Cell, Vol. 94, 61–71, July 10, 1998, Copyright ©1998 by Cell Press

The Initiator Function of DnaA Protein Is Negatively Regulated by the Sliding Clamp of the *E. coli* Chromosomal Replicase

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Summary

The β subunit of DNA polymerase III is essential for negative regulation of the initiator protein, DnaA. DnaA inactivation occurs through accelerated hydrolysis of ATP bound to DnaA; the resulting ADP-DnaA fails to initiate replication. The ability of β subunit to promote DnaA inactivation depends on its assembly as a sliding clamp on DNA and must be accompanied by a partially purified factor, IdaB protein. DnaA inactivation in the presence of IdaB and DNA polymerase III is further stimulated by DNA synthesis, indicating close linkage between initiator inactivation and replication. In vivo, DnaA predominantly takes on the ADP form in a β subunit–dependent manner. Thus, the initiator is negatively regulated by action of the replicase, a mechanism that may be key to effective control of the replication cycle.

Introduction

Initiation of chromosomal replication is a critical event in the regulation of the cell cycle in prokaryotes and eukaryotes. In *Escherichia coli*, a round of replication commences with the binding of the initiator protein, DnaA, to the unique chromosomal origin (*oriC*) (Kornberg and Baker, 1992; Skarstad and Boye, 1994; Messer and Weigel, 1996).

Reconstitution of replication from *oriC* with defined components led to the identification of a progression of steps in the initiation process that includes: (i) the cooperative binding of approximately 20 DnaA protein molecules to *oriC* to form an initial complex, (ii) a transition from the initial complex to an open complex in which a region of tandem AT-rich 13-mer repeats denatures so that single-stranded (ss) DNA is exposed, and (iii) the formation of a prepriming complex upon the introduction of DnaB helicase into the forks of the melted DNA where it further expands the region of ssDNA. Subsequently, DnaG primase forms a mobile complex with DnaB helicase of the prepriming complex and synthesizes RNA primers. A homodimer of the β subunit of DNA polymerase (pol) III is loaded, with the aid of the γ complex (a subassembly of pol III), as a so-called "sliding clamp" onto the primed DNA (Kelman and O'Donnell, 1995; Herendeen and Kelly, 1996; Baker and Bell, 1998). With the complete assembly of pol III holoenzyme, synthesis of complementary DNA ensues.

An indication of the importance of DnaA protein (the *dnaA* gene product) as a molecular switch for initiation of chromosome replication is the behavior of cells bearing the mutant *dnaAcos* allele; at nonpermissive temperatures overinitiation at *oriC* occurs (Kellenberger-Gujer et al., 1978; Katayama, 1994; Katayama and Kornberg, 1994). Purified DnaAcos mutant protein is active for minichromosome replication in vitro, but unlike wild-type DnaA protein, it is defective for the high affinity binding of ATP and ADP (Katayama, 1994) and insensitive to the DnaA-inactivating factor (RIDA, *R*egulatory *I*nactivation of *Dna**A*; formerly named IdaA) (Katayama and Crooke, 1995). Wild-type DnaA protein has strong affinity for ATP and ADP (K_D of 0.03 and 0.10 μM, respectively). ATP-DnaA protein, but not the ADP-bound form, is able to initiate minichromosome replication in vitro. The ADP-bound form can form an initial complex but fails at opening the duplex DNA of *oriC* (Sekimizu et al., 1987; Crooke et al., 1993).

RIDA is an activity found in a soluble protein extract that inactivates wild-type DnaA protein in a manner dependent on ATP and DNA (Katayama and Crooke, 1995). This inactivating action is targeted to DnaA protein; other proteins involved in DNA replication are not affected. DnaA protein's loss of initiation activity is apparently specific for replication from *oriC*, since RIDA-treated DnaA protein is still able to assemble and function in an ABC primosome in which DnaA protein bound to a hairpin structure interacts with DnaB helicase to initiate replication of ssDNA (Masai et al., 1990; Marszalek and Kaguni, 1994). A reason that RIDA-treated DnaA protein is defective for initiating replication at *oriC* may be its inability to melt duplex DNA, a property similar to that of the ADP-bound form of DnaA protein (Katayama and Crooke, 1995). Thus, RIDA may hydrolyze ATP bound to DnaA protein, or enhance the weak intrinsic ATPase of DnaA protein, to yield replicatively inactive ADP-DnaA protein. The overinitiation phenotype seen in the *dnaAcos* mutant suggests that RIDA control of the adenine nucleotide form of the initiator may be critical for proper regulation of the frequency with which chromosomal replication is initiated.

Here, we demonstrate that RIDA activity is reconstituted with the β subunit of pol III and that RIDA-mediated inactivation of DnaA indeed results from a conversion of the adenine nucleotide form of DnaA protein. Formation of the β sliding clamp by the γ complex of pol III and inclusion of partially purified IdaB protein fraction are necessary for RIDA activity, and concomitant DNA synthesis stimulates this IdaB-dependent inactivation of DnaA protein. Additionally, we find that β subunit in vivo functions to control the cellular distribution of DnaA protein between the ATP and ADP forms. Cumulatively,

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these data suggest that components of the chromosomal DNA replisome interact with the initiator protein in a way that negatively regulates initiation at *oriC*.

Results

DnaA protein's capacity to initiate minichromosomal replication can be inactivated by an activity termed RIDA, which may act by stimulating the hydrolysis of DnaA-bound ATP (Katayama and Crooke, 1995). To examine this possibility, [α-^{32}P]ATP-bound DnaA protein was incubated with a cell extract containing RIDA, immunoprecipitated, and radiolabeled adenine nucleotides were analyzed by thin layer chromatography (Kurokawa et al., 1998). When the cell extract was fractionated by either anion exchange (DE52) chromatography or gel filtration, the ability to inactivate DnaA protein for *oriC* replication and an activity that promotes the hydrolysis of DnaA-bound ATP coeluted with similar increases in specific activities (Kurokawa et al., 1998). As such, RIDA was assumed to be responsible for the stimulated hydrolysis of DnaA-bound ATP, and this was the activity monitored during the further purification of RIDA.

