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Once in a lifetime: strategies for preventing re-replication in prokaryotic and eukaryotic cells

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DNA replication is an extremely accurate process and cells have evolved intricate control mechanisms to ensure that each region of their genome is replicated only once during S phase. Here, we compare what is known about the processes that prevent re-replication in prokaryotic and eukaryotic cells by using the model organisms Escherichia coli and Schizosaccharomyces pombe as examples. Although the underlying molecular details are different, the logic behind the control mechanisms is similar. For example, after initiation, crucial molecules required for the loading of replicative helicases in both prokaryotes and eukaryotes are inactivated until the next cell cycle. Furthermore, in both systems the β-clamp of the replicative polymerase associates with enzymatic activities that contribute to the inactivation of the helicase loaders. Finally, recent studies suggest that the control mechanism that prevents re-replication in both systems also increases the synthesis of DNA building blocks.

Introduction

Genomic DNA is organized differently in prokaryotic and eukaryotic cells. The bacterium Escherichia coli contains a single circular chromosome and replication is initiated bi-directionally from a fixed origin (oriC; Fig 1). Consequently, a single initiation event will ensure replication of the entire 4.6 Mbp genome in a process that is completed when the two divergent replication forks collide at the opposite side of the circular chromosome.

In eukaryotic cells, the genomic DNA is distributed between multiple chromosomes that are contained within the nucleus. At S phase, replication is simultaneously initiated from many different origins that are scattered throughout the genome (Fig 1), and replication is completed when all replication forks have either met a convergent fork from an adjacent origin or reached the telomeres at the end of the chromosomes.

In the fission yeast Schizosaccharomyces pombe, many potential replication origins are found in intragenic regions of the 12.5 Mbp genome distributed across three chromosomes. However, only a limited subset of these is used in a given S phase. Furthermore, the specific origins that actually fire vary from one S phase to another, suggesting that origin selection occurs by a stochastic mechanism.

Replicating once, and only once

Despite these differences in organization, both cell types are faced with the challenge of ensuring that the entire genome is replicated once, and only once, in any given S phase. At face value, this problem seems to be different depending on whether cells have a single origin or many scattered ones. However, under optimal nutritional conditions, E. coli cells are able to grow with a doubling time that is much shorter than the time required for replication and segregation of the chromosome (S + G2 phases). Consequently, initiation of replication occurs one, two or even three generations before cell birth, depending on the growth rate (Cooper & Helmstetter, 1968).

Fast-growing cells are therefore born with chromosomes containing several active origins of replication, and such cells are also able to coordinate initiation at multiple—but identical—origins (Fig 1).

In both E. coli and fission yeast, initiation of replication is coupled to cell growth and is triggered by a specific signal that is generated when the cell has obtained a critical mass. Once activated, each replication origin is inhibited from re-firing until the next S phase (see below). Furthermore, in eukaryotic cells, passive replication by an incoming fork also prevents an origin that has not yet fired from firing until the next S phase. Together, these mechanisms ensure that the entire genome is replicated only once in each cell cycle.

Mechanisms of initiation

Initiation of replication in both E. coli and S. pombe occurs by a series of discrete steps. First, the origin recognition complex (ORC) is formed by the recruitment of replication factors to origin sequences. Subsequently, loading of the replicative helicase converts the ORC into a pre-replicative complex (pre-RC)—a process often referred to as ‘licensing’. This paves the way for loading of the polymerase itself; in both organisms, the crucial step seems to be loading of the helicase.
Our understanding of pre-RC formation is largely based on studies of Cdc6, the budding yeast orthologue of Cdc18, but we anticipate that the mechanism in fission yeast is similar. The presence of Cdc18 represents the next step in the initiation process. In fission yeast, the pre-RC stage and the transition to replication proceeds immediately by the loading of two or three DNA polymerase III holoenzymes on the origin (McInerney et al., 2007). When dNTPs are present, replication can then commence (Herrick & Sclavi, 2007).

The spatial arrangement of DnaA protein domains involved in nucleotide binding, DNA binding and oligomerization is similar to the fission yeast initiation factor Cdc18, and it has been suggested that the helical DNA-binding domain could direct similar functioning AAA+ domains to their respective origins (Erzberger et al., 2002). It is worth noting that both DnaA and Cdc18 can switch between active and inactive configurations depending on the nature of the bound nucleotide, and that this molecular switch is one of the determinants for initiation control.

