Replication cycle-coordinated change of the adenine nucleotide-bound forms of DnaA protein in *Escherichia coli*

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Introduction

In *Escherichia coli*, chromosomal replication is initiated in a nucleoprotein complex including the oriC region and DnaA protein. In the initiation complex of oriC that seems to contain 20–40 DnaA molecules the duplex DNA in the AT-rich 13mer sequence repeat is opened. On the exposed single-stranded region, replicative helicase (the dnaB gene product) is loaded and forms a mobile complex with primase (the dnaG gene product), and then following primer RNA synthesis complementary DNA strands are replicated by DNA polymerase (pol) III holoenzyme (Kornberg and Baker, 1992).

The initiation step of DNA replication is tightly regulated during the cell division cycle (Donachie, 1968; Helmstetter, 1996). For this regulation, initiation activity of DnaA protein has to be controlled precisely (Zyskind and Smith, 1992; Boye et al., 1996). Certain mutations in the dnaA gene cause overinitiation of chromosomal replication at its restrictive temperature and cell proliferation is inhibited (Kellenberger-Gujer et al., 1978; Katayama and Nagata, 1991; Katayama and Kornberg, 1994; Katayama et al., 1997a,b). The wild-type DnaA protein tightly binding ATP (K<sub>D</sub> of 30 nM) is active for opening of the oriC DNA duplex and leads to the following initiation reaction; one binding ADP is inert (Sekimizu et al., 1987). Unlike the wild-type form, DnaAcos protein, a mutant DnaA form causing overinitiation, lacks affinity for ATP and ADP, whereas its activity for initiation is sustained (Katayama, 1994; Katayama and Crooke, 1995), probably because amino acid substitutions in DnaAcos protein render its conformation active for initiation without the binding of ATP. In another case of overinitiation (Kaguni, 1988), and GroE affects folding conformation of proteins by direct interaction (Van Dyk et al., 1989; Sigler et al., 1998). These findings led to the notion that initiation is profoundly controlled by change in the nucleotide form of DnaA protein in vivo.

The hydrolysis of ATP bound to DnaA protein is accelerated by interaction with the β-subunit (the dnaN gene product) of pol III and so far unidentified IdaB protein (Katayama et al., 1998). We named this negative control RIDA (for regulatory inactivation of DnaA) since this system seems to be necessary to restrain the initiation frequency only once per chromosome per cell cycle by timely inactivation of the initiator DnaA. Unlike the wild-type form, DnaAcos protein is insensitive for this reaction due to its defective affinity for adenine nucleotides (Katayama, 1994; Katayama and Crooke, 1995).

pol III<sup>+</sup>, a subassembly of pol III excluding the β-subunit, consists of two complexes and a dimer of one subunit (Maki et al., 1988; Kelman and O’Donnell, 1994, 1995; Herenden and Kelly, 1996; Baker and Bell, 1998). One complex that has activity for polymerization of nucleotides and 3′ to 5′ exonuclease is pol III core, which contains each single peptide of three subunits (α, ε and θ); the β-subunit dimer binds pol III core dimer, and γ-complex contains five subunits (γ, δ, δ′, χ and ψ). The β-subunit is processed with the aid of γ-complex to form a ‘β-clamp’; the β-subunit dimer which encircles DNA (or primed DNA). The γ-complex has activity for loading of β-subunit onto DNA. β-clamp slides on DNA and ensures high processivity of DNA synthesis by pol III (Kornberg and Baker, 1992).
For RIDA, the β-clamp loaded on DNA, but not the unloaded form, is active (Katayama et al., 1998). Our in vitro study further revealed that the rate of β-subunit-dependent inactivation of DnaA protein is significantly enhanced by concomitant DNA synthesis (Katayama et al., 1998). These observations suggested that after the initiation reaction DnaA protein is inactivated by β-clamp formed for assembly of the polymerase, and thus RIDA is important for the coordinated control of DnaA activity for the replication cycle (Katayama and Sekimizu, 1999).

We also examined the nucleotide form of DnaA protein in vivo, under conditions where DnaA protein was supplied from the gene cloned on pBR322, for precise detection; in this case, the dnaN gene that encodes the pol III β-subunit was necessary for abundance of the ADP-bound DnaA molecules in vivo (Katayama et al., 1998). In the present work, an improved assay quantitatively detects nucleotides bound to DnaA protein that is derived only from the chromosomal gene. Under these conditions, we had in vivo evidence that the ADP-bound DnaA molecules are abundant in growing cells, and formation of the ADP-bound molecules depends on genes for DNA replication, including dnaB, dnaC and dnaG. Thus, we suggest that RIDA is tightly coupled with the polymerase function in vivo. This means that the initiation reaction is controlled by a direct feedback pathway where DnaA function is repressed by cross-talk between the initiator and the functioning replisome.