RIDA Involves Three Components

When proteins in the fraction III (DE52 fraction) were separated by gel filtration (Superdex-200), the activity capable of hydrolyzing DnaA-bound ATP was found in one major peak with a corresponding molecular mass of ca. 150 kDa (fraction IV-150), as previously reported (Katayama and Crooke, 1995) (Figures 1A and 1B).

Although the activity in the fraction III is rather stable, the activity in fraction IV-150 is not. However, we found that addition of the column fractions corresponding to a molecular mass exceeding 500 kDa (fraction IV-500) restored stability (data not shown) and, at the same time, greatly stimulated the activity in the fraction IV-150 (Figure 1B); the stimulation efficiency was 18-fold (data not shown). During further purification of fraction IV-150, fraction IV-500 was included in assays. A minor peak of activity around ca. 300 kDa may arise from overlapping traces of activities in fractions IV-150 and IV-500 (Figure 1B). Eluting coincidently with the ATPase activity in each peak was the ability that inactivates DnaA protein for *oriC* replication (data not shown).

Further purification of fraction IV-150 by cation exchange (Mono S) chromatography (Figure 1A) separated the components needed for hydrolyzing DnaA-bound ATP into two distinct fractions; one, named IdaB, was recovered in the flow-through fraction, while the other bound to the resin and eluted with 0.15 M NaCl (fraction V). Significant activity was seen only when the IdaB fraction and fraction V were mixed and assayed in the presence of fraction IV-500 (see below).

The β Subunit of Pol III Inactivates DnaA

The activity in fraction V was further purified through an anion exchange (Mono Q) column (Figures 1C and 1D). Results of microsequencing revealed that the amino-terminal sequence of a 40 kDa protein that coeluted with the activity coincided completely to that for the β subunit (DnaN protein) of pol III. Consistent with this identification is that the molecular mass of the β subunit is 42 kDa (Ohmori et al., 1984).

The β subunit was able to reconstitute the activity for promoting the hydrolysis of DnaA-bound ATP (Figure 2A). Highly (>90%) purified β subunit, obtained from an overproducing strain (Johanson et al., 1986), had a specific activity indistinguishable from fraction V (Figure 2A) and fraction VI (data not shown).

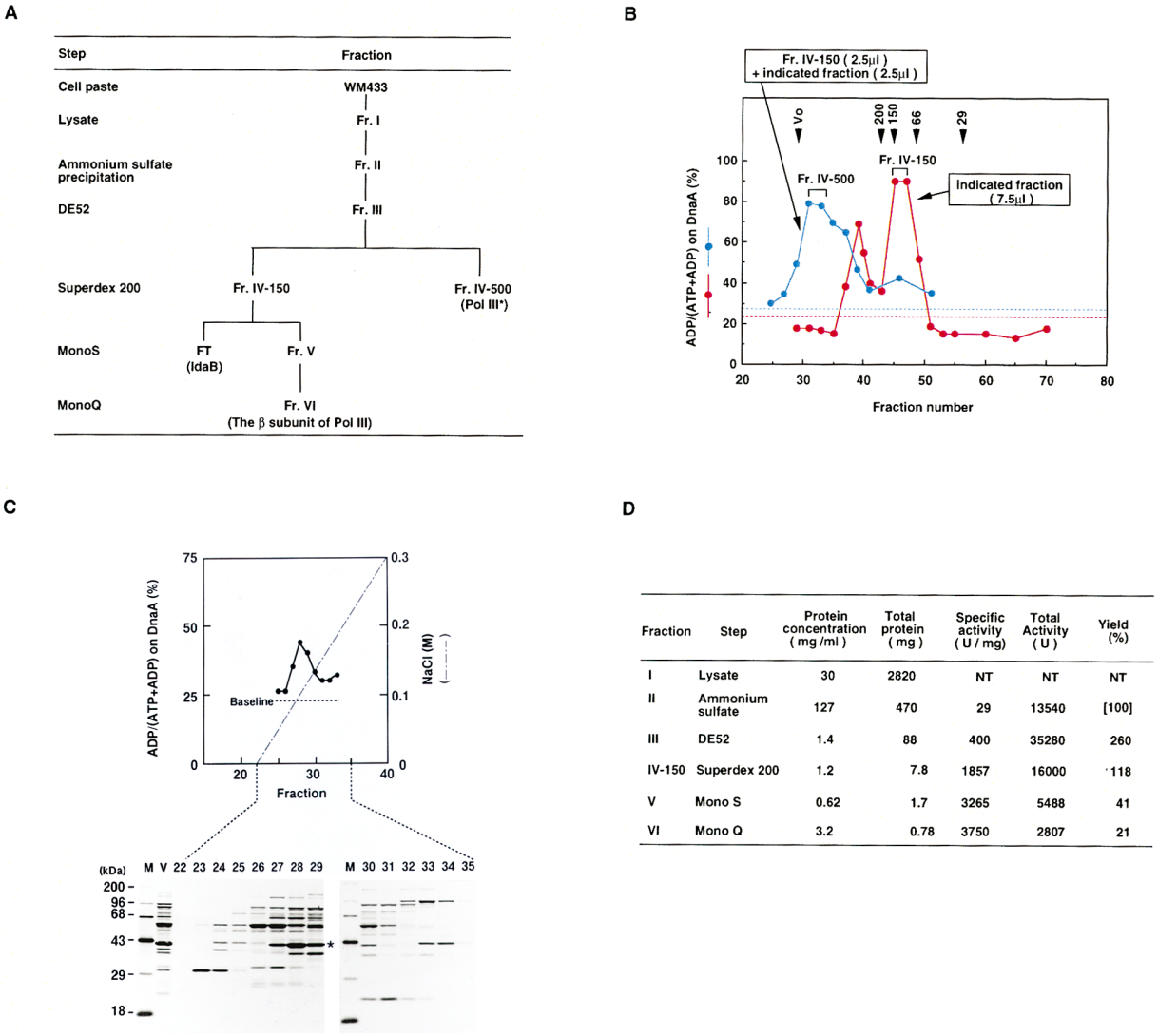
For further confirmation, we compared the RIDA activities of extracts prepared from a temperature-sensitive *dnaN* mutant cultured at permissive and nonpermissive temperatures (Figures 2B and 2C). The β subunit of *dnaN59* (Ts) cells denatures when the cells are grown at 42.5°C (Sakakibara and Mizukami, 1980; Burgers et al., 1981). Whereas the extract of cells having functional β subunit inactivated DnaA protein for minichromosomal replication (Figure 2B) and stimulated the hydrolysis of DnaA-bound ATP (Figure 2C), the extract from cells cultured at the nonpermissive temperature was much less efficient for both. When this extract was supplemented with purified β subunit, DnaA-inactivating capacity was clearly restored (Figures 2B and 2C). These results indicate that β subunit is necessary for inactivating DnaA protein by generating the ADP form. The loss of ca. 50% of DnaA residual activity seen with 7.5 μg of Fr. II prepared from cells grown at the nonpermissive temperature (Figures 2B and 2C) may arise from an additional DnaA-inactivating factor; possibly related to this is that Fr. II prepared from stationary phase cells can inactivate DnaA protein, even in the absence of DNA (unpublished data).

