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

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The ATP-bound but not the ADP-bound form of DnaA protein is active for replication initiation at the *Escherichia coli* chromosomal origin. The hydrolysis of ATP bound to DnaA is accelerated by the sliding clamp of DNA polymerase III loaded on DNA. Using a culture of randomly dividing cells, we now have evidence that the cellular level of ATP–DnaA is repressed to only ~20% of the total DnaA molecules, in a manner depending on DNA replication. In a synchronized culture, the ATP–DnaA level showed oscillation that has a temporal increase around the time of initiation, and decreases rapidly after initiation. Production of ATP–DnaA depended on concomitant protein synthesis, but not on SOS response, Dam or SeqA. Regeneration of ATP–DnaA from ADP–DnaA was also observed. These results indicate that the nucleotide form shifts of DnaA are tightly linked with an epistatic cell cycle event and with the chromosomal replication system.

Keywords: cell cycle/DnaA/DNA polymerase/DNA replication/sliding clamp

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 *E.coli* chromosome 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_D 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 (Katayama and Nagata, 1991), where *dnaA46* mutation and oversupply of GroE chaperone are necessary, a similar mechanism for overinitiation is conceivable because DnaA46 protein lacks affinity for ATP (Hwang and 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*, 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; Herendeen 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 τ -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 [^{32} P]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

Fig. 1. Immunoprecipitation of DnaA protein. Cells were labeled with [^{32} P]orthophosphate (0.4 mCi/ml) in TG medium. Immunochemically precipitated materials were separated by thin-layer chromatography and detected with BAS2000 (Fujix). (A) Results of thin-layer chromatography are shown. KH5402-1 [*dnaA*⁺] and NA001 [*dnaAcos*] were grown at 42°C. KP245 [*dnaA*⁺] and KP7364 [Δ *dnaA*] were grown at 37°C. Exponentially growing cells were harvested at an optical density (A_{660}) of 0.2, and lysates (750 μ l) were prepared from an aliquot (2 ml) of the culture (see Materials and methods). Materials isolated by anti-DnaA antiserum (A) or pre-immune serum (C) were analyzed. Migration positions of ATP, ADP and AMP are indicated by arrows. (B) The indicated amounts of ATP-bound DnaA protein as the competitor were added to cleared lysates (750 μ l) that were prepared from a culture of KH5402-1 and contained 0.1 mM ATP and ADP (see Materials and methods). Nucleotides recovered by immunoprecipitation were separated by thin-layer chromatography, quantified and determined for amounts using a standard [^{32} P]orthophosphate solution. (○), ADP; (●), ATP.

(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 [*dnaN⁺*] and KA474 [*dnaN59*] were grown at 28°C in TG medium including [³²P]orthophosphate up to an optical density (A_{660}) 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

Table I. Genes for DNA replication are necessary for adenine nucleotide control of DnaA

Strain (relevant genotype)	ATP form of DnaA (%) after incubation at 42°C for				
	0 (min)	15	30	30 (+ CM)	60
HC194 (<i>dnaN59</i>) [pSTV28]	17	n.t.	60	n.t.	75
HC194 (<i>dnaN59</i>) [pKW001-3]	26	n.t.	22	n.t.	11
KA489 (<i>dnaN59 recA::Tn10</i>)	32	n.t.	67	n.t.	75
KA487 (<i>dnaN59 seqA1::tet</i>)	15	n.t.	50	n.t.	n.t.
KA486 (<i>dnaN59 dam-13::Tn9</i>)	16	n.t.	60	n.t.	n.t.
ME6299 (<i>dnaB⁺</i>)	32	n.t.	22	n.t.	n.t.
ME5491 (<i>dnaB43</i>)	33	n.t.	82	n.t.	n.t.
DG75 (<i>dnaCG⁺</i>)	29	18	24	n.t.	14
PC2 (<i>dnaC2</i>)	37	n.t.	68	30	76
KA488 (<i>dnaC2 seqA1::tet</i>)	38	n.t.	76	35	70
PC7 (<i>dnaC7</i>)	30	n.t.	72	33	n.t.
PC3 (<i>dnaG3</i>)	32	62	84	n.t.	n.t.
AX729 (<i>dnaX⁺</i>)	23	n.t.	17	24	n.t.
AX727 (<i>dnaX2016</i>)	25	n.t.	78	27	n.t.
AZ5157 (<i>ftsZ⁺</i>)	34	n.t.	28	n.t.	n.t.
AZ5159 (<i>ftsZ84</i>)	35	n.t.	30	n.t.	n.t.

