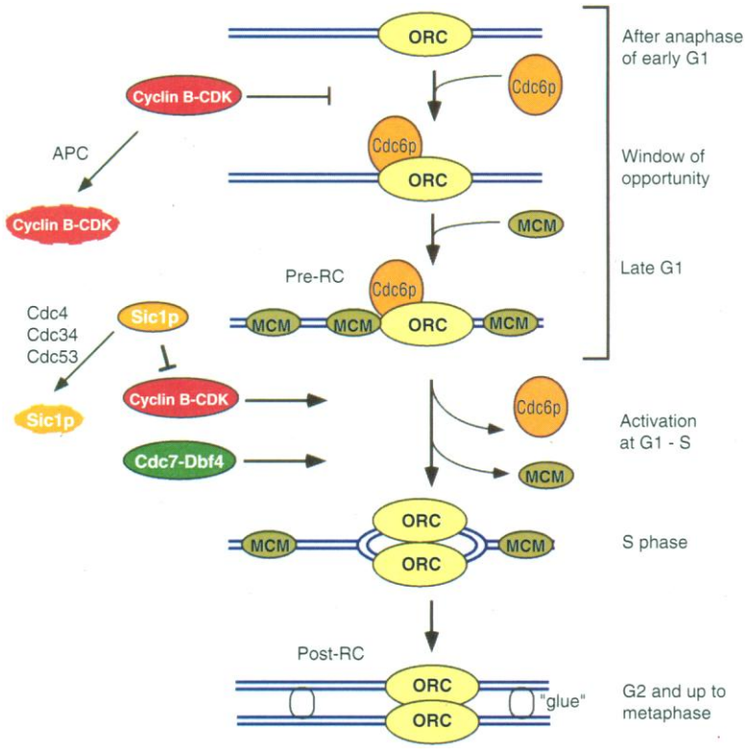

Fig. 2. Proposed model for the chromosome replication cycle in eukaryotes. ORC is bound to the chromosomes throughout the cell cycle. The ordered assembly of the pre-RC containing ORC, Cdc6p, and the MCM proteins occurs during a window of opportunity that is defined by the state of cyclin-CDK activity. Initiation of replication requires cyclin-CDK and Cdc7p-Dbf4p activities. After initiation, a post-RC is formed and cyclin-CDK activity blocks assembly of the pre-RC.

licensing has been blocked by treatment with a protein kinase inhibitor. RLF-M is a complex that contains some (and perhaps all) of the MCM proteins, but the identity of the RLF-B has not been determined (*57*). The MCM proteins interact with each other to form a multi-protein complex (*57*, *59*, *64*), but the subunit composition of the complex has not been determined.

The MCM proteins become bound to chromatin during late mitosis and remain there until they are gradually removed as S phase progresses (*59*, *64*, *65*). This is observed clearly in cultured *Xenopus* or mammalian somatic cells where the MCM proteins localize to the chromatin in the G1 phase of the cell cycle, but not to sites of ongoing DNA replication. These observations are consistent with a role for the MCM proteins in helping to establish a competent state for chromosome replication in the G1 phase. Recently, a pathway for assembly of a competent state for DNA replication has been demonstrated by studies using *Xenopus* egg extracts (*35*–*37*). The interaction of the MCM proteins with a nuclear structure that is probably chromatin is dependent on the *Xenopus* Cdc6p, and the interaction of Cdc6p with chromatin is in turn dependent on the prior association of the ORC with the nuclear structure (Fig. 2). Apart from ORC, however, the assembly of the various protein complexes on to the replicator DNA has not been demonstrated.

CDKs and Cyclins: Regulating Competency

The picture that has emerged recently is that the competent state of G1 chromosomes requires the establishment, during a window of the cell cycle, of a pre-RC, that probably contains ORC, Cdc6p, the MCM complex and other proteins. This window corresponds to the time when the Cdc6p can function to establish the pre-RC (*51*, *52*). It is now clear that active cyclin-dependent kinase (CDK) complexes function both to activate DNA replication and block reassembly of the pre-RC after initiation. Indeed, the state of activity of the mitotic cyclin-CDKs defines the window of opportunity.