We found that ATP-bound DnaA molecules are formed in a manner depending on de novo protein synthesis. The abundance of the ADP form of DnaA protein seen in growing cells means that RIDA is efficient in vivo and the ATP–DnaA level can be limited. In addition, we observed a temporal rise of the ATP–DnaA level in a synchronized culture, indicating that DnaA is activated in a cell-cycle-dependent manner. Regeneration of the ATP form of DnaA from the ADP form was also detected under certain conditions. This regeneration may be necessary for the rise in the ATP–DnaA level during the cell cycle.

**Results**

**ADP–DnaA is abundant in cells**

To assess the nucleotide forms of DnaA protein in vivo, cells were labeled with [32P]orthophosphate (Echols et al., 1961; Katayama et al., 1998). DnaA protein in cleared lysate was collected by immunoprecipitation, and nucleotides associated with the precipitates were analyzed by one-dimensional thin-layer chromatography (Figure 1).

Specificity in this assay for DnaA protein was supported by findings that immunoprecipitates prepared from dnaA-null and dnaAcos mutants did not yield significant signals corresponding to ATP or ADP, whereas those from wild-type dnaA strains did (Figure 1A). Since DnaAcos protein lacks the potential to bind ATP and ADP with a high affinity (Katayama, 1994), these results suggest that ATP and ADP recovered by this immunoprecipitation derive from those bound to the specific binding site of DnaA protein. Competition experiments supported the specificity and quantitative response of this assay (Figure 1B).

In exponentially growing wild-type dnaA cells, apparently the ADP form of DnaA protein is abundant and only 15–30% of DnaA molecules took on the ATP form (Figure 1A). Since randomly dividing cells were used here, these values can be considered as average levels in cells at various cell cycle stages (see below).

**Production of ADP–DnaA is dnaN dependent**

Consistent with in vitro evidence that stimulated hydrolysis of ATP bound to DnaA depends on the β-subunit (the


**Fig. 2.** Role of DnaN and de novo protein synthesis in the control of adenine nucleotide forms of DnaA. KA473 (dnaN59) and KA474 (dnaN59) were grown at 28°C in TG medium including [32P]orthophosphate up to an optical density (A600) of 0.2, and shifted to 42°C. At the indicated time, an aliquot (2 ml) was removed, and DnaA-bound nucleotides were analyzed. (A) Results of thin-layer chromatography were visualized by radiochemical imaging. Migration positions of ATP and ADP are indicated by arrows. C, control using pre-immune serum. (B) Recovered ATP and ADP in (A) were quantified, and relative amounts (%) of ATP are shown. ○, KA473; ●, KA474. (C) KA474 was similarly grown at 28°C and shifted up to 42°C in the presence (○) or absence (●) of chloramphenicol (CM; 150 μg/ml).

dnaN gene product) of pol III (Katayama et al., 1998), the dnaN gene was necessary to maintain abundance of the ADP form of cellular DnaA protein (Figure 2A and B).

When the culture of the dnaN59 [temperature-sensitive (Ts)] mutant was shifted from 28°C to a restrictive temperature of 42°C the content of ATP-bound DnaA molecules increased from 20% to >80% (Figure 2A and B). Conversely, in the wild-type dnaN strain the level of the ATP-form molecules did not increase with the temperature shift. A complementation test carried out with a plasmid (pKW001-3) bearing the intact dnaN cistron confirmed that the dnaN gene is obligate for maintaining the limited level of ATP-bound DnaA molecules (Table I, rows 1–2). These findings coincide with results obtained using a dnaN mutant bearing a dnaA-conveying pBR322 (Katayama et al., 1998).

Even when a recA-null mutation was present, the dnaN-dependent control of the nucleotide forms of DnaA protein was also seen (Table I, row 3). Therefore, the SOS response induced at 42°C in the dnaN mutant by arrest of replication fork progression is apparently non-essential for RIDA.

These findings revealed that the β-subunit of pol III is essential for RIDA in vivo, and suggest a direct or indirect interaction of the β-subunit with DnaA protein in vivo.

