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review

# Limiting DNA replication to once and only once

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**In *Escherichia coli* cells, the origin of chromosomal replication is temporarily inactivated after initiation has occurred. Origin sequestration is the first line of defence against over-initiation, providing a time window during which the initiation potential can be reduced by: (i) titration of DnaA proteins to newly replicated chromosomal elements; (ii) regulation of the activity of the DnaA initiator protein; and (iii) sequestration of the *dnaA* gene promoter. This review represents the first attempt to consider together older and more recent data on such inactivation mechanisms in order to analyze their contributions to the overall tight replication control observed *in vivo*. All cells have developed mechanisms for origin inactivation, but those of other bacteria and eukaryotic cells are clearly distinct from those of *E. coli*. Possible differences and similarities are discussed.**

## Introduction

Any living cell must strike a balance between, on the one hand, promoting the reactions that are required for proliferation and, on the other hand, limiting such multiple reactions that may be deleterious to cell growth. This review describes the mechanisms known to limit DNA replication, with emphasis on the bacterium *Escherichia coli*.

When supplied with adequate nutrients and supplements, cells grow in size, replicate their chromosomes and divide into two daughter cells, in a tightly regulated manner. It is important that all chromosomes are replicated before cell division occurs, but it is also important to make sure that they are replicated only once. All cells that have been investigated, both prokaryotic and eukaryotic, seem to obey the once-and-only-once doctrine of DNA replication during normal, exponential growth (Boye, 1991a), although the doctrine can be deviated from under certain physiological situations. In the bacterium *E. coli* there are at least three different mechanisms blocking the occurrence of multiple replication events. The *in vivo* significance of each of the three mechanisms is not clear, but in their presence multiple replication events are extremely rare.

## Chromosome replication in *E. coli*

*Escherichia coli* has one circular chromosome which is replicated from a fixed point, called *oriC*. Initiation of replication occurs only once per cell division cycle, and simultaneously at all origins present in a cell (Skarstad *et al.*, 1986). At initiation of replication, the initiator protein DnaA is bound to its five cognate binding sites within *oriC* and separates the two strands of the DNA helix in an AT-rich region of the origin (Figure 1B). The subsequent events include loading of the DnaB helicase into the open DNA structure, synthesis of an RNA primer and loading of the polymerase complex (Figure 1C) (Messer and Weigel, 1996). The intracellular availability of DnaA is the main regulator of replication initiation at *oriC in vivo* (Løbner-Olesen *et al.*, 1989), strongly suggesting that the formation of an appropriate DnaA–*oriC* complex is the critical step in replication control. Once sufficient DnaA activity has accumulated and initiation has occurred, the main challenge for the cell is to prevent this activity from re-initiating one of the new origins. Below we describe three strategies that have been shown to contribute to the inactivation of a recently initiated origin of replication in *E. coli*.

## Sequestration

The adenines of GATC sites in *E. coli* are methylated by Dam methyltransferase. The GATC sites on both strands are methylated but, during replication, unmethylated nucleotides are incorporated. Therefore, a region of hemimethylated DNA exists in the wake of the replication fork (Figure 1D and E). In general, Dam methyltransferase methylates these sites within less than a minute, but certain sites, e.g. *oriC* and the *dnaA* gene, remain hemimethylated for up to one-third of the cell cycle (Campbell