Cascades of initiation

As discussed above, rapidly growing E. coli cells contain many origins of replication that all fire simultaneously (Fig 1), and synchronous initiation presumably results from the release of the DnaA protein from the first initiated origin in a cell. This will momentarily increase the DnaA:oriC ratio for remaining ‘old’ origins and their initiation will follow in a cascade-like manner—known as the initiation cascade (Lobner-Olesen et al., 1994). Eukaryotic cells are faced with a similar problem: origins are selected by a stochastic mechanism, and therefore there is a risk that large chromosomal regions will occasionally remain unreplicated during any given S phase. Analogous to the initiation cascade model, it has been proposed that a crucial replication factor is rate limiting for initiation. As replication proceeds, this factor is released and can be redistributed to other origins, thereby increasing their probability of firing.
et al, 2000). The nature of this factor has not been established, but it would have to be a protein that is not degraded in the initiation process—that is, not Cdt1 or Cdc18 (see below).

**Mechanisms to prevent re-initiation**

In both *E. coli* and *S. pombe*, inactivation of the helicase loader proteins has a crucial role in preventing immediate re-firing of a recently
activated origin. In *E. coli*, the DnaA protein is the target for this regulation, whereas fission yeast cells regulate both Cdt1 and Cdc18. In both systems, inhibition of re-replication is accomplished both by physically preventing pre-RC assembly and by a reduction in the activity of AAA⁺ ATPase proteins. This is mediated by post-translational inactivation of the proteins and by the modulation of gene expression.

**Prevention of pre-RC assembly**

The *E. coli* origin of replication is rich in GATC sites, which is the substrate for the Dam methyltransferase. As methylation is a post-replication process, newly replicated origins are methylated on only one strand (Fig 2). These hemi-methylated origins are bound (sequestered) by SeqA, a protein with high affinity for hemi-methylated GATC sites (Lu et al., 1994). Sequestration renders the origin inaccessible to DnaA for approximately one-third of the generation time to prevent immediate re-initiation (Campbell & Kleckner, 1990; von Freiesleben et al., 2000).

Origin sequestration is instrumental not only in preventing the immediate re-initiation at an origin, but also in preparing the origin for the next round of initiation. During sequestration, the DnaA protein is only able to bind the high affinity sites R1, R2 and R4, to re-set the origin to the ORC stage (Nievera et al., 2006). Although sequestration lasts less than one generation, it ensures that successive initiations at the same origin are separated by a doubling time, because it provides a time window during which the origin cannot be initiated and the amount of DnaA-ATP is reduced to a level that cannot sustain initiation (see below). Consequently, a period of growth is necessary before origins of replication are released from sequestration and can re-initiate.

In eukaryotic cells, the increase in Cdk activity that initiates S phase has an additional function in preventing re-replication during S, G2 and M phases. The importance of this mechanism follows from the observation that G2 cells can be manipulated to follow from the observation that G2 cells can be manipulated to prevent immediate re-initiation (Campbell & Kleckner, 1990; von Freiesleben et al., 2000).

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**Post-translational inactivation of AAA⁺ proteins**

The activity of the DnaA protein is reduced during S phase by a process known as the ‘regulatory inactivation of DnaA’ (RIDA; Fig 2), in which the active ATP-bound DnaA protein is converted to the inactive ADP-bound form by ATP hydrolysis (Katayama et al., 1998). RIDA activity involves two proteins: the DnaA-related protein Hda (Kato & Katayama, 2001) and the β-clamp of the DNA polymerase (Pol III) holoenzyme (encoded by the *dnaA* gene; Katayama et al., 1998). These proteins form a complex even before the clamp is loaded onto the DNA (Kawakami et al., 2006). However, only the DNA-loaded β-subunit of Pol III in complex with the Hda protein stimulates the ATPase activity of DnaA to promote conversion of DnaA-ATP to the inactive DnaA-ADP (Su’etsugu et al., 2004). At the end of the initiation process, hydrolysis of DnaA-ATP by RIDA is accelerated because new replication forks are formed, and more β-clamps are loaded onto the DNA.