### De novo protein synthesis is necessary for generation of ATP–DnaA

De novo protein synthesis is necessary for replication initiation at oriC (McMacken et al., 1987). This suggests that a protein(s) necessary for initiation and/or one that activates such a factor is functional only for a short period after being translated. Overinitiation in certain dnaA mutants occurs in the presence of chloramphenicol (Kellenberger-Gujer et al., 1987; Katayama and Nagata, 1991; Katayama and Kornberg, 1994). Activity of DnaAcos, one such mutant protein, is resistant to RIDA, presumably providing a stable competence for initiation in cells (Katayama, 1994; Katayama and Crooke, 1995). Based on these findings and the features of RIDA, we

<table>
<thead>
<tr>
<th>Strain (relevant genotype)</th>
<th>ATP form of DnaA (%) after incubation at 42°C for</th>
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<tbody>
<tr>
<td></td>
<td>0 (min)</td>
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<tr>
<td>HC194 (dnaN59) [pSTV28]</td>
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</tr>
<tr>
<td>HC194 (dnaN59) [pKW001-3]</td>
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<tr>
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<tr>
<td>KA487 (dnaN59 seqA::Tet)</td>
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</tr>
<tr>
<td>KA486 (dnaN59 dam-13::Tn9)</td>
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<tr>
<td>ME6299 (dnaB+)</td>
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</tr>
<tr>
<td>ME5491(dnaB43)</td>
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</tr>
<tr>
<td>DG75 (dnaC++)</td>
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<tr>
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<tr>
<td>KA488 (dnaC2 seqA::Tet)</td>
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</tr>
<tr>
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</tr>
<tr>
<td>PC3 (dnaG3)</td>
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</tr>
<tr>
<td>AX729 (dnaX+)</td>
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<tr>
<td>AX727 (dnaX2016)</td>
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</tr>
<tr>
<td>AZ5157 (fixX+)</td>
<td>34</td>
</tr>
<tr>
<td>AZ5159 (fixZ84)</td>
<td>35</td>
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Cells were grown exponentially at 28°C and shifted to 42°C. Immunoprecipitation experiments were carried out as described in the legend to Figure 2 and Materials and methods. Additional independent experiments yielded similar results. The values shown as percentage include a measurement error of ±5%. n.t., not tested; +CM, incubated at 42°C in the presence of chloramphenicol (150 μg/ml).

The dnaX gene encodes the τ- and γ-subunits of pol III; the γ-subunit is synthesized by a translational frameshift (Tsuchihashi and Kornberg, 1990). dnaX2014 alters both subunits (Blinkova et al., 1993).
suggest that the ATP form of DnaA protein is transiently formed in a manner depending on concomitant protein synthesis, then by β-clamp formed after the initiation reaction; ATP bound to DnaA is hydrolyzed efficiently, generating the inactive, ADP-bound form (Katayama et al., 1998). A pathway regenerating ATP-DnaA from ADP-DnaA, if present in vivo, may also require de novo protein synthesis to function. Here, we asked whether generation of ATP-DnaA in vivo requires de novo protein synthesis (Figure 2C).

The exponentially growing dnaN59 mutant at 28°C was shifted to 42°C and incubated in the presence or absence of chloramphenicol. Assessing nucleotide forms of DnaA revealed that the increase in the ATP-DnaA level at 42°C was almost completely inhibited by chloramphenicol (Figure 2C). Therefore, generation of ATP-DnaA is largely dependent on de novo protein synthesis. This notion is consistent with findings that initiation of chromosomal replication requires concomitant protein synthesis.

**Genes responsible for chromosomal replication are required for RIDA**

Concomitant DNA replication by pol III accelerates the hydrolysis of DnaA-bound ATP (Katayama et al., 1998), presumably due to stimulation of β-clamp formation. To investigate the coupling of RIDA with chromosomal replication in vivo, we asked whether replication genes other than dnaN are necessary for control of the nucleotide forms of cellular DnaA protein (Table I).

We observed dramatic changes in levels of the DnaA forms in temperature-sensitive mutants of DnaB helicase, DnaC helicase-loader, DnaG primase, and pol III τ- and γ-subunits that are produced by a translational frameshift from the dnaX gene (Kornberg and Baker, 1992). The alleles of dnaB43, dnaC7 and dnaG3 inhibit the elongation reaction of chromosomal replication at 42°C, whereas the dnaC2 allele inhibits the initiation step (Wechsler and Gross, 1971). The dnaX2016 allele alters both τ- and γ-subunits (Blinkova et al., 1993). In all of these cases, upon shift-up to a restrictive temperature, the level of ATP-DnaA increased to ~70–80% in 30 min (Table I, rows 6–14). *De novo* protein synthesis was necessary for this increase. The basal level of ATP-DnaA in a dnaC2 mutant at 28°C was slightly higher than basal levels in other strains (Table I, row 9); DnaC2 protein may not be fully active even at this permissive temperature, and may slightly inhibit RIDA during chromosomal replication. As a control, ftsZ mutation, which inhibits septation of cells (Rothfield and Justice, 1997), yielded no significant change in the DnaA forms by temperature shift-up (Table I, rows 15 and 16).

These results indicate that negative regulation of the DnaA-nucleotide forms requires a concerted action of replication genes. In other words, RIDA seemed to depend on progression of the replication fork. Presumably the β sliding clamp is formed only at active replication forks, or progression of the replisome may be needed for efficient functioning of RIDA.