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 $\pm 5\%$. 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).

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 replicational 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

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 *rmhA* gene encoding RNaseHI

Fig. 3. *oriC*, *dam* and *seqA* are dispensable for the control of adenine nucleotide forms of DnaA. Cells were grown at 37°C in TG medium including [^{32} P]orthophosphate up to an optical density (A_{660}) of 0.2. Nucleotides bound to DnaA protein were analyzed. A, anti-DnaA antiserum; C, pre-immune serum; Ω, insertion mutation; Δ, deletion mutation. Migration positions of ATP and ADP are indicated by arrows. (A) KH5402-1 [wild-type control], YT411 [*rmhA::cat*], KA429 [*ΔoriC rmhA::cat*] and KA451 [*dnaA::Tn10 rmhA::cat*] were used. (B) KH5402-1, NA117 [*dam-13::Tn9*] and NA153 [*ΔseqA*] were used.

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 *rmhA* (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

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 cycle, 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 μ g/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

Fig. 4. Oscillation of the ATP-DnaA level in synchronized culture. PC2 [*dnaC2*] (A and B) and KA488 [*dnaC2 seqA1*] were grown in TG medium containing [^{32} P]orthophosphate at 30°C, as described in Materials and methods, up to an optical density (A_{660}) of 0.025, then shifted to 40°C. After incubation for 50 min at this temperature, 2 ml aliquots were immediately shifted down to 30°C by adding 2 ml of the same medium pre-warmed at 20°C, then incubated for the indicated time in the absence (A and C) or presence (B and D) of chloramphenicol (150 μ g/ml). The broken line indicates the ATP-DnaA content of each culture at 30°C. Similar results were obtained in 2–4 independent experiments and representative data are shown. Synchrony of the replication cycle was confirmed by pulse labeling using [^{3} H]thymidine according to Zhou *et al.* (1997).

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 is 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

Fig. 5. Regeneration of ADP-DnaA to ATP-DnaA. (A) KA474 [*dnaN59*] bearing pBR322 (■, □) and pHB10S (●, ○) were grown in TG medium including [³²P]orthophosphate at 28°C up to an optical density (*A*₆₆₀) of 0.2, shifted to 42°C, then incubated in the presence (open symbols) or absence (closed symbols) of chloramphenicol (CM; 150 µg/ml). pHB10 is a derivative of pBR322 carrying the *dnaA* cistron. (B) KA474 bearing pHB10S was similarly grown at 28°C up to an optical density (*A*₆₆₀) of 0.2, then chloramphenicol (150 µg/ml) was added. An aliquot of the culture was further incubated at 28°C (○), whereas individual aliquots (2 ml) were shifted to 42°C at the time indicated by an arrow, then incubated for 30 min (●) before being used for immunoprecipitation.

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 Leonard, 1987). We speculate that 70–80% of DnaA

Fig. 6. A schematic view of coupling of the replication cycle and the DnaA's nucleotide form shift. The doubling time of cells is >65 min (A) or <65 min (B). The x-axis indicates time and the y-axis the content of the ATP-bound form of DnaA per total DnaA molecules in a cell. The dotted line in (A) shows the case where accumulation of ATP-DnaA is stimulated by the regeneration reaction of ADP-DnaA. I, initiation of chromosomal replication; T, termination of one round of chromosomal replication; d, cell division; Seq, duration of *oriC* sequestration. See the text for details.

protein must be in the ATP form for firing the initiation reaction (Figure 4).

In cells growing at a doubling time of >65 min at 37°C , chromosomal replication is completed at or before initiation of the next round of replication (Zyskind and Smith, 1992; Helmstetter, 1996). As completion of chromosomal replication terminates RIDA, the ATP-DnaA molecules accumulate by *de novo* DnaA synthesis prior to the next round of initiation in slowly growing cells, whereas the regeneration system can stimulate this accumulation (Figure 6A). When the growth rate is very slow and there is a very long inter-initiation time, we speculate that a slow rate of protein synthesis allows for only a slow accumulation of ATP-DnaA molecules so as to harmonize the initiation timing with the cell cycle. In addition, post-replicative control restraining the ATP-DnaA level may be activated in a growth-phase-dependent manner by some unknown mechanism.

