Chromosoma (1996) 105:197–203

CHROMOSOMA
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Chromosoma Focus

Cell cycle control of S phase: a comparison of two yeasts

Joel A. Huberman

Department of Molecular and Cellular Biology,
Roswell Park Cancer Institute, Buffalo, NY 14263, USA
(e-mail huberman@acsu.buffalo.edu)

Received: 22 August 1996 / Accepted: 25 August 1996

Abstract. The mechanisms responsible for correct timing of DNA synthesis within the cell cycle and for limiting replication to one round per cell cycle are basically similar in the two model yeasts, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, despite many differences in detail. In both cases, the timing of initiation and the prevention of additional rounds are controlled by the activity levels of B-type cyclins. These similarities are likely to extend to other eukaryotic organisms.

Introduction

How initiation of DNA synthesis is regulated in the cell cycle – so that each chromosome is replicated once during each S phase – is a fascinating problem. Recent progress in this field has led to significant advances in our understanding, but it has also produced the potential for confusion owing to the sheer abundance of details being discovered, details that are frequently specific to a single organism. The purpose of this brief review is to sift through the details of S phase regulation in two model eukaryotic organisms, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, in order to separate out important conserved features and also to identify gaps in our understanding of this process in one or both yeasts. Although this review is specific for *S. cerevisiae* and *S. pombe*, it is likely that the features found to be conserved between these two yeasts are also present in other eukaryotic organisms.

To maximize brevity, I have minimized references to older or well-established papers. The missing references can be found in the bibliographies of the papers that I have cited here. I have been greatly aided in putting together this summary by recent reviews written by other authors (Diffley 1995; Muzi-Falconi et al. 1996b; Wuarin and Nurse 1996).

The points I shall discuss are summarized in Figs. 1 and 2, which show the key pathways involved in S phase regulation in *S. cerevisiae* and *S. pombe*, respectively. In these figures, the cell cycle phases are indicated on the outer black circle. The inner yellow and red circle indicates the type of protein complex present at replication origins (see next section). The cell cycle proportions should not be considered accurate. However, they are sufficiently realistic to make apparent the fact that G1 and S phase in *S. pombe* occupy a smaller portion of the cell cycle than in *S. cerevisiae*. Proteins and pathways of similar structure or function in the two yeasts are shown with matching colors in the two diagrams.

In this review all gene names are italicized, but protein names are not. I have named genes according to the conventions employed by *S. cerevisiae* and *S. pombe* geneticists. All dominant (usually wild type) *S. cerevisiae* gene names are in upper case, but recessive genes (usually mutants) are in lower case. All *S. pombe* gene names are in lower case. Specific proteins are named after the genes that encode them. Thus, the names of specific *S. cerevisiae* proteins are capitalized, while those of *S. pombe* proteins are lower case. The names of generic proteins (not named after specific genes) begin with a capital letter, followed by lower case.

Formation of prereplicative complexes

The presence during G1 of a large protein complex at replication origins was first suggested by chromatin