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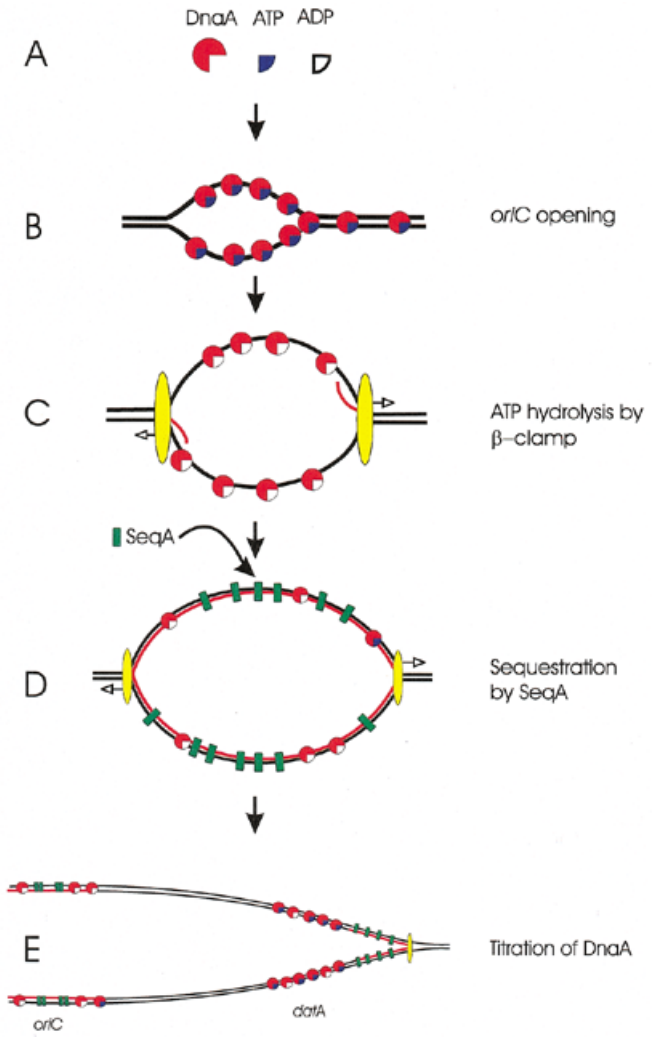


**Fig. 1.** Schematic representation of mechanisms to limit initiation of chromosome replication in *E. coli*. The DnaA protein can bind ATP or ADP (**A**), bind to *oriC* and separate the DNA strands (**B**). After loading of the DnaB helicase, primase, and the elongation machinery the DnaA-bound ATP may be reduced to ADP by the β-clamp, which is part of the polymerase complex [yellow ellipse, (**C**)]. It should be noted that this ATP hydrolysis may continue as long as the β-clamp is loaded and not only shortly after initiation. The nascent DNA strands are unmethylated (red) and the SeqA protein binds hemimethylated DNA (**D**) and sequesters hemimethylated *oriC*. SeqA remains bound at the hemimethylated *oriC* long after the replication fork has passed *datA*, which titrates a large amount of DnaA (**E**). Note the difference in scale in the different panels.

and Kleckner, 1990). Hemimethylated origins are not initiated *in vivo* (Russell and Zinder, 1987), but are perfectly good substrates *in vitro* (Landoulsi *et al.*, 1989; Boye, 1991b), arguing that some intracellular factor inactivates (sequesters) hemimethylated origins. This factor has been identified and named SeqA (Lu *et al.*, 1994; von Freiesleben *et al.*, 1994). The SeqA protein has a high affinity for fully methylated *oriC* and for hemimethylated DNA in general (Slater *et al.*, 1995; Brendler and Austin, 1999), and it binds specifically to the same two sites in *oriC*, irrespective of whether the DNA is hemi- or fully methylated (Skarstad *et al.*, 2000).

When *oriC* is replicated, it goes from being fully methylated to hemimethylated, a condition that is exploited by the cell as a kind of turnstile mechanism (Boye, 1991a): the origin cannot rapidly return to the fully methylated state because SeqA binds and sequesters the hemimethylated form (Figure 1D and E). However, this situation cannot persist, since *oriC* must eventually be remethylated and prepared for the next round of replication, but only after cell division has occurred. It is therefore important that the potential for initiation at *oriC* has been reduced by the time sequestration ends. The concentration of Dam methyltransferase influences both the duration of the hemimethylated state (Boye and Løbner-Olesen, 1990) and the minimum time between successive initiations of the same origin (von Freiesleben *et al.*, 2000).

Sequestration is absolutely required for efficient inactivation of fired origins. In *seqA* (Lu *et al.*, 1994; Boye *et al.*, 1996) and *dam* (Boye and Løbner-Olesen, 1990) mutant cells, origin refiring occurs at a significant rate, even in the presence of the two additional inhibitory mechanisms below. Therefore, sequestration can be considered as the first line of defence against over-initiation, providing a time window (eclipse) during which the initiation potential can be reduced by other means.