**oriC is unnecessary for the production of ADP–DnaA**

We next asked whether oriC is necessary for RIDA in vivo. A mutant defective in the rnhA gene encoding RNaseHI initiates chromosomal replication at alternative origins, oriKs (Kogoma, 1997). This oriC-independent chromosomal replication (constitutive stable DNA replication; cSDR) allows oriC-deleted mutants to grow.

Exponentially growing cells that lack oriC and rnhA (KA429) contained ADP-DnaA at a level similar to that in the wild-type counterpart (Figure 3A). This result is consistent with in vitro data showing that plasmid DNA excluding oriC is active for DnaN-dependent inactivation of DnaA protein (Katayama and Crooke, 1995; Katayama et al., 1998). Also, these data support the idea that

![Image](image-url)
rather than intermediates in the initiation reaction at oriC, β-clamp or functional replisome is necessary for RIDA.

**SeqA and Dam are unnecessary for the formation of ADP–DnaA**

The seqA gene is a negative modulator of the replicational initiation at oriC in that it represses irregular initiations at a certain level (Lu et al., 1994; von Freiesleben et al., 1994; Crooke, 1995; Boye et al., 1996). SeqA protein, with the aid of putative SeqB protein, specifically binds to oriC DNA that is methylated by DNA adenine methyltransferase (Dam) (Brendler et al., 1995; Slater et al., 1995; Shakibai et al., 1998). Semiconservative replication of fully methylated chromosomal DNA produces hemimethylated daughter duplexes and SeqA is needed to maintain the hemimethylated state of oriC for a certain duration (~10 min when the doubling time of cells is 30 min) by inhibiting re-methylation by Dam; in the seqA-null mutant, daughter strands are methylated rapidly and irregular initiations of chromosomal replication can occur (Lu et al., 1994). Inhibition of irregular initiation may result from SeqA binding to this site (Wold et al., 1998) and/or interaction of hemimethylated oriC with membrane (Ogden et al., 1988).

Moreover, SeqA protein seems to form a complex on a specific locus on the nucleoid in a manner dependent on Dam and DnaC protein (Hiraga et al., 1998). In this complex, SeqA protein might interact with the replisome, including the β-clamp, on replicated DNA in a hemimethylated form (Hiraga et al., 1998). Thus, we determined whether depletion of SeqA and Dam would affect the level of the nucleotide forms of DnaA protein in vivo.

Both the seqA-null and dam-null mutants maintained the ADP–DnaA content at a level similar to that seen in the wild-type counterpart (Figure 3B; Table I). In a dnaC2 seqA1 double mutant and a dnaN59 seqA1 double mutant, accumulation of the ATP-bound DnaA molecules was evident with temperature shift-up (Table I, rows 4 and 10). Thus, RIDA would appear to function independently of SeqA and Dam in vivo. These results coincide well with data obtained in vitro (Katayama and Crooke, 1995; Katayama et al., 1998).

**Coupling of RIDA with the chromosomal replication cycle**

To investigate whether change in the DnaA nucleotide forms is coupled with the replication reaction, we used a dnaC2 mutant (PC2) to synchronize initiation of chromosomal replication (Evans and Eberle, 1975; Withers and Bernander, 1998). In the dnaC2 mutant, initiation, but not progression of preformed replication forks, is inhibited at restrictive temperatures (>39°C). When this mutant is pre-warmed at 40°C until preformed replication forks complete the ongoing round of replication and then is shifted down to 30°C, two rounds of initiations occur synchronously in sequence with an interval of 20–30 min (Evans and Eberle, 1975; Zhou et al., 1997; our unpublished data). When chloramphenicol (150 μg/ml) is added at the time of temperature shift-down, the first, but not second, round of replication initiates (Evans and Eberle, 1975; Withers and Bernander, 1998; our unpublished data), which means that the first, but not second round initiation occurs independently of de novo protein synthesis. Certain steps preceding a DnaC-dependent step in initiation reactions are presumably completed at 40°C and in such steps a stage requiring de novo protein synthesis is included (Withers and Bernander, 1998).

When the replication cycle of PC2 cells was synchronized and the nucleotide forms of DnaA protein were assessed, oscillations of the levels of the forms were observed (Figure 4A). After incubation at 40°C, the level of ATP–DnaA increased to ~80% of the total nucleotide-bound DnaA molecules, as also shown in Table I. Upon shift-down to 30°C, the level of ATP–DnaA decreased rapidly, reaching the basal level within 20 min. This observation supports the idea that the start of chromosomal replication triggers RIDA.

With continued incubation, the level of ATP–DnaA was again increased, albeit temporarily (Figure 4A). This temporal increase in the ATP–DnaA level was not seen in the presence of chloramphenicol, a compound that inhibits the second round of initiation (Figure 4B). These observations not only support the notion that RIDA indeed depends on chromosomal replication, but also suggest that control of the nucleotide forms of DnaA is linked with an epistatic cell cycle event for timely firing of the initiation reaction.