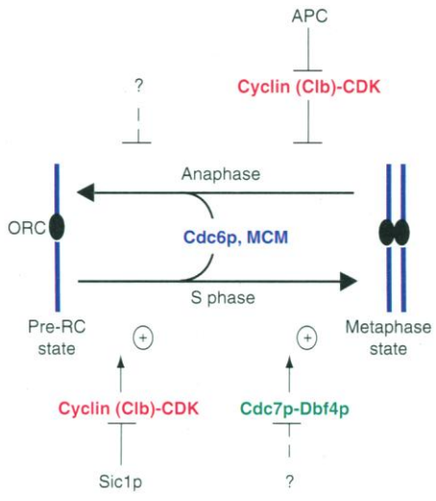

Fig. 3. Oscillating states of the chromosome replication cycle. See text for details. The dashed lines represent an additional pathway for controlling anaphase, or the existence of a pathway that regulates Cdc7p-Dbf4p protein kinase activity for entry into S phase.

The CDK (cdc2$^+$ protein) in *S. pombe* associates with the mitotic cyclin (cdc13$^+$) and kinase activity peaks during late G2 and early mitosis (*66*). Inhibition of the mitotic CDK activity by either inactivation of the CDK itself, by inactivation of the cyclin or by over-expression of the cdc2$^+$-cyclin inhibitor protein rum1$^+$, causes a block in mitosis, but interestingly, under these conditions, the cells enter multiple rounds of DNA replication (*50*, *67*). These observations provide evidence that, in addition to their role in activating mitosis, the mitotic cyclin–CDKs also function to block the initiation of DNA replication. But paradoxically, cyclin-CDK activity is required for the initiation of DNA replication (*66*).

Further insight into the role of the mitotic-like cyclin-CDK complexes has come from studies in *S. cerevisiae*. The S-phase cyclins (Clb5p and Clb6p) are apparently required for the initiation of DNA replication because removing them causes a delay in the onset of S phase (*68*). Without these cyclins, initiation becomes dependent on the true mitotic Clb1 through 4–CDK activities that are present later in the cell cycle. The activity of the Clb5-CDK (and other mitotic cyclin-CDKs) is blocked specifically by the inhibitor Sic1p, which is present in cells from late mitosis until shortly after START (*69*, *70*). Thus it is necessary to get rid of the Sic1p to activate the S-phase cyclin–CDK and allow initiation of DNA replication. This is accomplished by the ubiquitin–dependent proteolysis of Sic1p after START that requires the Cdc34p, Cdc53p, Cdc4p, and Skp1p proteins. Furthermore, the G1-phase cyclin–CDKs (Cln1p-Cdc28p, Cln2p-Cdc28p) that respond to cell growth signals are essential for marking Sic1p for degradation (*70*–*73*).

After DNA replication, the Clb-CDK complexes block the establishment of the pre-RC and hence re-initiation of DNA replication (*74*). This may be accomplished by the binding of the Clb-CDK complexes either to the Cdc6 (or Cdc18$^+$) proteins, to the ORC, or to both (*15*, *17*, *50*, *51*). Consistent with these observations, if the Sic1p was expressed in the G2 phase of the cell cycle for a period of time, then the pre-RC could be established because cyclin-CDK activity was suppressed (*74*). Strikingly, when the Sic1p was then removed from these G2 cells, initiation of DNA replication occurred without cells progressing through mitosis. Now the G2 nucleus was rendered competent for DNA replication! Deletion of the Sic1p CDK inhibitor is not lethal, but the initiation of DNA replication becomes uncoupled from other cell cycle events such as cell budding (cytokinesis) and the initiation of DNA replication occurs very soon after cell exit from mitosis (*72*).

The cyclins and CDKs appear to have multiple roles in the control of the chromosome replication cycle. They ensure coordination of S phase with other cell cycle events by; first, activating DNA replication; second, preventing re-replication by blocking formation of the pre-RC in G2; and third, allowing establishment of metaphase. By performing these multiple functions, they ensure that mitosis occurs after the DNA has been replicated only once per cell cycle. After metaphase has been established correctly, chromosome separation occurs and the cycle is reset when the mitotic cyclins are degraded by the anaphase promoting complex (APC) and ubiquitin dependent proteolysis (*73*). For an unknown reason, mutations in either the APC proteins or a ubiquitin hydrolase can cause over-replication of the genome (*75*).