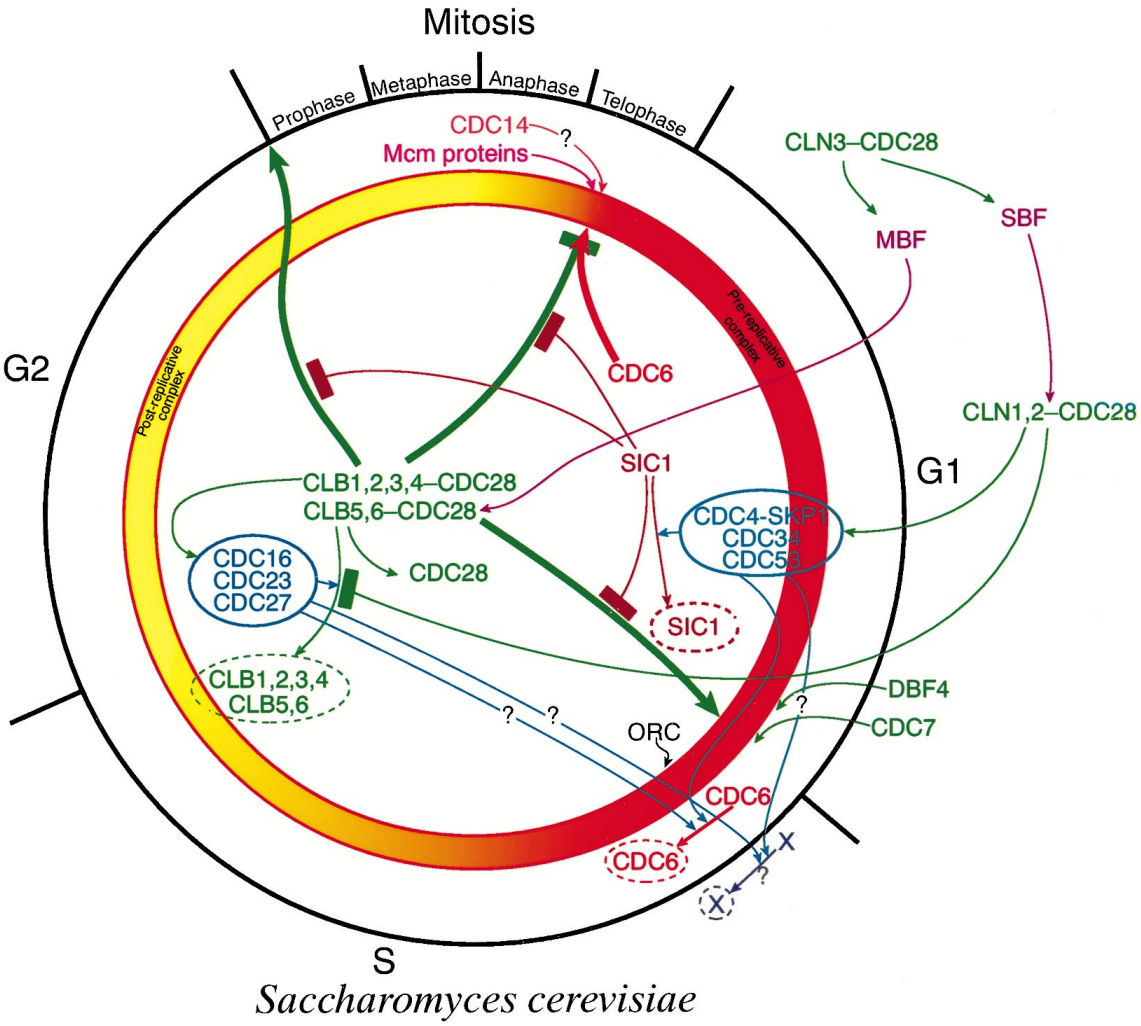

Fig. 1 Cell cycle regulation of DNA replication in *Saccharomyces cerevisiae*. The *black outer circle* represents the phases of the cell cycle. The *inner circle* represents changes in the proteins bound to a typical replication origin during the cell cycle. *Red* indicates that the origin is bound by a "prereplicative complex" (pre-RC), consisting of ORC plus additional proteins (see text). *Yellow* indicates the postreplicative state, when only ORC binding is detectable. In the remainder of the diagram, each group of proteins enclosed in a *solid oval* is thought to be part of a multi-protein complex. Proteins enclosed in *dashed ovals* are degraded by proteolysis. *Arrowheads* indicate stimulation; *small solid rectangles* indicate inhibition. *Thicker lines* indicate processes that appear to be more important or more fundamental for replication control within the cell cycle

footprinting studies from the Diffley laboratory (Diffley et al. 1994; Diffley 1995; Cocker et al. 1996), which showed that the proteins bound at *S. cerevisiae* replication origins change during the cell cycle. For the origins studied, which are thought to fire near the beginning of S phase, the footprint during the bulk of S phase, during all of G2, and during the pre-anaphase stages of mitosis is very similar to the footprint produced in vitro by purified origin recognition complex (ORC) binding to naked origin DNA (Diffley et al. 1994). However, from late mitosis until the G1/S transition, the footprint is broader, with fewer hypersensitive regions, suggesting that additional proteins bind at this time (Diffley et al. 1994). The time during which the suggested "prereplicative complex" (pre-RC) is present is indicated by the red portion of the inner circle in Fig. 1. The yellow portion represents the time when the "postreplicative complex," which may consist simply of ORC, is present. Abundant evidence, discussed below, suggests that establishment of pre-RCs is an essential step in the initiation process.

The CDC6 protein, which is required for replication, is essential for formation and maintenance of pre-RCs (Diffley 1995; Cocker et al. 1996). CDC6 is synthesized in late mitosis and early G1; another burst of synthesis takes place near the G1/S boundary in cells with prolonged G1 phases, and CDC6 disappears during S phase (Piatti et al. 1995).