**Coupling of DNA replication with RIDA in the absence of SeqA**

When a dnaC2 seqA1 double mutant is used for similar temperature shift experiments, a single burst of replication initiations occurs within 20 min after shift-down from 40°C (Bogan and Helmstetter, 1997; our unpublished data). Lack of the SeqA function perhaps shortens the interval between two successive rounds of initiation,
resulting in such concordant initiations (Bogan and Helmstetter, 1997). This system will facilitate further investigation of the coupling of the replication cycle with the change of DnaA's nucleotide forms.

After exponentially growing cells of the dnaC2 seqA1 mutant were incubated at 40°C for 50 min the level of the ATP form of DnaA was elevated to ~70% (Figure 4C). When the temperature was shifted down to 30°C, the ATP–DnaA level decreased rapidly and reached the basal level within 15 min; the secondary increase in the ATP–DnaA level was not seen (Figure 4C).

After the double mutant pre-warmed at 40°C was shifted down to 30°C in the presence of chloramphenicol, results similar to those obtained in a dnaC2 mutant were obtained (Figure 4B and D). Pulse-labeling experiments using [3H]thymidine, under these conditions, showed that only one burst of replication occurs within 20 min after temperature shift-down (data not shown).

These results support the notion that change in the DnaA form is coordinated with the chromosomal replication cycle.

**Regeneration pathway from ADP–DnaA to ATP–DnaA**

To elucidate the mechanism involved in the increase of the ATP–DnaA level seen in the synchronized cell culture (Figure 4A), we further examined the possibility of the presence of a pathway regenerating the ATP–DnaA molecules from the ADP–DnaA molecules *in vivo*. Based on an assumption that the regeneration pathway is temporarily induced in a cell-cycle-dependent manner, and requires *de novo* protein synthesis, we considered that genetically altered gene expressions may render this regeneration pathway constitutive and independent of *de novo* protein synthesis.

We detected the regeneration of ATP–DnaA in the presence of overexpressed DnaA protein, which affects transcriptional expression of some genes (Messer and Weigel, 1997), provokes irregular replication of minichromosome during the cell cycle (Atlung et al., 1987; Pierucci et al., 1987, 1989), induces extra initiations at the chromosomal oriC region (Atlung et al., 1987), and reduces the average cell volume when initiation of chromosomal replication occurs (Løbner-Olesen et al., 1989; Skarstad et al., 1989). The regeneration was examined in a dnaN59 mutant incubated at 42°C in the presence of chloramphenicol; under these conditions, the ATP form of DnaA is obtained only by regeneration from the ADP form due to inhibition of *de novo* DnaA synthesis, and if the ATP–DnaA molecules do regenerate, the generated ATP–DnaA molecules are stably present as RIDA is inhibited by a defect in the dnaN gene.

Using this system, we observed significant activity for regeneration of the ATP form of DnaA in cells bearing phB10S, a pBR322 derivative carrying a dnaA cistron (Figure 5). Since pBR322 had no influence on the ATP–DnaA level in the presence of chloramphenicol, oversupply of DnaA protein apparently induced the regeneration pathway (Figure 5A). This regenerating activity was maintained constantly for at least 90 min in the presence of chloramphenicol (Figure 5B).

Immunoblotting experiments indicated that the content of DnaA molecules was elevated ~5-fold by introduction of pHB10S (data not shown). Stable expression of the regeneration activity by DnaA oversupply may possibly be related to the untimely initiations at *oriC*.

**Discussion**

**The nucleotide form of DnaA in the cell cycle**

Our evidence shows that the nucleotide form of DnaA protein *in vivo* is carefully controlled during the cell cycle of *E. coli*. The content of the ATP-bound form of DnaA protein is maintained at a low level, and probably only around the time of replicational initiation does it increase to ~80%. A rapid decrease in the ATP–DnaA level after initiation in a synchronized culture coincides with the proposal that the hydrolysis of ATP bound to DnaA protein is accelerated by interaction with the β-clamp formed at the replication fork. We suggest that β-clamp formation is a key signal for the timely switching of the initiator forms so as to repress extra initiations that can be provoked independently of the cell cycle regulation.

