Fenner Conference

Cell cycle control of chromosomal DNA replication

STEPHENV DALTON

Department of Biochemistry, University of Adelaide, Adelaide, South Australia, Australia

Summary Accurate replication and segregation of chromosomal DNA is essential for high-fidelity transmission of genetic information from generation to generation. Eukaryotic cells typically replicate by first duplicating their chromosomes during the S phase followed by their segregation between two daughter cells during the M phase. Over recent years, advances in our understanding of this process at the molecular level have been incredibly rapid. The present review will focus on molecular control of DNA replication and the mechanisms which operate to ensure that once replicated, chromosomes are not rereplicated in the same cell cycle.

Key words: cell cycle, DNA replication, S phase.

Introduction

Chromosomal DNA replication occurs during a phase of the cell cycle known as the S phase. Although the length of the S phase can vary significantly between different cell types, several absolute controls exist that dictate when and how many times in a cell cycle DNA replication can occur. First, chromosomes must be duplicated once and only once in each cell cycle. This obviously implies that once chromosomes have been replicated, further duplication in the same cell cycle is not permitted. In only a few exceptional circumstances can this rule be breached. For example, Drosophila polytene chromosomes and polyploidy in mouse embryonic trophectoderm arise from multiple rounds of DNA replication without an intervening mitosis. It is now clear that hard-wired mechanisms operate that ensure that before a cell can re-replicate its chromosomal DNA, it must first pass through mitosis. This ensures that replicated chromosomes are segregated to daughter cells by establishing an interdependency between the S and M phases. The emerging picture is one of a tight interdependency between the S phase and the M phase, such that each phase cannot proceed until being preceded by the other. This ‘S–M’ control ensures that general cell cycle events are maintained in the correct order and that genome transmission occurs faithfully from generation to generation. In recent years our understanding of these processes has advanced very rapidly and a molecular explanation for the interdependency between the S and M phases has emerged.

Saccharomyces cerevisiae and more complex metazoans such as Xenopus laevis, and mammalian cells in culture. The requirement that a cell must first pass through the M phase before it can replicate its DNA was demonstrated in cell fusion experiments by Rao and Johnson.1 These experiments essentially showed that after the S phase, activities in G2- and M-phase cells precluded further rounds of DNA replication and that passage through the M phase was required to reverse the inhibition and to re-establish a replication-competent state. The significance of this at the molecular level is discussed later in this review. Similar controls operate that prevent entry into mitosis until DNA has been completely replicated. This is generally referred to as the DNA replication checkpoint2 and ensures that cells do not segregate unreplicated chromosomes, once more emphasizing the tight interdependency between chromosome replication and segregation during the cell cycle.

The Xenopus egg extract system has been particularly valuable in defining the relationship between passage through the M phase and the re-establishment of replication-competency in the ensuing cell cycle. The replication licensing factor (RLF) model emerged from Ron Laskey’s laboratory3,4 as a way to explain the obligatory coupling between the S and M phases. These studies implied that breakdown of the nuclear envelope during the M phase was necessary in order to ‘license’ chromatin for replication. This was convincingly demonstrated by showing that replicated G2 nuclei (those which had already undergone an S phase and contained replicated DNA) from HeLa cells, in the presence of Xenopus egg extract, could re-replicate their DNA if they were permeabilized by treatment with mild detergents. According to the RLF model, as the nuclear envelope disassembles during each mitosis, a fresh supply of cytosolic ‘licensing activity’ would gain access to chromatin, placing it in a replication-competent state. This adequately explains the requirement that a cell has to pass through the M phase before replicating DNA. In order to accommodate the problem that replication occurs only once in each cell cycle, it was predicted that RLF activity...
was either inactivated or destroyed immediately after initiation of replication, thereby blocking further rounds of replication in the same cell cycle. Hence, the RLF model explained how the interdependency between the S and M phases could be established, and second, how replication could be restricted to once in each cell cycle. Until recently, the exact biochemical activity underlying the RLF phenomenon was poorly understood. Recent advances made by researchers working on the replication problem in *Xenopus* and *Saccharomyces* have given us a good understanding as to how this process is controlled.