It is not yet known whether pre-RCs exist in *S. pombe*, but since a key S-phase regulatory protein in *S. pombe*, the cdc18 protein, is similar in structure to CDC6, it seems likely that pre-RCs are also present in *S. pombe*. Unlike CDC6, however, cdc18 is not synthesized until mid-G1, after the cdc10-sct1 transcription factor has been activated (Nishitani and Nurse 1995; Muzi-Falconi et al. 1996a). Consequently, if cdc18 is essential for formation of pre-RCs in *S. pombe*, as it is in *S. cerevisiae*,

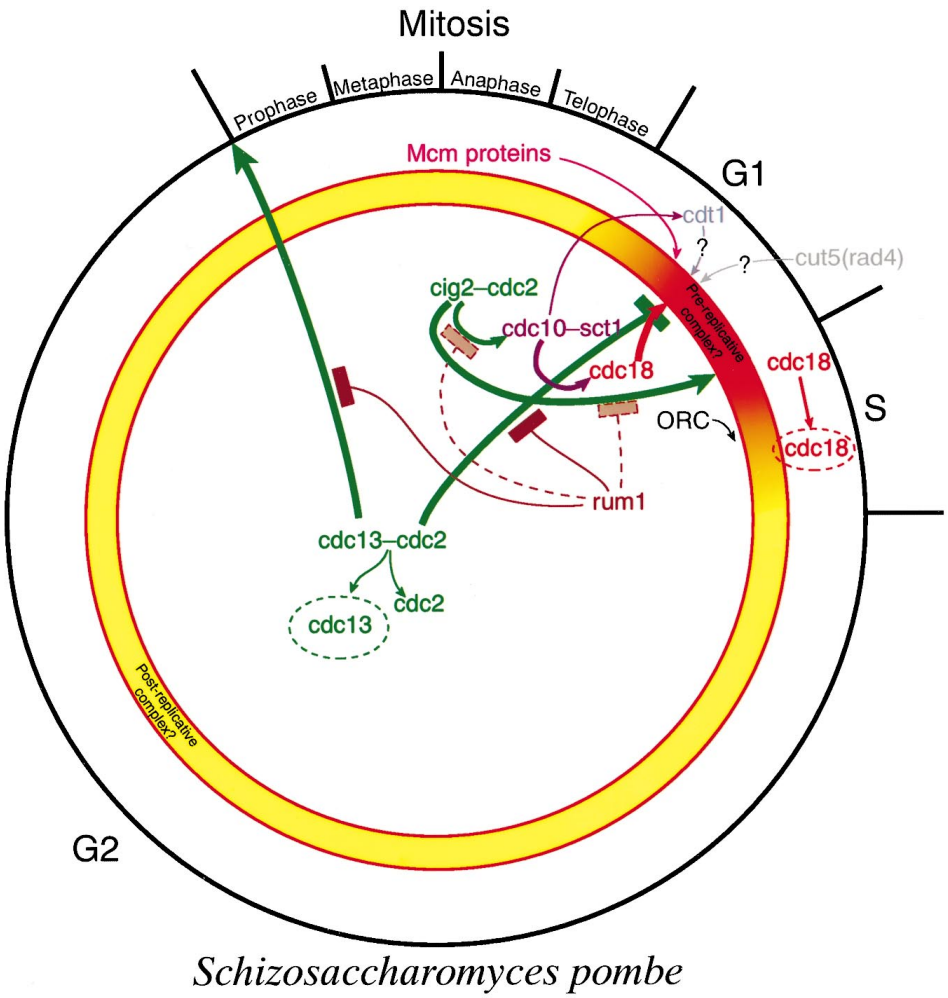

Fig. 2 Cell cycle regulation of DNA replication in *Schizosaccharomyces pombe*. The symbols have the same meanings as in Fig. 1. Proteins with functions similar to *S. cerevisiae* proteins are shown with the same colors as the corresponding *S. cerevisiae* proteins (see Fig. 1)

then formation of *S. pombe* pre-RCs would not be completed until mid-G1 (fully red portion of the inner circle in Fig. 2). Like CDC6, cdc18 is unstable and disappears during S phase (Nishitani and Nurse 1995; Muzi-Falconi et al. 1996a), and so, presumably, do *S. pombe* pre-RCs.