In fission yeast, the two helicase-loader proteins Cdc18 and Cdt1 also become inactivated after initiation of replication, but here this is accomplished by physical degradation rather than biochemical inactivation. The increase in Cdk activity that brings about S phase also causes phosphorylation of Cdc18, which targets the protein for SCF-mediated ubiquitination and subsequent degradation by proteolysis (Fig 2; Jallepalli et al., 1997). The importance of this regulation is clear from the fact that ectopic over-production of Cdc18 causes massive re-initiation of DNA replication (Nishitani & Nurse, 1995).

The Cdt1 protein also becomes degraded after successful initiation of DNA replication, but this process does not require Cdk. Instead, Cdt1 is targeted for degradation by a different E3 ubiquitin ligase, the Cullin4–Ddb1–Roc1 complex (Ralph et al., 2006). Interestingly, Cdt1 ubiquitination is tightly coupled to its function in initiation by means of two different mechanisms. First, substrate recognition requires a specific adaptor protein, the WD40-repeat protein Cdt2, which becomes transcriptionally induced when cells enter S phase (Liu et al., 2005). Second, Cdt1 only becomes ubiquitinated when it is associated with the proliferating cell nuclear antigen (PCNA) processivity clamp of the polymerase (Fig 2; Arias & Walter, 2006; Jin et al., 2006; Nishitani et al., 2006; Senga et al., 2006). Presumably, Cdt1 molecules are consumed when they have been actively engaged in initiation. Therefore, enzymatic activities that negatively regulate helicase-loader proteins seem to associate with the processivity clamp in both *E. coli* and *S. pombe*.

**Modulation of gene expression**

*E. coli* does not seem to regulate DnaA activity by degrading the protein; however, in addition to RIDA, a second mechanism for reducing DnaA activity in the post-initiation period exists. This method uses the sequestration mechanism to reduce expression of the *dnaA* gene. On replication, the *dnaA* gene promoter region, which is rich in GATC sequences, is hemi-methylated and sequestered for the same time period as the origin of replication. Sequestration of the *dnaA* promoter completely blocks transcription of the *dnaA* gene (Campbell & Kleckner, 1990). As the *dnaA* gene is close to the origin, sequestration of *dnaA* is virtually coincident with sequestration of oriC, and de novo DnaA synthesis is prevented during the origin sequestration period (Fig 2). In cells in which origin and *dnaA* gene sequestration no longer coincide, DnaA synthesis continues during origin sequestration. In such cells, re-initiations occasionally occur at some origins within the same cell cycle (Riber & Løbner-Olesen, 2005).

Transcription of the genes encoding the helicase-loader proteins Cdc18 and Cdt1 also oscillates in fission yeast and is high in late mitosis and G1 (Hofmann & Beach, 1994; Kelly et al., 1993). However, this is actively controlled by the cell-cycle-regulated MBF transcription factor complex rather than by an intricate system that monitors ongoing replication.

**Titrating DnaA to reservoir sites**

During origin sequestration, replication generates new DnaA protein-binding sites outside oriC. These titrate DnaA protein away from the origin and, in the absence of de novo DnaA synthesis (Campbell & Kleckner, 1990), efficiently reduce the intracellular concentration of DnaA protein available for initiation (Fig 2). The *E. coli* chromosome contains a hierarchy of 308 evenly distributed R-type DnaA boxes with different affinities for the DnaA protein. The *datA* locus, which contains five R-type DnaA boxes, seems to have the highest DnaA-binding capacity, and might bind to several hundred molecules of DnaA protein associated with either ATP or ADP. The *datA* locus is
located approximately 470Kbp away from oriC and is replicated within the period of origin sequestration during which no new DnaA protein is synthesized. This generates a sink for free DnaA protein (Kitagawa et al., 1998).

**Coupling nucleotide synthesis to chromosome replication**

In most cells, the intracellular concentration of DNA precursors (dNTPs) is low and can only sustain limited chromosome replication unless they are continuously synthesized to match the demand of ongoing replication forks. Upregulation of dNTP synthesis in S phase is carefully controlled because imbalances between the four individual nucleotide pools, as well as balanced deviation from the normal level, are mutagenic (reviewed by Mathews, 2006). In both *E. coli* and fission yeast, dNTPs are synthesized from their corresponding NTPs exclusively by the ribonucleotide reductase (RNR) complex. RNR is a heterodimeric tetramer consisting of two large and two small subunits. RNR activity is the rate-limiting step in dNTP synthesis.