Based on the finding of oscillation of the ATP–DnaA level in a synchronized culture, we propose a model in which initiation occurs at the time when the ATP–DnaA content reaches a certain level (Figure 6). This model can explain the cause of the simultaneous initiations of minichromosomal and chromosomal replication during the cell cycle (Leonard and Helmstetter, 1986; Helmstetter and Loenard, 1987). We speculate that 70–80% of DnaA
DnaA level may be activated in a growth-phase-dependent manner by some unknown mechanism. In slowly growing cells, the content of the ATP-bound form of DnaA per total DnaA molecules in a cell shows a slow accumulation of ATP–DnaA molecules so as to control the ATP–DnaA level for timely initiation. In rapidly growing cells, the ATP–DnaA level can increase to a threshold for firing the next round of initiation by the simultaneous action of these two mechanisms. These events enable both the recycling of ADP–DnaA molecules and de novo synthesized DnaA molecules that will bind ATP preferentially. We suggest that such mechanisms are located downstream of epistatic cell-cycle-dependent signaling, and are needed to control the ATP–DnaA level for timely initiation.

**DNA replication-dependent RIDA in vivo**

Our results support the idea that RIDA is tightly coupled with chromosomal replication in vivo. As β-clamp is formed at the primed site and slides, associated with pol III*, on the replicating DNA region (Kelman and O’Donnell, 1994, 1995), our finding suggests that the DnaA-bound ATP hydrolysis occurs in a manner coupled with DNA synthesis by pol III in vivo, as previously noted in vitro.

For this coupling, at least two mechanistic models are conceivable based on the finding of a requirement for dna genes other than dnaN for RIDA. The first one is that the replisome sliding on DNA is required for RIDA. As the number of β-clamps is much lower than the number of DnaA molecules in a cell (1000–2000/cell; Sekimizu et al., 1988; Katayama and Kornberg, 1994), the β-clamp should be in repeated use for interactions with many different molecules of DnaA. For this cycling, rapid sliding or movement, in addition to the presence of the β-clamps along with DNA strands may be needed; only when traveling as a part of the replisome can the β-clamp interact with many DnaA molecules efficiently and repeatedly.

The second putative mechanism is that even in dna mutants other than dnaN the β-clamp is dissociated at the restrictive temperature, and RIDA is inhibited. During one round of chromosomal replication ~3000 pieces of Okazaki fragment are expected to be formed (Wu et al., 1992). This means that unloading of the β-clamp for recycling has to occur efficiently (Kelman and O’Donnell, 1994, 1995).

The β-clamp is formed by the γ-complex, which seems to bind selectively at the RNA primer–template single-stranded DNA junction (Kelman and O’Donnell, 1994, 1995; Hingorani and O’Donnell, 1998). In addition to the role of the clamp loader, the γ-complex has the function of unloading the preformed β-clamp (Kelman and O’Donnell, 1994, 1995; Yao et al., 1996). Thus, the following mechanisms are conceivable. On the lagging strand, loss of priming activity by DnaG results in loss of location for loading of the β-clamp. After unloading of the β-clamp by completion of the Okazaki fragment, sliding clamps disappear on the lagging strand. When DnaB helicase is inactivated at a restrictive temperature, the replication fork does not proceed and thus single-stranded region is not supplied for priming by DnaG primase and the result is loss of the β-clamp, as mentioned above. Although the function of DnaC in the elongation reaction of chromosome replication is still not clear, arrest of replication fork progression results in situations similar to the case of the defect in DnaB.

After dissociation of the β-clamp from the lagging strand, the β-clamp on the leading strand may be unloaded by the γ-complex as follows. The ATP-bound form of
γ-complex associates with the β-dimer and then, concomitantly with clamp loading, ATP is hydrolyzed to dissociate the γ-complex from the clamp. When the lagging strand is ongoing the cycle of γ-complex–β-dimer interaction should occur efficiently. Arrest of the replication fork can stabilize the ATP-bound form of the γ-complex, and this can result in interaction with β-clamp on the leading strand, thus causing its unloading.

Inactivation of τ- and γ-subunits by dnaX2016 mutation should inhibit clamp loading. A possible mechanism for unloading of preformed β-clamps in this case is that it occurs by an unidentified factor or by the β-subunit of the γ-complex. This subunit, as a single protein, interacts with the β-dimer (Naktinis et al., 1995) and dissociates the β-clamp by opening its dimer interface (Turner et al., 1999).

Negative modulations of initiation by SeqA, datA and RIDA
When initiation was synchronously induced in the dnaC2 mutant (PC2) by temperature shift-down from 40 to 30°C, 15–20 min were needed for the level of ATP–DnaA to decrease to the baseline (Figure 4). Based on this observation, we consider that, during the cell cycle, sequestration of oriC by SeqA protein plays a role in preventing the occurrence of extra initiations by the residual ATP–DnaA molecules present temporarily after initiation (Figure 6). The duration of the origin sequestration is ~10 min when the doubling time of cells is ~30 min (Lu et al., 1994).