Initiation of chromosomal DNA replication occurs at specific origins

The first and most crucial level of control which determines when and how many times in a cell cycle DNA replication occurs, lies at the initiation step. Up until recently, remarkably little was known about the control mechanisms that influence initiation of chromosomal DNA replication in eukaryotes. This contrasts with the vast amount of information available on the replication of other genetic elements in eukaryotes such as DNA tumour viruses, in particular SV40. This problem has been largely solved due to analysis of DNA replication in *Saccharomyces*, where specific initiation sites (replication origins) have been identified, each generating a single bi-directional replication fork in each S phase. The strategy of initiating chromosomal DNA replication from a sequence-specific origin was of course established many years ago in bacteria, but because the size of eukaryotic chromosomes is considerably larger, a higher level of complexity is inevitably involved. Even so, features of initiation in eukaryotes are likely to be similar to those which have been shown to exist in the bacterial 'replicon'. Such features include origin recognition by regulatory proteins, localized DNA unwinding and recruitment of replication enzymes.

Between 250 and 300 origins are thought to operate in a normal *Saccharomyces* S phase, although not all origins are synchronously activated. In fact, there is significant disparity regarding when replication forks emerge from different origins. This allows some origins to be designated as 'early' firing, and others as 'late' firing. The factors determining when in the S phase an origin fires are not so much inherent to the origin itself but to the position of the origin within a chromosome. 'Late' firing origins are often associated with telomeric or transcriptionally silent chromatin, but transplacing a 'late' origin to the position of an 'early' origin results in it becoming reprogrammed to an 'early' state. Hence, the timing of origin activation appears to be context dependent and heavily influenced by local chromatin structure.5,6

A detailed analysis of the DNA sequences making up origins has been made, revealing some striking features.7,8 In general, all yeast origins are 100–200 b.p. in length and contain a degenerate 11-b.p. element [5'(A/T)TTFA(T/C)(A/G)TTTA(T/A)T] known as the ARS consensus sequence (ACS). This element is absolutely required for origin activity and is found in all yeasts origins characterized to date. A B-element, located 3' to the ACS, is also required to make up a functionally active chromosomal origin of replication. B-element DNA sequences are quite variable, but generally consist of either two or three sub-elements (B1, B2, and B3), which can be functionally redundant. Recruitment of transcription factors (Abf1 being one example) may be important for B-element function *in vivo*.9 Alone, the ACS or B-elements are not sufficient for origin activity and must act in combination to be functionally active. It is helpful to consider replication origins as being structurally and functionally modular, a feature reminiscent of transcriptional promoters where each module serves as a docking site for regulatory proteins. A similar scenario appears to operate in the control of origin function.

The molecular nature of replication origins in metazoans has yet to be fully established, although information generated in yeast should accelerate understanding in this area. A number of trends are emerging which suggest that although initiators in metazoans are likely to be more complex than in yeast, the general principles will be similar. It is still unclear, for example, if metazoans use short, genetically definable, origins of replication (as in *Saccharomyces*) or use more elaborate mechanisms. One possibility that has arisen from studies on the amplified dihydrofolate reductase (DHFR) locus in Chinese hamster ovary (CHO) cells is that replication initiates from a broad zone containing several potential origins which act in concert.10 The same may also be true for the initiator associated with the β-globin gene, thus making genetic characterization of initiators generally difficult. Other reports focusing on an initiation zone associated with the human lamin B2 gene, however, suggest that some origins may be in the order of 100 b.p. in length.11