In the absence of cdc18 activity, the *S. pombe* checkpoint that normally inhibits mitosis until replication is complete does not function, and cells go through a lethal mitosis with 1N DNA content (the "cut" phenotype) (Kelly et al. 1993). This behavior could be explained if pre-RCs are required to generate the signal that replication is incomplete; in the absence of cdc18, pre-RCs could not be made, and no signal indicating incomplete replication would be generated (Kelly et al. 1993). Two additional proteins – cdt1 (Hofmann and Beach 1994) and cut5 (also called rad4) (Fenech et al. 1991; Saka and Yanagida 1993; Saka et al. 1994) – are known to share with cdc18 the property of being essential both for replication and for generation of the incomplete replication checkpoint signal. It is possible that one or both of these proteins may also be involved in formation and/or maintenance of pre-RCs.

The six members of the Mcm protein family appear to be essential for initiation in both yeasts (reviewed in Kearsey et al. 1996). *Xenopus* homologues of these proteins are components of the machinery that "licenses" replication, limiting it to one round per cell cycle. In *S. cerevisiae* and in animal cells, Mcm proteins bind to chromatin during G1 phase, roughly the period when pre-RCs are present (reviewed in Kearsey et al. 1996), suggesting that Mcm proteins might be components of pre-RCs. However, the large stoichiometric excess of Mcm proteins over pre-RCs in *S. cerevisiae* cells suggests that, even if Mcms are components of pre-RCs, they may in addition bind to other chromatin regions (Diffley 1995). Chromatin binding of *S. pombe* Mcm proteins has, to my knowledge, not yet been directly tested. Since at least one of the *S. pombe* Mcm proteins is present in the nucleus in early G1 prior to cdc10-sct1 activation (Maiorano et al. 1996), it is possible that, if *S. pombe* Mcms are components of pre-RCs at origins, they may bind to origins independently of cdc18 in late mitosis and early G1 (as suggested by the gradient from yellow to red, from late mitosis to early G1, in Fig. 2).

Other G1 events

During G1 a decision is made concerning whether to continue with preparations for replication and cell divi-

sion or to exist from the cell cycle. In this review, I shall not deal with that important decision. Instead, I shall assume that the decision has been made to continue cycling – to replicate DNA and then divide – permitting me to focus on the events in G1 that culminate in the initiation of DNA synthesis.

In both yeasts, early preparations for replication (presumably formation and/or maintenance of pre-RCs) are inhibited by high concentrations of a cyclin-dependent kinase (Cdk: CDC28 in *S. cerevisiae* and cdc2 in *S. pombe*) complexed with a B-type cyclin (CLB1–CLB6 in *S. cerevisiae*; cdc13 in *S. pombe*). The B cyclins are present at very high concentrations during early mitosis. Their destruction by targeted ubiquitin-mediated proteolysis during anaphase permits the formation of pre-RCs. B-cyclin proteolysis in *S cerevisiae* depends on a protein complex that contains CDC16, CDC23 and CDC27. In *S. cerevisiae*, at least, the potential for B-cyclin proteolysis continues until late G1 (Amon et al. 1994). In both yeasts, an inhibitor specific for B cyclins (SIC1 in *S. cerevisiae* and rum1 in *S. pombe*) also helps to keep the Cyclin B-Cdk activity low during most of G1.

At the end of G1 Cyclin B-Cdk activity must rise, because it is required for triggering initiation of replication. Consequently, both yeasts have developed mechanisms that permit Cyclin B-Cdk activity to increase in late G1. In *S. cerevisiae*, and presumably also in *S. pombe*, targeted B cyclin proteolysis is turned off. However, the two yeasts deal with their Cyclin B-Cdk inhibitors in different ways. In *S. cerevisiae*, the same G1 cyclins that inhibit B-cyclin proteolysis also stimulate SIC1 proteolysis. In *S. pombe*, a B-type cyclin is produced that is relatively resistant to rum1 inhibition. Additional G1 events also differ between the two yeasts. Therefore, it is helpful to keep in mind, as one reads more about the details of G1 events in these two yeasts in the next few paragraphs, that the goals of these G1 events are the same: to keep Cyclin B-Cdk activity low in early G1 and to permit it to rise in late G1.