The RNR subunits of *E. coli* are encoded by the nrdAB operon, and nrdAB expression is adjusted to DNA synthesis (reviewed by Herrick & Sclavì, 2007). Transcription of nrdAB is induced at the time of initiation by a DnaA-independent mechanism (Jacobson & Fuchs, 1998). Superimposed on this cell-cycle regulation is modulation of transcription by the DnaA protein (Augustin et al., 1994). DnaA was initially reported to stimulate nrdAB transcription (Jacobson & Fuchs, 1998) although a recent study indicates that DnaA–ATP—but not DnaA–ADP—is an efficient repressor of its transcription (Gon et al., 2006). The nrdAB expression level is therefore determined by the DnaA–ATP:DnaA–ADP ratio.

The RIDA-imposed variation in DnaA–ATP:DnaA–ADP ratio throughout the cell cycle (Kurokawa et al., 1999) could therefore couple dNTP synthesis to the elongation step of chromosome replication. Before initiation, when the cellular DnaA–ATP:DnaA–ADP ratio is high (Kurokawa et al., 1999), DnaA regulation would favour nrdAB repression. After initiation, RIDA is accelerated, resulting in a reduced DnaA–ATP:DnaA–ADP ratio and consequently an increase in nrdAB transcription. Therefore, the RNR level is increased in S phase, resulting in an increased synthesis of dNTPs to match the demand from the ongoing replication forks.

Precursor synthesis in eukaryotic cells is also adjusted to ongoing DNA replication by the regulation of RNR activity; however, the molecular basis is different. In *S. pombe*, transcription of the gene encoding the large subunit (Cdc22) is cell-cycle regulated (Fernandez Sarabia et al., 1993). In addition, assembly of RNR is actively prevented outside S phase by the presence of the RNR inhibitor protein Spd1 (Liu et al., 2003). When cells enter S phase, Spd1 is degraded by the same pathway that downregulates the Cdt1 helicase loader—that is, the Cullin4–Ddb1–Roc1 E3 ubiquitin ligase and the adaptor protein Cdt2 (Holmberg et al., 2005; Liu et al., 2005). It is unclear whether the degradation of Spd1—similar to the degradation of Cdt1—is coupled to PCNA.

**Perspectives**

The development of the eukaryotic type of genome organization—with multiple chromosomes and many scattered origins of replication—was probably important for the expansion of genome size that allowed the development of complex organisms. Taken at face value, control of replication seems to be organized differently in prokaryotic and eukaryotic cells; however, the control mechanisms found in the two systems seem to regulate the same steps in the process. First, in both prokaryotes and eukaryotes the crucial step in the establishment of a replication origin is loading of the replicative helicase. This process is mediated when the concentration of the helicase–loading AAA+ ATPases builds up to a certain threshold in the cell. In *E. coli*, this seems to be the rate-limiting step; loading of the replicative polymerase and initiation immediately follows. In *S. pombe*, further progress requires the action of S-phase-activating kinases. Second, once an origin of replication has fired, re-firing is prevented for a period of time. In both systems, this is accomplished by a combination of physical modification of the origin and/or associated protein factors (by sequestration or by phosphorylation), such that the helicase loader cannot access it, and by removing the helicase-loader activity. Eukaryotic cells literally get rid of the protein by switching on ubiquitin-mediated degradation. The prokaryotic cell does not have this option and therefore it is dependent on several other methods of reducing the active concentration of the helicase loader, such as through hydrolysis of its bound ATP, binding of the loader to unproductive sites or downregulation of its expression. The development of ubiquitin-mediated protein degradation made these mechanisms redundant.

In this review, we have attempted to draw parallels between the basic mechanisms that prevent re-replication in two simple unicellular model organisms. Failure to restrict replication to once per cell cycle leads to DNA damage through the generation of double-stranded breaks and can result in development of tumours (reviewed by Arias & Walter, 2007). It is therefore not surprising that metazoans have evolved additional mechanisms—such as inactivation of Cdt1 by Geminin binding—to minimize the likelihood of untimely replication initiations.

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