When the doubling time is near or less than 65 min, accumulation of ATP-DnaA requires efficient regeneration of ADP-DnaA to ATP-DnaA and/or temporal inhibition

of RIDA during ongoing DNA replication (Figure 6B). We speculate that even when the growth rate is very rapid, 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 *in vivo*. 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 δ -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, even at the basal level. This number of ATP-DnaA molecules is sufficient to form the initiation complex at *oriC* *in vitro*. 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 (Chiaromello 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

Table II. Bacterial strains

Strain	Relevant genotype ^a	Source or reference
KA473	<i>tnaA::Tn10</i> ^b	this study
HC194	KA473 <i>dnaN59</i> (Ts) <i>tnaA</i> ⁺	Sakakibara and Mizukami (1980)
KA474	KA473 <i>dnaN59</i>	this study
KA484	KA473 <i>dnaN59 tnaA</i> ⁺ <i>zid-131::Tn5 seqA1::tet</i>	this study
KA486	KA473 <i>dnaN59 tnaA</i> ⁺ <i>zid-131::Tn5 dam-13::Tn9</i>	this study
KA489	KA473 <i>dnaN59 tnaA</i> ⁺ <i>zid-131::Tn5 recA::Tn10</i>	this study
ME6299	HfrP ₄ X8	NIG collection; Carl (1970)
ME5491	ME6299 <i>dnaB43</i> (Ts) <i>thy</i>	NIG collection; Carl (1970)
DG75	<i>leuB6 thyA47 deoC3 rpsL153</i>	Carl (1970)
PC2	DG75 <i>dnaC2</i> (Ts)	T.Ogawa; Carl (1970)
PC3	DG75 <i>dnaG3</i> (Ts)	Wechsler and Gross (1971)
PC7	DG75 <i>dnaC7</i> (Ts)	Carl (1970)
KA488	DG75 <i>dnaC2 seqA1::tet</i>	Carl (1970)
KH5402-1	<i>thyA</i> ^c	Murakami <i>et al.</i> (1987)
YT411	KH5402-1 <i>rnhA::cat thyA</i>	Katayama and Nagata (1991)
KA429	KH5402-1 <i>ΔoriC1071::Tn10 rnhA::cat thyA</i>	Katayama and Nagata (1991)
KA451	KH5402-1 <i>dnaA750::Tn10 rnhA::cat thyA</i>	this study
NA001	KH5402-1 <i>dnaAcos</i>	Katayama <i>et al.</i> (1997a)
NA117	KH5402-1 <i>dam-13::Tn9 thyA</i>	Katayama <i>et al.</i> (1997a)
NA153	KH5402-1 <i>ΔseqA1::tet thyA</i>	Katayama <i>et al.</i> (1997a)
KP245	<i>thyA</i> ^d	Miki <i>et al.</i> (1978)
KP7364	KP245 <i>ΔdnaA::spec rnhA::kan</i>	T.Miki; Katayama <i>et al.</i> (1998)
AX729	<i>str thi lac</i>	Ginés-Candelaria <i>et al.</i> (1995)
AX727	AX729 <i>dnaZX2016</i> (Ts)	Ginés-Candelaria <i>et al.</i> (1995)
AZ5157	<i>leu-63::Tn10</i> ^e	S.Hiraga
AZ5159	AZ5157 <i>ftsZ84</i> (Ts)	S.Hiraga

^aAm, amber; Ts, temperature sensitive.

^bOther genetic markers are HfrC *thyA metB uhp-1 rel-1 tnA22 T2*(?) (λ^+).

^cOther genetic markers are *ilv thr thrA*(Am) *trpE9829*(Am) *metE deo supF6*(Ts).

^dOther genetic markers are *trp his metB lac gal tsx*.

^eOther genetic markers are *ara*(Am) *lac*(Am) *galK*(Am) *galE trp*(Am) *ilv his thyA supF81*(Ts).

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 pSTV28 (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 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 lysozyme.

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 lysozyme is omitted. Buffer MB is the same as buffer LA except that NaCl, ammonium sulfate and lysozyme 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 μ l) in buffer LA were prepared as described (Katayama *et al.*, 1998), and mixed with anti-DnaA antiserum (5 μ l) that had been incubated in 100 μ l of a cleared lysate (30 mg protein/ml) of KP7364 [Δ *dnaA*] for 30 min at 0°C. Protein A-Sepharose (60 μ l; 50% slurry in buffer MB) was added, and mixed by tapping and rotation for 30 min at 2°C. Precipitates were isolated by centrifugation (1500 g for 1 min at 2°C), and washed twice in chilled buffer LB (1 ml). Washing of precipitates was similarly repeated using chilled buffer MA and then buffer MB. After removal of the final wash solution (buffer MB), materials in the precipitates were extracted in solution (20 μ l) 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 KH5402-1 and KA474 in this assay was ~60% of the total cellular DnaA protein determined by immunoblotting (data not shown).

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