Cdc7-Dbf4: Another Pathway?

The initiation of DNA replication also requires the activity of another protein kinase, Cdc7p-Dbf4p (*76*–*79*). This kinase may represent a cyclin-CDK independent regulatory pathway that activates the competent G1 state. Mutations in the *CDC7* gene block the initiation of DNA replication even though the cells have passed through START and Clb-CDKs are activated. This gene product encodes a protein kinase whose activity depends on Dbf4p and fluctuates throughout the cell cycle, being activated at the G1- to S-phase boundary (*78*, *80*, *81*). The Cdc7 protein kinase is conserved in *S. pombe* and mammals (*82*). The targets of the Cdc7p-Dbf4p protein kinase are not known, but the MCM proteins have been implicated as candidates (*61*). There is genetic evidence that Cdc7p interacts with ORC and that Dbf4p interacts with the origin of DNA replication (*28*, *83*). The Dbf4p also interacts with the Cdc5p, another protein kinase that participates in the control of late mitotic events (*84*). This latter observation raises another potential link between the

initiation of DNA replication and the separation of sister chromatids during M phase.

Oscillating States of S and M

Once initiation of DNA replication has been accomplished, the control over this process does not stop. As discussed in the accompanying review (*85*), there are checkpoint controls that oversee the progression through S phase and link the completion of S phase to entry into M phase. These controls may require some of the above mentioned proteins.

For the chromosome replication cycle in eukaryotes, there are two important states that need to be established; one is the competent state for initiation of DNA replication (pre-RC) and the other is the alignment of the chromosomes at the metaphase plate during mitosis (Fig. 3). The cell cycle regulatory machinery ensures that both states cannot be established at the same time. Moreover, there are two separate transitions that occur during the cell cycle; first is the transition from the pre-RC at individual origins of DNA replication to a post-RC–a transition that leads to new sister chromatids–and second, the metaphase-to-anaphase transition that causes separation of the duplicated chromatids (horizontal arrows, Fig. 3). Again, these transition events cannot occur at the same time. This situation has been likened to the two states of a piston in a reciprocating steam engine (*51*).

The competent G1 replication state cannot be established when the factors that are necessary to establish metaphase are active in the cell (such as the mitotic CDK-cyclins). Logic would predict that the reciprocal might be the case, that metaphase cannot be established when factors necessary to form the pre-RC are present. Perhaps the Cdc6-cdc18^{+} proteins or RLF-B fit this bill. Superimposed on this simple picture are the two states of the mitotic cyclin–CDK kinase activity. When the activity is on, transition from the pre-RC to the post-RC can occur and metaphase can be established. But activation of a CDK-cyclin is not sufficient for replication to occur and another activating pathway, the Cdc7p-Dbf4p protein kinase pathway, is necessary. On the other hand, when the mitotic CDK-cyclin activity is off, the pre-RC can be established and exit from mitosis can occur. But the metaphase-to-anaphase transition still needs another pathway that leads to the degradation of the sister-chromatid "glue" that holds the sister chromatids together (*73*, *86*). The degradation of Pds1p in *S. cerevisiae* or the cut2^{+} protein in *S. pombe* represent components of this pathway.

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Cell Cycle Checkpoints: Preventing an Identity Crisis

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Cell cycle checkpoints are regulatory pathways that control the order and timing of cell cycle transitions and ensure that critical events such as DNA replication and chromosome segregation are completed with high fidelity. In addition, checkpoints respond to damage by arresting the cell cycle to provide time for repair and by inducing transcription of genes that facilitate repair. Checkpoint loss results in genomic instability and has been implicated in the evolution of normal cells into cancer cells. Recent advances have revealed signal transduction pathways that transmit checkpoint signals in response to DNA damage, replication blocks, and spindle damage. Checkpoint pathways have components shared among all eukaryotes, underscoring the conservation of cell cycle regulatory machinery.