The datA locus, an ~950 bp DNA segment containing five DnaA boxes located at 94.7 min on the genome map, seems to have the potential to bind 370 DnaA molecules in vivo (Kitagawa et al., 1996, 1998). Deletion of this locus causes an asynchronous phenotype of chromosomal replication due to extra initiations. The ATP–DnaA level never reached below 10% at the basal level, so far as examined (Figures 1–5; Table I). Since the number of DnaA molecules in a cell is 1000–2000 (Sekimizu et al., 1988; Katayama and Kornberg, 1994), at least 100–200 molecules of DnaA would take on the ATP form. These observations suggest that the regeneration machinery will function.

If all the residual ATP–DnaA molecules are free in the cytosol and accidentally localize around oriC, extra initiations could occur. We suggest that the datA locus restricts localization of DnaA molecules to restrain the number of molecules freely accessible for oriC. In addition, part of the DnaA molecule binds to other DnaA boxes (Roth and Messer, 1998) and may localize on the membrane surface, based on its own affinity (Sekimizu et al., 1988; Garner et al., 1998).

Regeneration of ATP–DnaA from ADP–DnaA
Interaction of DnaA protein with vesicles of acidic phospholipid such as cardiolipin and phosphatidylglycerol decreases its affinity for ATP and ADP in vitro. This enables regeneration of ATP–DnaA from ADP–DnaA by exchange of nucleotides, e.g. ADP bound to DnaA is released by phospholipid vesicles, and if ATP is present at a high concentration of ~5 mM, DnaA binds this (Sekimizu and Kornberg, 1988). A decrease in pgsA gene expression, which is necessary for synthesis of cardiolipin and phosphatidylglycerol (Gopalakrishnan et al., 1986), was suggested to inhibit initiation at oriC (Xia and Dowhan, 1995). This genetic study suggests that a regeneration pathway from ADP–DnaA to ATP–DnaA by phospholipids functions to maintain the initiation capacity of cells; whether or not this system is induced in a cell-cycle-dependent manner remains to be determined.

We presented here the first, direct evidence for the presence of a regeneration pathway of ATP–DnaA in vivo (Figure 5). Under the conditions we used, detection of regeneration required an oversupply of DnaA protein, which suggests that DnaA protein itself is related to regulation for this pathway. We speculate that this regenerating activity is induced only for a short period during the cell cycle, hence the activity was too low to be detected in randomly dividing cells where DnaA is not oversupplied (Figure 2C). Cells in the medium for labeling with [32P]phosphate grow at a doubling time of 65–80 min at 28–30°C. This slow rate may also explain why the regeneration pathway was too weak to be detected in cells without DnaA oversupply. In certain strains the cellular content of DnaA protein is increased proportionally to the growth rate (Chiaramello and Zyskind, 1989). Oversupply of DnaA protein may mimic certain physiological conditions in the case of a faster growth rate, thus leading to activation of the regeneration pathway. Another notion is that an increase of target molecules increases the likelihood that the regeneration machinery will function.

In randomly dividing cells bearing pHB10S the level of ATP–DnaA is kept to ~20% of the total nucleotide-bound DnaA molecules in a dnaN-dependent manner (Figure 5). This means that the regeneration pathway is less efficient than RIDA, and suggests that cell-cycle-dependent temporal inhibition of RIDA is required for increases in ATP–DnaA when chromosomal replication is ongoing, or that the regeneration activity is more potent in rapidly growing cells than detected here. Given that the binding stoichiometry of adenine nucleotide per DnaA is ~0.4 (Sekimizu et al., 1987), the recovery of DnaA from KA474(pHB10S) was calculated to be ~30% (data not shown); probably, a significant amount of DnaA molecules stick on membrane and sediment into an insoluble fraction at preparation of cleared lysates. Taking this and a loss of DnaA molecules during washing into account, the value seems to reflect a considerably large part of DnaA molecules in this strain.

Compared with the wild-type cells, cells bearing pHB10S contain ~5-fold elevated amounts of DnaA, yet grow without causing severe overinitiation, such as is seen in the dnaAcos mutant (Kellenberger-Gujer et al., 1978; Katayama and Kornberg, 1994). Whereas in the overinitiating dnaAcos cells the mutant DnaA molecules seem to take on a predominantly initiation-competent form (Katayama, 1994; Katayama and Crooke, 1995), in cells bearing pHB10S only 20% of DnaA molecules apparently take on the ATP form. These observations suggest that rather than the cellular concentration of the ATP–DnaA molecules itself, the ratio of the ATP- and ADP-forms of DnaA is a more important determinant for the occurrence of initiation of chromosomal replication.

Roles of the sliding clamp in cell cycle regulation
The sliding clamp plays various roles in cells by associating with different proteins for DNA replication, DNA
modification, DNA replication-dependent transcription, chromatin structuring, and a check point control induced by DNA damage (Kelman and O’Donnell, 1994; Baker and Bell, 1998; Kelman and Hurwitz, 1998; Tsurimoto, 1998; Shibahara and Stillman, 1999). Therefore, an unknown protein binding to the β-clamp has an important role in the cell-cycle-dependent regulation of RIDA.