Pol III* Renders the β Subunit Competent for Inactivation of DnaA

Pol III holoenzyme (900 kDa) consists of ten different polypeptides (Maki et al., 1988; Herendeen and Kelly, 1996): pol III core (170 kDa) is a complex of the α (polymerase), ϵ (3′ to 5′ exonuclease), and θ subunits; the τ subunit forms a complex with a dimer of pol III core (pol III', 400 kDa; McHenry, 1982) and binds the γ complex (Dallmann and McHenry, 1995; Onrust et al., 1995); the γ complex (200 kDa), which has weak affinity to DNA and contains the γ, δ, δ', χ, and ψ subunits, interacts with the β subunit to direct its loading onto DNA (Wickner, 1976; Maki and Kornberg, 1988; Stukenberg et al., 1991; Reems et al, 1995); and pol III* is the assembly composed of the γ complex, τ subunit, and pol III core dimer. The complete pol III holoenzyme contains pol III* and the β subunit.

With this newly defined role of β subunit, fraction IV-500 (Figure 1A), which has a large molecular mass exceeding 500 kDa, was examined to see whether its capacity to stimulate β subunit–dependent hydrolysis of DnaA-bound ATP is derived from a subassembly of pol III holoenzyme. Highly purified pol III* (pol III lacking the β subunit; Lasken and Kornberg, 1987) had a specific activity for the stimulation of the β subunit (Figures 3A and 3B) similar to that of the fraction IV-500.

Immunoblot analysis revealed that the ratio of pol III α subunit to γ subunit in the fraction IV-500 was approximately 1:1 (data not shown), the same ratio as that seen for pol III* (Maki et al., 1988; Onrust et al., 1995). This supports that the entity in the fraction IV-500 responsible for stimulation of the β subunit is mainly pol III*.



Fraction	Step	Protein concentration (mg /ml)	Total protein (mg)	Specific activity (U / mg)	Total Activity (U)	Yield (%)
I	Lysate	30	2820	NT	NT	NT
II	Ammonium sulfate	127	470	29	13540	[100]
III	DE52	1.4	88	400	35280	260
IV-150	Superdex 200	1.2	7.8	1857	16000	118
V	Mono S	0.62	1.7	3265	5488	41
VI	Mono Q	3.2	0.78	3750	2807	21

Figure 1. Isolation of the β Subunit of Pol III

(A) Purification scheme. Fr., fraction; FT, flowthrough.

(B) Gel filtration chromatography. Proteins in fraction III (11.5 mg, 8.2 ml) were concentrated by ammonium sulfate precipitation and resolved with a Superdex 200 HR10/30 column. Each fraction (7.5 μl; red circle) and each fraction (2.5 μl; blue circles) mixed with a pool of fraction IV-150 (2.5 μl) were assessed for the ability to promote hydrolysis of DnaA-bound ATP in the presence of RF I (supercoiled) form of *oriC* plasmid (M13E10; 200 ng; 35 fmol as circles). The weak, intrinsic ATPase activity of DnaA protein alone is shown (red broken line), as well as that for DnaA protein mixed with the pool of fraction IV-150 (2.5 μl; blue broken line). The elution positions of protein standards and their molecular mass (kDa) are indicated. Vo, void volume.

(C) Anion-exchange chromatography. Fraction V (135 μg, 0.5 ml) was loaded onto a MonoQ PC1.6/5 column (SMART system, Pharmacia Biotech), and bound proteins were eluted with a NaCl gradient (1 ml). Portions (0.25 μl) of fractions (30 μl) were assayed for hydrolysis of DnaA-bound ATP in the presence of fraction IV-500 (290 ng) and IdaB fraction (1.5 μg); baseline (broken line) indicates the hydrolysis of DnaA-bound ATP in the absence of fraction V. Proteins in fraction 22–35 (3.0 μl each) were separated by SDS-polyacrylamide (10%) gel electrophoresis and visualized with silver staining. A protein of 40 kDa (indicated by asterisk) coeluted with the activity to promote hydrolysis of DnaA-bound ATP. M, molecular mass protein standards; V, fraction V.