During early G1 phase in *S. cerevisiae*, the G1 kinase, CLN3-CDC28, promotes the synthesis of two transcription factors, MBF and SBF, each of which stimulates the transcription of a set of cell-cycle-regulated genes (Dirick et al. 1995). MBF activates many genes, including the genes encoding the CLB5 and CLB6 cyclins. Note that there are 6 B cyclins in *S. cerevisiae*, CLB1–CLB6. Under normal conditions, CLB5 and/or 6 are responsible for activating DNA synthesis, but when the genes encoding these two cyclins are deleted, any of the remaining 4 B cyclins can play the role (Schwob and Nasmyth 1993; Schwob et al. 1994).

Among the genes activated by SBF are those encoding the G1 cyclins, CLN1 and CLN2. After binding to CDC28 to generate active kinases, CLN1 and CLN2 have two effects important for progression into S phase: (1) they inhibit the targeted proteolysis of B cyclins mediated by CDC16, CDC23 and CDC27 and (2) they stimulate the targeted proteolysis of SIC1 by CDC4-SKP1, CDC34 and CDC53 (Dirick et al. 1995; Bai et al. 1996). The net result of all these actions is an increase in the level of CLB5,6-CDC28 kinase activity sufficient to permit initiation of DNA replication.

A major difference between G1 in *S. cerevisiae* and G1 in *S. pombe* is the nature of the cyclins that drive progression through G1. Although all cyclins are structurally related, the *S. cerevisiae* G1 cyclins form a subfamily of their own, distinct from the B cyclin subfamily. In contrast, the *S. pombe* cyclins capable of promoting passage through G1 – cig2, cig1 and cdc13 – are members of the B cyclin subfamily. Although cig2 is the B cyclin normally responsible for initiating replication, that role can also be assumed by cig1 or cdc13 (which are not normally present in high concentration until G2) (Fisher and Nurse 1996; Mondesert et al. 1996). Another major difference is the specificity of the Cyclin B-Cdk inhibitor. Whereas SIC1 of *S. cerevisiae* appears to inhibit all Cyclin B-Cdk complexes, rum1 of *S. pombe* can inhibit cdc13-cdc2 completely, cig2-cdc2 only partially, and cig1-cdc2 not at all (Correa-Bordes and Nurse 1995).

The cig2-cdc2 kinase accumulates during G1, activating the cdc10-sct1 transcription factor, which stimulates transcription of the *cdc18* gene among others. Eventually, it accumulates sufficiently to overcome partial inhibition by rum1 and to stimulate initiation of replication (Jallepalli and Kelly 1996; Martín-Castellanos et al. 1996; Fisher and Nurse 1996; Mondesert et al. 1996).

Triggering replication

In *S. cerevisiae*, the actions of at least two protein kinases, CDC7 and CLB5,6-CDC28, are required in late G1 to trigger initiation of replication. CDC7 is not a member of the family of cyclin-dependent kinases (Cdks), but, like the Cdks, to become active it must associate with a protein partner, DBF4, whose abundance is cell-cycle regulated and is greatest in late G1 (Chapman and Johnston 1989; Jackson et al. 1993). CDC7 activation also requires phosphorylation by an as yet unindentified kinase, possibly a Cdk (Yoon et al. 1993).

Evidence from a 1-hybrid assay suggests that the DBF4 protein associates with replication origins (Dowell et al. 1994). Presumably it does so in late G1, when it is most abundant, and subsequently recruits CDC7 to origins. The critical substrate(s) of the CDC7 kinase have not yet been identified, but are likely to be proteins present at origins.

The other kinase whose action is essential to trigger S phase is the Cdk, CDC28, associated with a B-type cyclin. As in the case of CDC7-DBF4, the critical substrate(s) of the CLB5,6-CDC28 kinase have not yet been identified.

The importance of CLB5,6-CDC28 action for progression into S phase is underscored by the fact that, in the presence of normal amounts of the Cyclin B-CDC28 inhibitor, SIC1, there is insufficient residual CLB5,6-CDC28 activity to permit initiation of replication. For this reason, proteolysis of SIC1 at the end of G1 is essential for initiation of replication (Schwob et al. 1994).

The order, relative importance and interdependence of the CLB5,6-CDC28 and CDC7-DBF4 phosphorylat-

ions are not yet known. Under normal conditions, both are essential. However, a cellular mutation, *bob1*, has been discovered in which the requirement for CDC7-DBF4 is eliminated (Jackson et al. 1993). In this mutant, phosphorylation by CLB5,6-CDC28 is still required (Jackson et al. 1993), suggesting that phosphorylation by CLB5,6-CDC28 may act downstream of CDC7-DBF4.