## Regulating the activity of DnaA

The DnaA protein binds the nucleotides ATP and ADP with high affinity (Figure 1A; Sekimizu *et al.*, 1987). Both forms of the protein bind *oriC* and other sites with DnaA boxes, but ATP-DnaA is required to form an initiation complex that promotes strand separation and initiation of replication. The ATP form is therefore the active form of the DnaA protein, while the ADP form is inactive. Mutations in DnaA that affect binding or hydrolysis of ATP may cause the protein to be constitutively active, leading to over-initiation and even lethality (Katayama and Kornberg, 1994; Mizushima *et al.*, 1997). Studies of one such mutant protein (DnaAcos), which over-initiates at high temperature, revealed that the over-initiation was due to a failure to inactivate DnaA, strongly suggesting that this inactivation is required *in vivo*. The mutant protein did not bind ATP and was therefore insensitive to normal inactivation by hydrolysis. The inactivating switch for DnaA turned out to be the β-subunit of the replication complex, together with a so far unidentified factor called IdaB (Katayama *et al.*, 1998), and the two factors appear to work in concert. The β-subunit forms a multimeric, doughnut-shaped molecule, a so-called sliding clamp, which encircles the DNA and dramatically improves the processivity of DNA polymerase III. Efficient inactivation of wild type DnaA by hydrolysis of its bound ATP requires the β-subunit to be loaded onto DNA as a sliding clamp (Figure 1C), and the inactivation is further stimulated by ongoing DNA replication. By this mechanism the capacity for initiation of replication may be reduced as soon as one round of replication is underway. However, in the absence of sequestration and with excess DnaA activity the eclipse period is virtually non-existent (Bogan and Helmstetter, 1997), suggesting that the DnaA is not all inactivated, or is not

inactivated fast enough, to prevent another round of replication from being started.

The lifetimes of ATP- and ADP-DnaA *in vitro* are ~1 h. However, when DnaA is bound to DNA in the presence of anionic phospholipids the nucleotides can be released immediately and fresh nucleotide may bind DnaA (Sekimizu and Kornberg, 1988). Thus, in the presence of phospholipid and excess ATP, the inactive ADP form of DnaA can be rejuvenated to the active form.

A marked reduction of the intracellular ATP-DnaA level around the time of initiation further suggests that the ATP/ADP exchange has a biological function *in vivo* (Kurokawa *et al.*, 1999). The DnaA protein is frequently found associated with the membrane (Newman and Crooke, 2000) and cells lacking anionic phospholipids cannot perform DnaA-dependent initiation at *oriC*, arguing, first, that the membrane is important for the function of DnaA *in vivo* and, secondly, that the DnaA-membrane interaction may facilitate rejuvenation of inactive DnaA.

## Regulating the level of free DnaA

*The datA locus*. The affinity of DnaA for its cognate boxes is relatively high and the protein is mostly bound to DNA when DnaA boxes are available. The chromosome contains a hierarchy of ~300 DnaA boxes with different affinities for the DnaA protein. Initiation of replication can be seen, at least in part, as a competition for active DnaA protein between the boxes in *oriC* and in other DnaA-binding regions. There are five regions of the chromosome which bind DnaA protein with particularly high affinity (Roth and Messer, 1998). The locus with the highest DnaA-binding capacity appears to be *datA* (Kitagawa *et al.*, 1996), which can titrate 8-fold more DnaA protein than the region spanning both *oriC* and the neighbouring gene *mioC*, even though the two regions (*datA* and *oriC*/*mioC*) contain the same number of DnaA boxes. The reason for the difference in DnaA-binding capacity is not known, but may at least partly be explained by a competition between DnaA and SeqA for binding to *oriC* (von Freiesleben *et al.*, 2000), whereas SeqA is not expected to bind much to *datA*, since it contains few GATC sites. Because *datA* is located close to *oriC*, it has been suggested to play a pivotal role in controlling replication initiation (Kitagawa *et al.*, 1998). As the *datA* region is replicated it will titrate twice the amount of DnaA protein, thereby drastically reducing the intracellular concentration of free DnaA protein at this point in the cell cycle (Figure 1E).

There is direct evidence that *datA* has an important regulatory function *in vivo*. First, over-initiation is observed when *datA* is removed from the chromosome (Kitagawa *et al.*, 1998). Secondly, introduction of additional intracellular copies of the *datA* locus is limiting for initiation (Kitagawa *et al.*, 1998) and high *datA* copy numbers totally shut down intiation from *oriC* (our unpublished data). We conclude that the function of *datA* is required as a sink for DnaA protein for normal regulation of initiation. However, normal replication control does not require *datA* to be located close to *oriC*. This suggests that the timing of *datA* replication is not critical, as long as sequestration is active. It is not known how or whether *datA* replication can contribute to initiation control if its replication occurs after the sequestration period is over.