(D) Purification table. Fraction I was prepared from 80 g of frozen cell paste of WM433[*dnaA204*]. Fraction IV-150 was assayed in the presence of fraction IV-500 (290 ng). Fractions V and VI were assayed in the presence of fraction IV-500 (290 ng) and IdaB fraction (1.5 μg). Only 26% of fraction IV-150 was used for preparation of fraction V, and thus the values of fractions V and VI were corrected by a factor of 4.4. One unit (U) of activity corresponds to a 2-fold stimulation in the hydrolysis of ATP bound to DnaA protein (1 pmol) when assayed for 20 min at 30°C.

The γ Complex of Pol III Is Essential for the Inactivation of DnaA Protein

Isolated γ complex was found to efficiently replace pol III* in the stimulation of the hydrolysis of DnaA-bound ATP, whereas pol III core, τ subunit, and pol III' (a complex of pol III core and the τ subunit) could not (Table 1, Experiment 1). In addition, their presence did not inhibit the DnaA-inactivating function of the γ complex. The molar ratio of γ complex to β subunit needed for the optimal activity was 1:10 (data not shown), suggesting that the γ complex may recycle in the process of DnaA protein inactivation.

Omission of purified β subunit or γ complex led to a loss of the accelerated hydrolysis of DnaA-bound ATP

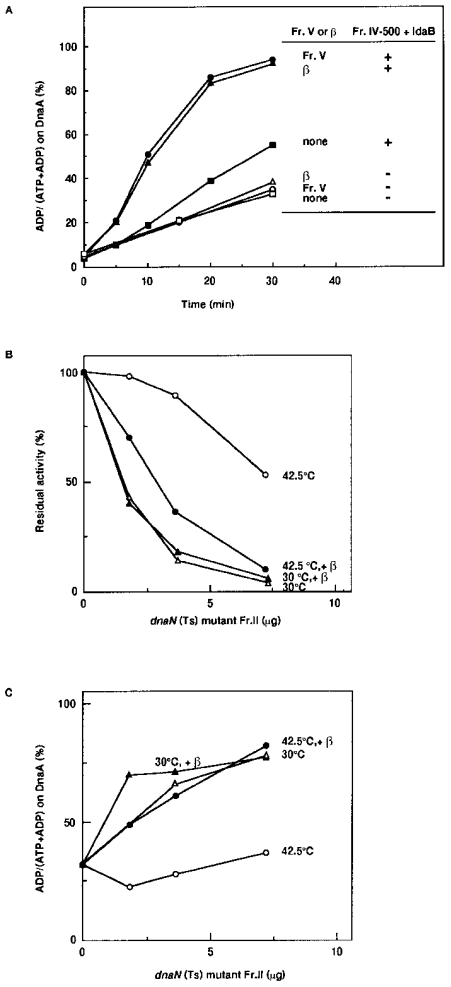


Figure 2. Pol III β Subunit Is Necessary for DnaA Inactivation

(A) Conversion of ATP-DnaA protein to the ADP form. Purified DNA polymerase III β subunit or fraction V (100 units each, as β-subunit activity for ssDNA replication) were examined for their ability to promote hydrolysis of ATP bound to DnaA protein. The reaction mixtures contained fraction IV-500 (290 ng), IdaB fraction (1.5 μg), and M13E10 RF I DNA (200 ng). Mixtures were incubated at 30°C. β-subunit activity (units) was determined in an assay for replication of G4 ssDNA, as described (Maki et al., 1988). Specific activities of β subunit and fraction V were 1.4×10^6 and 4.4×10^5 units/mg, respectively.

(B and C) In vitro complementation of *dnaN* activity. Protein extracts (Fr. II) were prepared from the *dnaN59* (Ts) mutant that was (42.5°C) or was not (30°C) cultured at the nonpermissive temperature for 30 min prior to being harvested. The extracts were added to DnaA protein inactivation reaction mixtures (10 μl) containing 2.0 pmol of unlabled DnaA protein (B) or [α-^{32}P] ATP-bound DnaA protein (C), and incubated at 30°C for 25 min in the presence of M13mp19 RFI DNA (400 ng) (see Experimental Procedures). Where indicated, purifed β subunit (0.81 pmol as dimer) was also included. (B) Following the DnaA inactivation reactions, portions (5 μl) were transferred to in vitro DNA synthesis complementation reactions (25 μl, 20 min). DnaA protein activity remaining after the first incubation (Residual activity) is shown. (C) Hydrolysis of [α-^{32}P] ATP bound to DnaA protein in 5 μl portions was measured following the inactivation reaction.

(Table 1, Experiment 2), indicating that both components of the holoenzyme are required for the activity. This agrees with the small but significant activity of γ complex

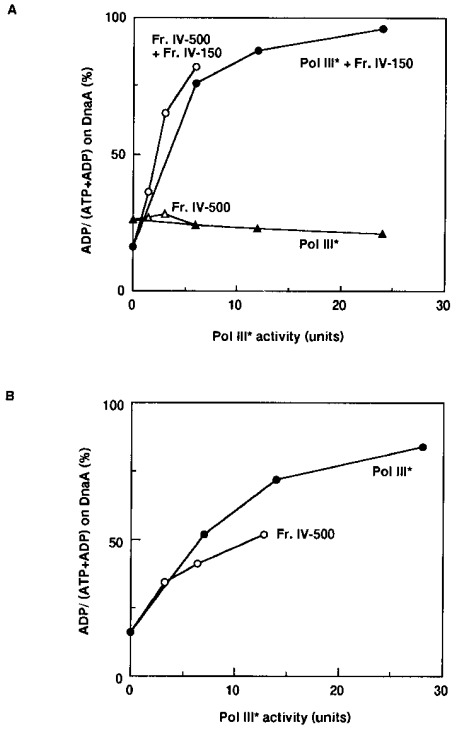


Figure 3. Pol III* Promotes the β Subunit for DnaA Inactivation

Purified pol III* and fraction IV-500 were assayed for their capacity to stimulate hydrolysis ATP bound to DnaA protein in the presence of M13E10 RFI DNA (200 ng) (see Experimental procedures). Pol III* activity (units) was determined in an assay for replication of G4 ssDNA, as described (Maki et al., 1988).