Entry into the S phase requires an S phase Cdk activity

The cell cycle can be described as a series of biochemical events orchestrated by a family of protein kinases, known as cyclin-dependent protein kinases (Cdk). The Cdk control the timing and order of cell cycle phases and are clearly important in ensuring that events such as the S phase and M phase occur in the correct temporal order. As with other phases of the cell cycle, a phase-specific Cdk activity is required for entry into the S phase. The Cdk activity necessary for the S phase in *Saccharomyces* consists of Cdc28p (the Cdk catalytic subunit) and one of two B-type cyclin subunits (Clb5,6p).12 Activation of the S phase Cdk in budding yeast, and probably other eukaryotes, requires ubiquitin-mediated proteolysis of an associated inhibitor molecule (in *Saccharomyces* this is Sic1p).13 Following proteolysis of Sic1p, the S phase Cdk is switched on and then activates substrates required for initiation of replication (S phase entry). Some of these substrates have been tentatively identified and will be discussed later.

Although the S phase Cdk is activated at the beginning of the S phase, it does not remain active throughout the replicative phase. Other waves of Cdk activity, associated with B-type cyclins, are activated as the Clb5,6p-dependent kinase is switched off. These Cdk activities persist throughout the remainder of the cell cycle and eventually collapse as cells exit mitosis (Fig. 1). Because initiation of replication from ‘late’ firing origins occurs in the absence of
Clb5p, it is likely that other B-type Cdk activities play a role in catalysing late S phase events (elongation, termination and activation of origins). The big dilemma is how replication origins are prevented from being reactivated for a second or third time in the same S phase. This is especially intriguing because the controls that activate origin activity must still be on in late S phase but, nevertheless, refiring of early origins is clearly not permitted. Quite obviously, there must be negative controls that prevent origins firing multiple times.

Cdk prevent multiple rounds of DNA replication in each cell cycle

The problem of how DNA replication occurs only once in each cell cycle has been partially resolved by studies in the fission yeast, *Schizosaccharomyces pombe*, where genetic approaches have been used to study the sequential coupling of the S and M phases. As will be discussed, Cdk are implicated as central regulators.

In fission yeast, the Cdk required for entry into mitosis consists of a Cdk catalytic subunit (Cdc2) and a B-type cyclin regulatory subunit, Cdc13. Inactivation of this kinase is then required to exit mitosis and for entry into G1. Cells with an inactive mitotic kinase fail to enter mitosis but also continue to replicate their DNA multiple times.14,15 This is compelling evidence that Cdk activity plays a role in controlling the frequency of DNA replication within a cell cycle. In normal circumstances, different B-type Cdk activities can be envisioned to inhibit re-replication through the S, G2 and M phases by mechanisms that will be dealt with later. Following completion of the M phase, these inhibitory signals are inactivated, placing the cell in a replication-competent state. These observations are of special significance because they provide a molecular basis for the original observations of Rao and Johnson,1 who described passage through mitosis as being the defining point in re-establishing replication competency.

So far, two important principles of general cell cycle control have been dealt with: (i) the interdependency between the S phase and the M phase; and (ii) that chromosomal DNA replication must be tightly controlled so that it occurs once and only once in each cell cycle. Both of these processes are controlled by the action of Cdk. Having established these principles at the conceptual level, the remainder of the present paper will deal with the molecular basis behind how replication is controlled and the possible ways in which Cdk control initiation of replication.