As an analogy to DnaA-β-clamp interaction, there may be an interaction of the sliding clamp proliferating cell nuclear antigen (PCNA) with ORC (origin recognition complex) and/or Cdc6p that form a pre-replicative complex needed for initiation of chromosomal replication in Saccharomyces cerevisiae (Stillman, 1996). ATP binding to ORC is necessary for affinity of ORC for the autonomously replicating sequence (Dutta and Bell, 1997). Mutational analysis of the putative ATP binding site of Cdc6p suggests that ATP binding is necessary for assembly of the pre-replicative complex (Wang et al., 1999; Weinreich et al., 1999). Whether or not the sliding clamp can stimulate hydrolysis of the bound ATP remains unclear.

Materials and methods

Bacterial strains, plasmids, media and buffers

Bacterial strains used are listed in Table II. Plasmid pKW001-3 is a derivative of a low-copy vector pST28 (Takara Biochemicals) bearing a PCR-cloned dnaN cistron at the multi-cloning site (H.Kawakami, K.Sekimizu and T.Katayama, unpublished). The structure of pHB10S is a derivative of a low-copy vector pSTV28 (Takara Biochemicals) bearing a constitutive lac cistron at the multi-cloning site (H.Kawakami, K.Sekimizu and T.Katayama, unpublished). The structure of pHB10S is described (Katayama et al., 1998), except that the medium contained 0.4 mCi/ml [32P]orthophosphate. From aliquots (2 ml), cleared lysates (750 μl) in buffer LA were prepared as described (Katayama et al., 1998), and mixed with anti-DnaA antiserum (5 μg/ml) in buffer MA and then buffer MB. After removal of the final wash solution (buffer MB), materials in the precipitates were extracted in solution (200 μl) containing 1 M HCOOH and 5 mM each of ATP, ADP and AMP. Radiolabeled nucleotides were separated by thin-layer chromatography and quantified as described (Katayama et al., 1998). Given that the binding stoichiometry of adenine nucleotide per DnaA is ~0.4 (Sekimizu et al., 1987), the recovered DnaA protein from DnaA is ~0.4 (Sekimizu et al., 1987), the recovered DnaA protein from buffer LA except that 500 mM NaCl is included. Buffer MA is the same as buffer LA except that 500 mM NaCl is included and lysosome is omitted. Buffer MB is the same as buffer LA except that NaCl, ammonium sulfate and lysosome are omitted.

**Determination of cellular levels of the adenine nucleotide forms of DnaA protein**

Cells were labeled with [32P]orthophosphate in TG medium as described (Katayama et al., 1998), except that the medium contained 0.4 mCi/ml [32P]orthophosphate. From aliquots (2 ml), cleared lysates (750 μl) in buffer LA were prepared as described (Katayama et al., 1998), and mixed with anti-DnaA antiserum (5 μg/ml) in buffer MA and then buffer MB. After removal of the final wash solution (buffer MB), materials in the precipitates were extracted in solution (200 μl) containing 1 M HCOOH and 5 mM each of ATP, ADP and AMP. Radiolabeled nucleotides were separated by thin-layer chromatography and quantified as described (Katayama et al., 1998). Given that the binding stoichiometry of adenine nucleotide per DnaA is ~0.4 (Sekimizu et al., 1987), the recovered DnaA protein from buffer LA except that 500 mM NaCl is included. Buffer MA is the same as buffer LA except that 500 mM NaCl is included and lysosome is omitted. Buffer MB is the same as buffer LA except that NaCl, ammonium sulfate and lysosome are omitted.

**Materials and methods**

**Bacterial strains, plasmids, media and buffers**

Bacterial strains used are listed in Table II. Plasmid pKW001-3 is a derivative of a low-copy vector pST28 (Takara Biochemicals) bearing a PCR-cloned dnaN cistron at the multi-cloning site (H.Kawakami, K.Sekimizu and T.Katayama, unpublished). The structure of pHB10S is as described (Katayama et al., 1998). Growth media were used were described by Sambrook et al. (1989), except for TG medium (Echols et al., 1961). Buffer LA contained 50 mM HEPES–KOH (pH 7.6 at 1 M), 250 mM NaCl, 30 mM ammonium sulfate, 0.1 mM each of ATP and ADP, 5 mM Mg(OAc)₂, 1 mM EDTA, 0.005% Triton X-100 and 5 mg/ml lysosome.

Buffer LB is the same as buffer LA except that 500 mM NaCl is included. Buffer MA is the same as buffer LA except that 500 mM NaCl is included and lysosome is omitted. Buffer MB is the same as buffer LA except that NaCl, ammonium sulfate and lysosome are omitted.

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