(A) The reaction mixture in the presence or absence of fraction IV-150 (1.6 μg) was incubated at 30°C for 20 min. Fraction IV-150 contains β subunit and IdaB.

(B) The reaction mixture containing purified β subunit (4 pmol) and IdaB fraction (1.5 μg) was incubated at 30°C for 15 min.

found (data not shown) in the fraction IV-150 obtained during gel filtration (Figures 1B and 1D).

β Subunit Must Be in the Form of a Sliding Clamp to Inactivate DnaA Protein

The ATP-dependent action of γ complex promotes the formation of the β sliding clamp, the ring-shaped β subunit dimer that encircles DNA (Herendeen and Kelly, 1996). The formation of this β clamp occurs on primed DNA, RF II (nicked circular) DNA, and under certain conditions, also on the RF I form of DNA (Stukenberg et al., 1991; M. O'Donnell, personal communication). Furthermore, following formation of the β clamp, γ complex is released from this complex (Maki and Kornberg, 1988; Stukenberg et al., 1991; Naktinis et al., 1995). Thus, the roles of γ complex and the soluble versus sliding clamp forms of β subunit in the inactivation of DnaA protein were examined (Table 1, Experiment 3).

When β subunit, γ complex, ATP, and IdaB were incubated in the presence of RF I *oriC* DNA, followed by gel filtration separation of β_2-DNA complexes from γ complex and unbound β subunit, the DNA-containing void-volume fraction possessed the activity for accelerating the hydrolysis of DnaA-bound ATP (Table 1, Experiment 3, line 1). Approximately 70% of the DNA was recovered in this fraction, and quantitation of β subunit

Table 1. Activation of β Subunit for Interaction with DnaA Protein

Experiment 1. Requirement of the γ Complex Function

Proteins Added				ADP/(ATP+ADP) on DnaA (%)
Core	τ Subunit	γ Complex	Pol III*	
−	−	−	−	27
+	−	−	−	24
−	+	−	−	27
−	−	+	−	99
+	+	−	−	23
+	−	+	−	98
−	+	+	−	97
+	+	+	−	99
−	−	−	+	96

Experiment 2. Necessity of γ Complex and β Subunit for Hydrolysis of ATP Bound to DnaA Protein

Factors Omitted	ADP/(ATP+ADP) on DnaA (%)
None (complete)	89
β subunit	27
γ complex	27
β subunit, γ complex	28
β subunit, γ complex, IdaB	26

Experiment 3. β Subunit Loaded onto DNA by γ Complex Is Able to Interact with DnaA Protein

1st Incubation	2nd Incubation	ADP/(ATP+ADP) on DnaA (%)	
Factors Omitted	Factors Added	Before Spin	After Spin
None (complete)	None	91	68
ATP	None	89	27
γ complex	γ complex	85	28
β subunit	β subunit	90	28
β subunit, γ complex	None	34	25
M13E10	M13E10	92	31
M13E10	None	8	NT

(Experiment 1) Pol III core (0.3 pmol), γ complex (0.3 pmol), τ subunit (0.3 pmol as dimer), and pol III* (0.13 pmol) were examined for their ability to promote hydrolysis of ATP bound to DnaA protein in the presence of [α-^{32}P] ATP-DnaA protein (1.0 pmol), β subunit (0.98 pmol as dimer), IdaB fraction (1.5 μg), M13E10 DNA (200 ng; 35 fmol as circles), and ATP (2 mM). Reactions (25 μl) were incubated for 20 min at 30°C, and nucleotides bound to DnaA protein were assessed (see Experimental Procedures). For the formation of pol III', pol III core and τ subunit were incubated at 0°C for 1 hr before being added to the above reactions.
(Experiment 2) The complete reaction (30 μl) includes M13E10 (200 ng), β subunit (2.4 pmol as dimer), γ complex (0.5 pmol), IdaB fraction (1.5 μg), and [α-^{32}P] ATP-DnaA protein (1.0 pmol). The buffer conditions of the system for reconstituted replication of minichromosomes were used except that ATP (2 mM) is the only nucleotide included. After incubation at 30°C for 20 min, nucleotides bound to DnaA protein were determined. Similar experiments using the buffer conditions as for Experiment 1 yielded similar results.
(Experiment 3) The complete reaction (first incubation; 40 μl) included the amounts of M13E10 DNA, β subunit, and γ complex as for Experiment 2; for certain reactions components in this first incubation were omitted, as indicated. Samples were incubated at 30°C for 30 min in the buffer conditions as for Experiment 2. Half of each sample from the first incubation (Before Spin) was added to a DnaA inactivation reactions using the same buffer conditions as the first incubation (second incubation); the other half was filtered with a spin column (0.5 ml bed volume of Sephacryl-S400 HR, Pharmacia Biotech) and the void-volume (After Spin) was collected and added to a DnaA inactivation reaction as above (second incubation). DnaA inactivation reactions (second incubation; final volume of 30 μl) contained [α-^{32}P] ATP-DnaA protein (1.0 pmol), IdaB fraction (1.5 μg), and 2 mM ATP and were supplemented with the indicated factor. Following the second incubation (30°C, 20 min) nucleotides bound to DnaA protein were determined.

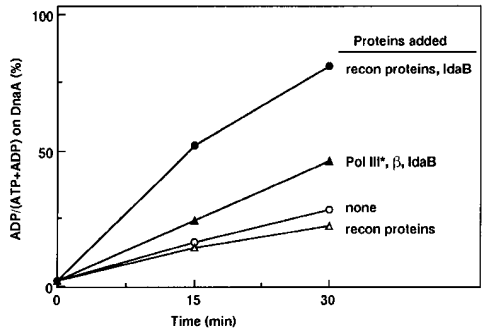
suggested that around five β dimers were loaded onto each circle of plasmid DNA. γ complex in the void-volume fraction was below the level of detection of clamp-loading function (<4% of input; <0.6 γ complex/plasmid).