Assembly of origins into pre-initiation complexes during the G1 phase

Understanding the initiation step has been the key to resolving the question of how DNA replication is controlled...
at the molecular level. Based on our knowledge of bacterial origins of replication, a sensible prediction was that changes in the activity of replication origins during the cell cycle would be controlled by specific origin-binding proteins. John Difley’s laboratory (Imperial Cancer Research Fund, London, UK) began looking at this possibility using in vivo footprinting as a method to detect and characterize protein assemblies which formed over replication origins. This approach led to several key observations. First, the footprint that formed over origins changed in a cell cycle-regulated manner. Although part of the origin (ACS) appeared to be occupied throughout the cell cycle, additional components formed at the origin during G1, then disappeared during the S phase, coinciding with initiation events at specific origins. Quite remarkably, the G1-specific footprint activities did not reappear until cells had passed through the M phase into G1. These were highly significant observations for several reasons. First, it suggested that replication origins become functionally competent during G1 by the recruitment of regulatory proteins (the complex formed prior to initiation during G1 is known as the pre-replicative complex (pre-RC)). Second, the assembly of these complexes during G1 was significant because it coincided with the time when cells become replication competent after the completion of the M phase. Finally, the disassembly of pre-RC provided a way to explain why origins do not fire multiple times in the same S phase. After the initiation event, a single bi-directional replication fork is generated, leaving behind a ‘spent’ origin. In the post-replicative state only the ACS remains occupied but significantly, the origin is incapable of firing again in the same cell cycle. With this information, the origin could be viewed as a docking site for a multiprotein replicative machine, which formed during G1 and following activation during the S phase would be disassembled, thus precluding further initiation events. The key to this regulation is that the pre-RC is prevented from reforming until completion of the M phase. Several questions remained unanswered by this model, however. Even though pre-RC quite clearly formed during the S phase, the trigger mechanism that activated them was undefined and it was also unclear how these complexes disengaged from origins. Moreover, what prevented the reformation of pre-RC throughout the remainder of the S phase and during the G2 and M phases? To answer these questions, a thorough understanding of the events occurring at replication origins was necessary.

The origin recognition complex

The first step in understanding how replication origins function was to gain information on how their activity is controlled at different points of the cell cycle. This initially focused on the identification of proteins that made up the pre-RC. The first component of the pre-RC to be characterized was an activity that appeared to bind the ACS (and part of the B-element) throughout the cell cycle. This activity was purified as a hexameric protein complex consisting of 120, 72, 62, 56, 53 and 50-kDa subunits, designated the origin recognition complex (ORC). Genetic evidence has gone on to show that the ORC is essential for origin activity in vivo. For example, mutations in the gene encoding the 72-kDa subunit, Orc2p, are defective in the initiation of replication. As would be expected, genes encoding subunits of the ORC are all essential for cell viability. Because the ORC remains bound to replicators throughout the cell cycle, it is thought to serve as a docking site for other regulatory factors which assemble at origins during G1.

Cdc6p, MCM and the pre-replicative complex

The CDC6 gene was identified by genetic analysis as being essential for DNA replication in Saccharomyces. A special role for Cdc6p in coordinating the S phase with the M phase was later implied following the observation that when depleted of Cdc6p, cells not only fail to undergo DNA replication but enter mitosis and segregate unduplicated chromosomes. Hence, Cdc6p plays a critical role in ensuring that mitosis does not proceed until DNA replication occurs. Similar observations have been made in fission yeast, where failure to synthesize the Cdc6p-related protein Cdc18p in late G1 causes cells to enter mitosis without first passing through the S phase.

Cdc6p levels are controlled at the level of transcription and protein stability. The CDC6 gene is transcribed during early G1, accounting for periodic accumulation of its mRNA during this period. Disappearance of Cdc6p during the S phase results from its phosphorylation-dependent degradation via ubiquitin-mediated degradation. Failure to degrade Cdc18 in fission yeast results in re-replication of DNA without an intervening mitosis. This again emphasizes the central role of Cdc6p-type proteins in restricting replication to once per cell cycle. The implication therefore is that Cdc6p plays a major role in coordinating the S phase with the M phase. The link between Cdc6p activity, initiation of replication and Cdk activity comes from the observation that proteolysis of Cdc6p requires prior phosphorylation at its N terminus by S phase Cdk activity.

The question of how Cdc6p restricts initiation of replication to once only per cell cycle is now beginning to be answered. Several studies have shown that Cdc6p must execute its role in G1 before the activation of the S phase Cdk activities. This makes sense, because the S phase Cdk trigger Cdc6p degradation, but it does not answer the question of how Cdc6p works. This problem has recently been simplified by experiments demonstrating that Cdc6p assembles into complexes with the ORC by directly binding to replication origins during early G1. Cdc6p in fact appears to be the key element in establishing the formation of functionally competent replication origins during G1 by facilitating the recruitment of other pre-RC factors, including MCM. A later step in pre-RC assembly involves the recruitment of Cdc45p, in what appears to be an S phase Cdk-dependent step. Cdc45p has previously been shown to be essential for initiation of replication by assembly into complexes with MCM; this assembly almost certainly involves Cdc6p. The current picture (Fig. 1) is one where Cdc6p assembles at origins with the ORC, followed by recruitment of MCM proteins, followed by Cdc45p. Hence, assembly of pre-RC complexes occurs in a sequential manner during G1.
Activation of origins and disassembly of pre-RC