The cell cycle is a collection of highly ordered processes that result in the duplication of a cell. As cells progress through the cell cycle, they undergo several discrete transitions. A cell cycle transition is a unidirectional change of state in which a cell that was performing one set of processes shifts its activity to perform a different set of processes. A current focus of cell cycle research concerns how these transitions are coordinated to occur at a precise time and in a defined order. In principle, the ordering of cell cycle events could be accomplished by requiring the next event to physically require the completion of the previous event, much like building a house—the roof cannot go up until the walls are built. This has been referred to as a substrate-product relationship (*1*). Alternatively, dependency could be established by positive or negative regulatory circuits, and this appears to be the predominant mechanism. An example of a pathway of cell cycle events that is subject to positive and negative control is shown in Fig. 1A. A negative circuit is shown leading from b to a step in the d to e pathway. A positive circuit, shown linking events b and c, cannot be easily distinguished from a substrate-product relationship and depends upon the biochemical function of the step in question. These regulatory circuits are surveillance mechanisms that monitor the completion of critical cell cycle events and allow subsequent cell cycle transitions to occur. There are two classes of regulatory circuits, termed here intrinsic and extrinsic. Intrinsic mechanisms act in each cell cycle to order events. Extrinsic mechanisms are induced to act only when a defect is detected. Both mechanisms may use the same components to enforce cell cycle arrest. An example of how some of these circuits are integrated into a typical cell cycle is shown in Fig. 1B. These pathways are of considerable interest because their loss leads to reduced fidelity of cell cycle events such as chromosome duplication and segregation. Such alterations decrease the reproductive fitness of unicellular organisms and in multicellular organisms may lead to uncontrolled proliferation and cancer.

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Checkpoint is the name given to a particular subset of these intrinsic or extrinsic mechanisms (*1*). A checkpoint is a biochemical pathway that ensures dependence of one process upon another process that is otherwise biochemically unrelated. A null allele in a checkpoint gene results in a loss of this dependency and, thus, checkpoints are inhibitory pathways. This definition of a checkpoint is broad and can apply to many situations that occur in multicellular organisms, particularly during development. However, its most common usage is in reference to control of cell cycle transitions. The word checkpoint conjures visions of both a place (a border) and a process (examination) and this duality has led to some confusion. The word is often used in a manner suggesting that checkpoints are points in the cell cycle or are cell cycle transitions, but the usage is best restricted to refer to the biochemical pathway that ensures dependency. For example, the DNA-damage checkpoint is the mechanism that detects damaged DNA and generates a signal that arrests cells in the G1 phase of the cell cycle, slows down S phase (DNA synthesis), arrests cells in the G2 phase, and induces the transcription of repair genes. The position of arrest within the cell cycle varies depending upon the phase in which the damage is sensed. Whether the loss of a checkpoint has an immediate consequence for an organism during a normal cycle depends on the particular pathway and the inherent timing of the processes themselves. Timing and checkpoints can act as redundant controls to ensure the proper order of events. Thus, there are no constraints on whether checkpoints are essential or inducible (extrinsic).

The first indications that the cell cycle was not controlled strictly by a substrate-product relationship came from cell fusion experiments in *Physarium polycephalum* that showed that timing of mitotic entry could be influenced by the ratio of the nuclear volume to cytoplasmic volume (*2*). Similar experiments with mammalian cells showed that when cells in S and G2 phases of the cycle were fused, the G2 nucleus delayed mitotic entry until the S-phase nucleus finished DNA replication; then both nuclei synchronously entered mitosis (*3*). This was interpreted to mean that S-phase nuclei produced an inhibitor of mitosis. The first example of a dependency relationship relieved by mutation was from bacterial studies. DNA damage and certain mutations cause a block to septation resulting in filamentation of *Escherichia coli* (*4*), and mutations in the *recA*, *lexA* and *sulA*(*sfiA*) genes relieve this septation block (*5*). SulA is an inhibitor of septation induced in response to DNA damage as part of the SOS response (*6*) controlled by *recA* and the repressor *lexA*. In eukaryotes, cells from hu-