However, when either ATP, γ complex, or β subunit was omitted prior to the gel filtration step, and then the missing component was added back to the void-volume fraction, accelerated hydrolysis of DnaA-bound ATP was not observed; only 30% of DnaA protein was found in the ADP form (lines 2–4), the level of ADP-DnaA protein produced by the slow DNA-dependent, intrinsic ATPase of DnaA protein (lines 5 and 6). When DNA was omitted, only 8% of the ATP-DnaA was converted into the ADP form (line 7), confirming the necessity for the interaction of DnaA protein or β subunit (or both) with DNA for DnaA inactivation. Taken together, these results suggest that the β sliding clamp generated by the ATP-dependent action of γ complex, and not the non–DNA-bound form of β subunit, is the form capable of inactivating DnaA protein. Furthermore, the essential role of the γ complex in DnaA inactivation is that of a clamp loader, since after the formation of the β sliding clamp, γ complex is dispensable.

DNA Replication Further Stimulates the Inactivation of DnaA Protein

We found that inactivation of DnaA protein is further enhanced by the concomitant DNA synthesis action of pol III in an *oriC* plasmid replication system (Figures 4A and 4B) reconstituted with highly purified proteins

A



B

	ADP/ (ATP+ADP) on DnaA (%)		
	oriC$^+$		*oriC* ΔLMR
Proteins added	+dNTP	-dNTP	+dNTP
(none)	30	26	27
Recon proteins	20	16	17
Pol III*, β, IdaB	40	42	39
Recon proteins, IdaB	83	28	22

Figure 4. Concomitant DNA Replication Enhances Inactivation of DnaA Protein

(A) Indicated factors and DnaA protein (1 pmol) were added to a reconstituted *oriC* plasmid replication system, incubated at 30°C, and the ratio of the nucleotide forms of DnaA protein was determined. "Recon proteins" indicates the inclusion of a mixture of DnaB helicase, DnaC, DnaG primase, SSB, HU, gyrase, pol III*, and β subunit of pol III* (see Experimental Procedures). IdaB fraction (1.5 μg) was included, as indicated.

(B) Conditions were as above, except for the presence or absence of dNTPs, and the use of an intact (*oriC*$^+$) or mutant (ΔLMR) *oriC* plasmid (lacks the 13 mer region). Reactions were incubated at 30°C for 30 min. When an intact *oriC* plasmid and dNTPs were used, approximately 200–300 pmol of DNA was synthesized. IdaB did not inhibit or stimulate DNA replication.

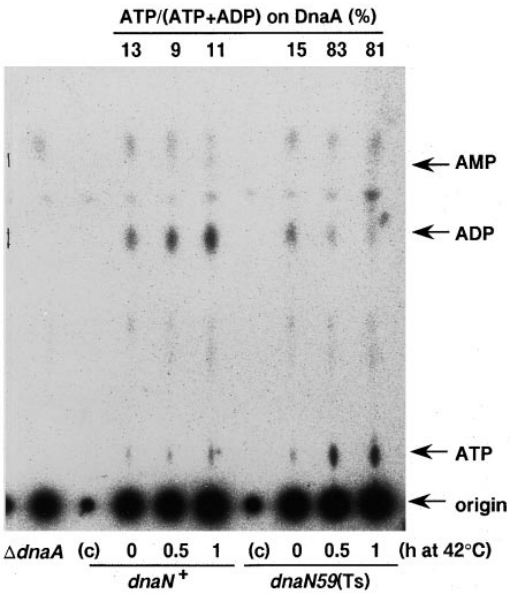


Figure 5. Functional β Subunit Is Needed for Inactivation of DnaA Protein In Vivo

Cells were metabolically labeled with ^{32}P-orthophosphate. DnaA protein in lysates was isolated by immunoprecipitation, and nucleotides bound to DnaA protein were assayed by TLC (see Experimental Procedures). The Δ*dnaA* mutant, grown at 37°C, serves as an indicator of the immunoprecipitation's specificity for DnaA protein. The *dnaN*(Ts) mutant and its wild-type parent (*dnaN*$^+$) carried pHB10S and were grown at 30°C and then shifted to 42°C. After incubation for the indicated time at the higher temperature, portions of the cultures were harvested. (c) marks samples where preimmune serum was used as a negative control. Migration positions of AMP, ADP, and ATP are shown.

(Ogawa et al., 1985; van der Ende et al., 1985; Crooke, 1995a). In these conditions, the hydrolysis of DnaA-bound ATP was less efficient than that observed in the conditions used in Figure 3 but was significantly stimulated in a manner dependent on the inclusion of both the proteins that support *oriC* plasmid replication and IdaB (Figure 4A). This reconstituted replication system synthesized approximately 200 pmol of DNA, and the IdaB fraction did not adversely affect DNA synthesis (data not shown). The effect of the reconstituted replication system per se on the rate of hydrolysis of DnaA-bound ATP was minimal, being as slow as that when pol III and the IdaB fraction were missing.

The importance of DNA synthesis, rather than initiation at *oriC*, for the IdaB-dependent stimulated hydrolysis of DnaA-bound ATP was observed (Figure 4B). The stimulation was also dependent on the inclusion of dNTPs as well as a functional *oriC* sequence. An *oriC* plasmid, which is inert for initiation because the 13-mer region has been deleted and thus can not be replicated (Bramhill and Kornberg, 1988), was unable to stimulate DnaA inactivation. Similarly, a minichromosome possessing an intact *oriC* sequence, which can complete the initiation process but cannot be replicated in the absence of dNTPs, also failed to stimulate inactivation of DnaA protein. However, when dNTPs were included so that DNA synthesis was possible, greater than 80% of the DnaA protein was converted into the replicatively inert ADP form. Thus, the stimulation of DnaA inactivation is evidently dependent on DNA chain elongation and not solely on the act of initiating at *oriC*. Also, the stimulation is not apparently enhanced by conditions unique to the reconstituted replication system, since a similar enhancement was observed when a replicatively active, crude (fraction II) cell extract was utilized (data not shown).