Whether homologs of CDC7 and DBF4 are required to initiate replication in *S. pombe* is not yet known. Interestingly, in *S. pombe* overexpression of cdc18 appears to bypass both the known requirement for cig2-cdc2 and the possible requirement for homologs of CDC7 and DBF4, because it permits rereplication without an intervening mitosis (Nishitani and Nurse 1995; Muzi-Falconi et al. 1996a). In fact, overexpression of cdc18 permits uncontrolled reinitiation of replication even in the absence of protein synthesis (Nishitani and Nurse 1995), suggesting that cdc18 function (perhaps abnormal owing to overexpression) is sufficient for a very late step in the overall process of initiation.

S phase events

During S phase in *S. cerevisiae*, pre-RCs are converted to postreplicative complexes, apparently owing to loss of most or all proteins bound to the origin except for ORC (Diffley et al. 1994). Because all origins whose footprints have been examined to date are ones that fire in early S phase, it is not yet clear when this conversion takes place for late-firing origins. It seems likely that the conversion will prove to take place at the time during S phase when the origin fires. For that reason, the transition from pre-RCs to post-RCs is shown as a gradual transition (red to yellow) during S phase in Figs. 1 and 2.

Since most or all origins are associated with ORC during S phase (Diffley and Cocker 1992; Diffley et al. 1994), it is likely that new ORC binds to new origins within S phase, shortly after origin replication.

Resetting the clock

During S, G2 and early M phases, high levels of Cyclin B-Cdk kinase inhibit the formation of new pre-RCs and prevent rereplication. In *S. cerevisiae*, targeted proteolysis of the B cyclins takes place in anaphase and is mediated by the evolutionarily conserved "anaphase promoting complex (APC)," which contains CDC16, CDC23, CDC27 and perhaps additional proteins (Irniger et al. 1995; King et al. 1995; Zachariae and Nasmyth 1996). The resulting dramatic reduction in cyclin-Cdk activity is important for resetting many aspects of the cell cycle, not just those relating to DNA replication.

Overreplication

In both yeasts, mutations leading to loss of Cyclin B-Cdk activity during G2 permit rereplication of the genome, in some cases for multiple rounds, without an intervening mitosis. The types of mutations that permit such unregulated replication include certain Cdk mutations that appear to affect interactions with B cyclins (Broek et al. 1991), deletion of the gene(s) encoding the major B cyclins (Hayles et al. 1994), and inhibition of Cyclin B-Cdk complexes (Correa-Bordes and Nurse 1996; Dahmann et al. 1995).

Thus, high levels of Cyclin B-Cdk are needed to prevent initiation of replication during G2 and M phases. This suppression of abnormal initation may well be mediated by interactions between Cyclin B-Cdk and cdc18/CDC6, which prevent the formation of, or destabilize, pre-RCs. This conclusion is based on the observations that: (1) pre-RCs can be formed in G2/M if B cyclins are inhibited (Dahmann et al. 1995), (2) CDC6 and cdc18 are in vitro substrates for Cyclin B-Cdk (Piatti et al. 1996; Jallepalli and Kelly 1996), (3) inhibition of Cyclin B-Cdk leads to massive accumulation of cdc18 (Jallepalli and Kelly 1996), and (4) Cyclin B-Cdk physically associates with CDC6 (Piatti et al. 1996).

Based on these observations, it is tempting to speculate that one of the actions of Cyclin B-Cdk complexes may be to bind to and/or phosphorylate CDC6/cdc18, thereby simultaneously (1) rendering CDC6/cdc18 active for an essential, perhaps final, S phase promoting function and (2) rendering it incapable of forming pre-RCs, perhaps even marking it for destruction by proteolysis.

Although this simple speculation may ultimately prove to be correct, several results suggest that what is actually going on may be more complicated. One such result is the observation that overproduction of CDC6 appears incapable of inducing reinitiation without an intervening mitosis (Piatti et al. 1996), whereas, as mentioned above, overproduction of cdc18 in *S. pombe* is sufficient to induce reinitiation (Nishitani and Nurse 1995; Muzi-Falconi et al. 1996a). In fact, CDC6 is capable of stimulating the formation of pre-RCs only from late mitosis to a point in late G1 that roughly corresponds to the time when Cyclin B-Cdk activity becomes high enough to permit initiation of replication. When CDC6 expression is induced in later cell cycle stages, pre-RCs are not formed and DNA synthesis is not initiated (Piatti et al. 1996). It is not yet clear whether this apparent difference between the consequences of cdc18 and CDC6 overproduction reflects major differences between the functions of the two proteins or is simply a consequence of quantitative differences between the two yeasts in the efficiency with which Cyclin B-Cdk inhibits CDC6/cdc18 function.