*Sequestration of the dnaA gene.* The *dnaA* gene is replicated shortly after *oriC* and, like *oriC*, becomes hemimethylated and is sequestered by a SeqA-dependent mechanism (Lu *et al.*, 1994). This sequestration results in a transient suppression of *dnaA* gene transcription shortly after initiation (Theisen *et al.*, 1993). Thus, following *oriC* initiation, there is a period of time when no new DnaA protein is being synthesized. Since the chromosome is replicated during this period, the ratio between DnaA protein and DnaA boxes is reduced, resulting in a reduction of the initiation capacity. This temporary repression of *dnaA* transcription is not important in the presence of the other mechanisms, though, since the gene can be expressed continuously, from a heterologous promoter, without any effect on replication control (Løbner-Olesen *et al.*, 1989).

## Gram-positive bacteria

Sequestration, as it is described here, is dependent upon methylation for discrimination between old and new replication origins. However, replication control is equally tight in Gram-positive bacteria that do not possess a Dam-like methylation system or a SeqA homologue, e.g. *Bacillus subtilis* (Seror *et al.*, 1994). Presumably, these bacteria possess alternative, highly efficient regulatory mechanisms. In *bacilli, oriC* and *dnaA* are colocalized, representing a region with a high capacity to bind DnaA, particularly since SeqA is absent. Therefore, replication of this region will create a large sink for DnaA, which will effectively reduce the level of free DnaA. This mechanism may on its own represent the main form of replication control in Gram-positive bacteria. However, a separate regulatory mechanism that involves a strong interaction of *oriC* with the cell membrane may also be active (Firshein, 1989).

## Eukaryotic cells

In eukaryotic cells, the major mechanism behind the restriction of chromosome replication to once per generation is termed licensing (Blow and Laskey, 1988). The term reflects the notion that each origin of replication is, in M/$G_1$ phase, given a licence to replicate once, and the cells must pass through mitosis again to receive another licence. This mechanism is therefore distinct from sequestration. It involves assembly of the pre-replication complex, including loading of the minichromosome maintenance (MCM) proteins onto future replication origins, in late M or in $G_1$, at a time when cdk/cyclin activity is low. Later in the replication cycle, a pre-replication complex cannot be assembled or re-assembled, for two reasons. First, the factor which allows MCM loading, the Cdc6 protein, is phosphorylated and degraded in $G_1$ (Donovan *et al.*, 1997; Tanaka *et al.*, 1997). Secondly, some of the MCM proteins are phosphorylated in S phase (Coue *et al.*, 1996) and, in the yeast *Saccharomyces cerevisiae*, this may cause the MCM proteins to be transported to the cytoplasm, thereby denying them access to the replication origins. Thus, a licence to assemble the pre-replication complex is issued only when the cells pass through mitosis, after the cyclins have been degraded and the cdk/cyclin activity is abolished.

Licensing may be sufficient to prevent re-initiation from occurring, but the ATP/ADP binding properties of some of the eukaryotic initiation proteins probably also have regulatory roles and

may function in preventing re-initiation. The *S. cerevisiae* ORC and Cdc6 proteins, which are part of the pre-replication complex, both bind and hydrolyze ATP. Cdc6p requires ATP binding for proper replication complex assembly (Perkins and Diffley, 1998; Weinreich *et al.*, 1999). In this situation, the assembly and disassembly of the pre-replication complex may well be controlled by ATP hydrolysis (Lee and Bell, 2000).

## Conclusions

All cells investigated have built up a defense against replicating their chromosomes without purpose. However, the molecular details of the defense mechanisms vary between cell types. In *E. coli*, sequestration temporarily inactivates the origins and provides a time window during which other mechanisms can reduce the initiation potential. Titration of DnaA proteins by *datA* and reduction of DnaA activity by ATP/ADP exchange is operative during the sequestration period to reduce the initiation potential. Such mechanisms are important for optimal functioning and for genomic stability of individual cells, whether they are prokaryotic or eukaryotic.

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