β Subunit Is Required for Inactivation of DnaA Protein In Vivo

To determine whether the β subunit is involved in the inactivation of DnaA protein in vivo, the levels of ADP-DnaA versus ATP-DnaA protein in lysates prepared from cells with differing levels of functional β subunit were measured (Figure 5). Cells were metabolically labeled with ^{32}P-orthophosphate, shifted to an elevated temperature (42°C) for a period, lysed, and DnaA protein with tightly bound nucleotide was recovered by immunoprecipitation.

In cells having an intact *dnaN* gene, approximately 85% of the precipitated DnaA protein was in the ADP-bound form (Figure 5). In contrast, only 20% of the DnaA protein was as ADP-DnaA when a temperature-sensitive

dnaN59 strain cultured at 42°C was used. The small amount of the ADP form of DnaA protein detected in the *dnaN59* mutant grown at 42°C may persist from the period before the temperature shift, or it may be formed by residual activity of the mutant β subunit at 42°C. Thus, these results are consistent with the findings in vitro and indicate the essential role in vivo of β subunit for inactivation of DnaA protein.

Since newly synthesized DnaA protein will likely be in the ATP form, given that ATP is present at much higher cellular concentrations than ADP, these results suggest that the ATP-bound form of DnaA protein is rapidly converted to ADP-DnaA in a manner dependent on the *dnaN* gene product, the β subunit of pol III holoenzyme.

Discussion

Regulatory Inactivation of DnaA Protein Involves the β Clamp of Pol III: Interaction of the Initiator and Replicase

The replicatively active ATP-bound form of DnaA protein is converted to inactive ADP-DnaA by the β subunit of pol III and IdaB fraction. For β subunit to play its role in inactivating DnaA protein, formation of the sliding clamp by the pol III γ complex is needed (Table 1). Concomitant DNA synthesis further stimulates inactivation of DnaA protein (Figure 4). Thus, there seems to be three means of generating the ADP form of DnaA protein from ATP-DnaA: (i) the weak, DNA-dependent intrinsic ATPase activity of DnaA protein (Sekimizu et al., 1987); (ii) the reaction that depends on β subunit in the configuration of a sliding clamp and the IdaB protein (a protein postulated to be present in the IdaB fraction and is necessary for DnaA inactivation) (Figure 6A); and (iii) the enhanced conversion that occurs during concomitant DNA synthesis.

The first of these, DnaA protein's own ATPase activity, one which does not require a specific DNA sequence, millimolar levels of ATP, or other proteins (Sekimizu et al., 1987), may be integral to the other two mechanisms. Interaction of DnaA protein with the β clamp and IdaB may stimulate this intrinsic ATPase activity. The ADP-bound form of DnaA, generated from the ATP form by the intrinsic ATPase activity, is inactive for *oriC* replication (unpublished data).

The second mode is that of β clamp–dependent inactivation of DnaA protein (Figure 6A). The non–DNA-bound form of β subunit does not support inactivation of DnaA. This specificity for the form of β subunit loaded onto DNA may be a determinant that couples negative control of DnaA protein with DNA replication. β subunit may undergo a conformational change when loaded onto DNA and in conjunction with IdaB protein, interact with DnaA protein. Alternatively, the requirement for the β subunit being assembled onto DNA may be dictated by IdaB protein, which needs simultaneous interaction with DNA and β subunit for inactivation of DnaA to occur.

The rate at which DnaA protein is inactivated in an IdaB-dependent manner increases significantly with concomitant DNA synthesis. This indicates a coupling of DNA replication with inactivation of DnaA protein and suggests cross-talk between the initiator and replicase.

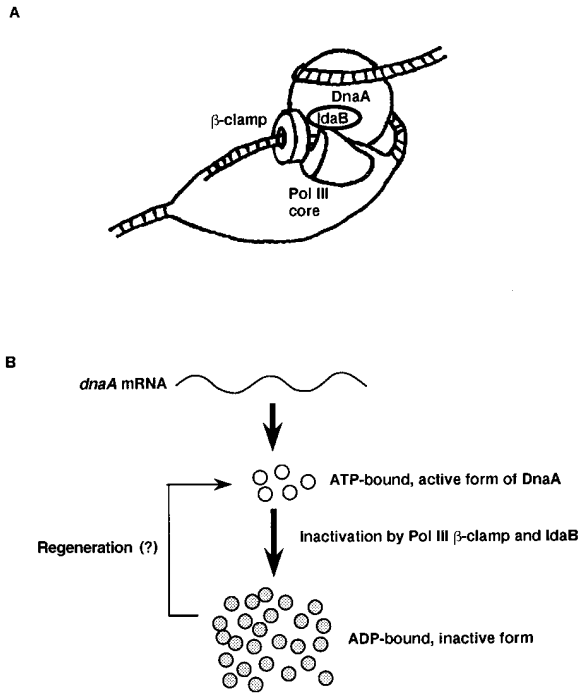


Figure 6. Regulatory Inactivation of DnaA Protein

(A) A model for the inactivation of DnaA protein at *oriC* by the β subunit sliding clamp. Only one polymerase is shown.

(B) A model for in vivo regulation of DnaA protein. Newly synthesized DnaA protein adopts the active ATP form but then is rapidly changed to the inactive, ADP form in a β subunit–dependent manner. Regeneration of the ADP form to the ATP form is slower than the conversion of ATP-DnaA into the ADP form.