Another result suggesting possible complications is the finding that mutants in two *S. cerevisiae* genes encoding components of APC, *CDC16* and *CDC27*, overreplicate their DNA despite the continued presence of high levels of Cyclin B-Cdk (Heichman and Roberts 1996). This overreplication resembles the overreplication induced in *S. pombe* by cdc18 overproduction (Nishitani and Nurse 1995; Muzi-Falconi et al. 1996a) and is distinct from the overreplication induced in *S. pombe* (Broek et al. 1991; Hayles et al. 1994; Correa-Bordes and Nurse 1995) or *S. cerevisiae* (Dahmann et al. 1995)

by reduction of Cyclin B-Cdk activity, in the sense that the overreplication resulting from these APC mutations does not produce discrete genome doublings (Heichman and Roberts 1996). Consequently, an attractive hypothesis (Heichman and Roberts 1996; Wuarin and Nurse 1996) is that APC activity is required for targeted proteolysis of CDC6 during S phase and that, in the absence of such proteolysis, CDC6 accumulates and, like its cousin, cdc18 in *S. pombe*, stimulates new initiation events. This hypothesis may ultimately prove to be correct, but several obstacles stand in the way of its immediate acceptance: (1) intentional overexpression of CDC6 does not appear capable of inducing overreplication (Piatti et al. 1996); (2) there is no evidence that APC is involved in CDC6 proteolysis; and (3) there is evidence that a different set of proteins – CDC4, CDC34, and CDC53 – *is* involved in CDC6 proteolysis (Piatti et al. 1996). Each of these obstacles may prove surmountable, but, until then, it is necessary to consider alternative possibilities as well. For example, an additional protein ("X" in Fig. 1) may be capable of triggering overreplication in the presence of high Cyclin B-Cdk activity, and the degradation of this protein by APC after initiation of replication may be essential for preventing overreplication in the normal cell cycle.

Many pathways leading to targeted proteolysis, including both the APC pathway and the CDC4, CDC34, CDC53 pathway, involve ubiquitination of the targeted proteins. Perhaps it is not surprising, then, that a mutation in the *DOA4* gene, which encodes a ubiquitin hydrolase, also induces over-replication (Singer et al. 1996). Identification of the critical substrates of DOA4, CDC16 and CDC27 is a crucial next step in figuring out what is going on. Are all three proteins involved in aspects of CDC6 proteolysis? Or are they involved in hydrolysis of different substrates (X in Fig. 1) that are, perhaps, unrelated in function to CDC6?

For all the pathways leading to overreplication described in this section, it would be interesting to know which of the steps required for normal replication are also required for overreplication. For example, does the overreplication resulting from loss of CDC16 function require CDC7 function? Does the overreplication induced by cdc18 overexpression require a moderately high level of Cyclin B-Cdk activity (similar to the level normally required for triggering S phase)? Do any of these overreplications require Mcm proteins?

What is licensing?

Since the time it was proposed in 1988, the "licensing" hypothesis of Blow and Laskey (Blow and Laskey 1988) has helped to shape our thinking about the mechanisms involved in limiting replication to a single round per cell cycle. These investigators suggested that, during passage through mitosis a substance necessary for replication (i.e., a "license" for replication) gains access to chromatin, that this license is destroyed by the subsequent act of replication itself, and that this license cannot be regenerated until the next passage through mitosis. Advances since 1988 in our understanding of replication control in *S. cerevisiae* and *S. pombe*, reviewed above, suggest that the licensing hypothesis is basically valid but can now be restated in a more sophisticated fashion: passage through mitosis reduces Cyclin B-Cdk activity sufficiently to permit formation of pre-RCs, which are essential for replication; preRCs are destroyed by replication; and the high level of Cyclin B-Cdk prevalent during G2 and early M phases prevents their reformation.

Recent experiments with *Xenopus* extracts have implicated the Mcm proteins in licensing (reviewed in Kearsey et al. 1996). It is to be hoped that future yeast experiments will contribute to clarification of the role of the Mcm proteins in this process.

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