How origins are activated following pre-RC assembly clearly involves an S phase Cdk as the trigger, but the exact sequence of events leading to the generation of a replication fork are largely unknown. Besides Cdk, the Cdc7p protein kinase and its regulatory subunit, Dbf4p, are important for origin activation through their activity on Cdc45p and various Mcm7 subunits. In vitro, Cdc7p phosphorylates MCM but the biochemical consequences of this are unclear. The major outstanding question to be resolved is how do the replicative polymerases get recruited to the origin so that elongation can occur? This will be the subject of intense investigation over the next year.

In vivo footprinting has shown us that as an origin ‘fires’ the pre-RC rapidly disassembles, or at least no longer occupies the origin. For Cdc6p, we know this involves its degradation. Presumably this signals MCM and Cdc45p into vacating the origin, perhaps through displacement, destabilization or (as will be discussed) through movement away from the origin with the replication fork. The key events leading to origin activation seem to involve phosphorylation of at least some pre-RC components. For example, phosphorylation of MCM and Cdc45p by Cdk/Cdc7 kinases are likely to be key steps that lead to recruitment of replicative polymerases and DNA unwinding at the origin.

The question of how the origin is prevented from refiring is a separate, but related one. Here it can be envisaged that after the pre-RC is disassembled, it can not reassemble due to the absence of Cdc6p, which does not reappear until passage through the M phase. This would be sufficient to preclude further rounds of initiation because disassembled pre-RC components such as the MCM and Cdc45p would not be re-recruited to replication origins. But what of the other pre-RC components; where do they go and what do they do? Until recently it was generally believed that MCM and Cdc45p dissociated from origins, no longer playing a role of any consequence during the cell cycle. One distinct possibility however, is that MCM and Cdc45p move with the replication machinery as part of the replication fork, suggesting a role in fork elongation. This is a provocative possibility because the MCM are proposed to have an associated helicase activity: a property consistent with them being components of the replication machinery.

Conclusions

Our understanding of eukaryotic chromosomal DNA replication has been accelerated markedly by genetic analysis in yeast. The key to restricting replication to once only per cell cycle lies with the ability to build functionally active replication origins during G1, and on the other hand, having mechanisms in place that allow them to function only once in each S phase. This is achieved by functionally inactivating origins after they have ‘fired’, by phosphorylating components of the replicative apparatus. Re-replication is blocked by persistent negative regulation provided by overlapping waves of B-type Cdk activity, a control reversed by passage through mitosis. This is indeed an elegant strategy by which the S and M phases are coordinated.

An encouraging aspect of this research is the possibility that many of these principles will apply to the control of chromosomal replication in higher eukaryotes. Homologues for almost all of the pre-RC components described in yeast have been identified in mammalian cells. Similar things can be said for Cdk and the general architecture of the cell cycle between all eukaryotes, especially the general aspects such as ‘S–M’ control. Although a complete understanding of replicons/origins has not been established in metazoans, it is likely that lessons learnt from bacteria and yeast will be generally applicable. The lack of functional data in these systems does not allow one to make firm conclusions yet, but it is likely that similar control mechanisms will exist in all eukaryotes and over the next few years a generic description of replication control in all eukaryotes should be available.

References


18 Piatti S, Lengauer C, Nasmyth K. Cdc6 is an unstable protein whose de novo synthesis in G1 is important for the onset of S phase and for preventing a ‘reductional’ anaphase in the budding yeast Saccharomyces cerevisiae. EMBO J. 1995; 14: 3788–99.