There is a possibility that this does not require direct DnaA–β clamp interaction but instead depends on interaction of DnaA protein with another protein. However, that IdaB is still required in addition to the proteins necessary for replication implies a common basis between the β subunit and replication-dependent modes of inactivating DnaA protein. Since isolated replication intermediates generated during *oriC* plasmid synthesis caused, in the presence of IdaB, elevated hydrolysis of DnaA-bound ATP (unpublished results), a fully assembled replisome may have enhanced interaction with DnaA protein. At a replication fork, pol III may interact with Primase (Zechner et al., 1992; Tougu and Marians, 1996) and other proteins. Perhaps related to DnaA inactivation are the observations that pol III at a replication fork interacts with DnaB helicase (Kim et al., 1996; Yuzhakov et al., 1996), and DnaB helicase associates with DnaA protein (Marszalek and Kaguni, 1994).

Coupling inactivation of the initiator with replicase function permits exquisite regulation of the chromosomal replication cycle. Immediately following initiation at *oriC*, a cell's capacity to initiate replication must rapidly be decreased so that extra, secondary initiations are prevented. DNA replication coupled to inactivation of DnaA protein would serve as a powerful mechanism to prevent reinitiations at recently replicated origins (Figure 6A). Once chromosomal replication is completed, this negative regulation is lost as disassembly of pol III occurs, and the capacity for DnaA protein to initiate DNA

synthesis can then increase in preparation for a subsequent round of replication (for detailed discussion, see below).

DnaA Inactivation In Vivo

Immunoprecipitation of intracellular DnaA revealed that ca. 80% of the protein was in the inactive, ADP-bound form, indicating that the inactivation of DnaA protein in vivo is efficient (Figure 5). Since the cellular concentration of ATP (ca. 3 mM) is much higher than that of ADP (ca. 250 μM) (Bochner and Ames, 1982), newly synthesized DnaA protein will bind ATP predominantly and be in the active form. However, efficient hydrolysis of the bound ATP will rapidly convert DnaA into the inactive ADP form (Figure 6B). This mechanism is in agreement with the observation that de novo protein synthesis is necessary for replication to be initiated at *oriC*; without de novo synthesis, the cellular content of ATP-DnaA molecules is rapidly depleted by efficient inactivation to levels too low to support initiation. Consistent with this is that mutant DnaAcos protein, which promotes initiations at *oriC* even in chloramphenicol-treated cells, is insensitive to RIDA (Katayama, 1994; Katayama and Crooke, 1995).

Although the direct participation of the β subunit in inactivating DnaA protein in vivo, as may occur in vitro, is still unclear, the essential role of the β subunit in the control of the adenine nucleotide forms of DnaA protein in vivo is evident (Figure 5). The *dnaN59* gene product degrades rapidly at restrictive temperatures in vivo (Burgers et al., 1981) (unpublished results), and concurrently the content of ATP-bound DnaA protein increases rapidly. As such, the inactivation pathway that depends on β subunit is the major or sole one in vivo.

The number of DnaA protein molecules per cell is about 1000–2000 (Sekimizu et al., 1988), an excess over the amount bound at *oriC*. However, the β clamp may still interact with DnaA protein that is not bound to *oriC*. Indeed, β clamp–dependent inactivation in vitro does not require the *oriC* sequence when replication is not coupled to inactivation (Katayama and Crooke, 1995) or when other replicons are used for initiating replication (unpublished results).

In *E. coli*, the *dnaN* cistron is adjacent to the *dnaA* cistron, comprising one operon (Ohmori et al., 1984). In about a dozen of the eubacterial species examined, the flanking arrangement of *dnaA* and *dnaN* is conserved (Yoshikawa and Ogasawara, 1991; Ogasawara and Yoshikawa, 1992). This marked evolutionary stability should be noted in light of the close link between these proteins described in this study.

Control of the cellular amount of the ATP form of DnaA protein is possible by modulating the β-clamp function and regulating regeneration of the ADP-bound form of the protein. The ATP form of DnaA protein seems to be slowly regenerated from the ADP form in vivo (unpublished results) (Figure 6B). In vitro, ADP bound to DnaA protein can exchange with free ATP through interaction of DnaA protein with acidic phospholipids (Sekimizu and Kornberg, 1988; Crooke et al., 1992; Garner and Crooke, 1996). Evidence of such a lipid-mediated pathway in the regeneration of DnaA protein has been observed in vivo (Xia and Dowhan, 1995).

Control of Initiation by β Clamp–Dependent Inactivation of DnaA and Sequestration of *oriC*

Initiations at *oriC* are affected by methylation of the origin by DNA adenine methyltransferase (Dam) (Messer and Noyer-Weidner, 1988; Zyskind and Smith, 1992; Crooke, 1995b). Semiconservative replication of the methylated DNA duplex yields hemimethylated daughter strands. Following initiation, the hemimethylated state of the *oriC* region persists for approximately one-third of the cell cycle, and during this period *oriC* is sequestered by SeqA protein (Brendler et al., 1995; Slater et al, 1995) such that secondary initiations are prevented (Campbell and Kleckner, 1990). In cells bearing a defect in the *seqA* gene, the hemimethylated state of *oriC* is maintained for a much shorter time, and secondary initiations occur (Lu et al., 1994; von Freiesleben et al., 1994).

In vitro, the β clamp–dependent inactivation of DnaA protein does not require SeqA protein (Katayama and Crooke, 1995). Also, in vivo certain *dnaA* mutants initiate excessively at *oriC*, even though the cells have intact Dam and SeqA functions (Kellenberger-Gujer et al., 1978; Katayama and Nagata, 1991; Katayama et al., 1997). This indicates that methylation control alone is not sufficient to completely prevent extra initiations. Moreover, in wild-type *seqA* cells additional mechanisms must exist to prevent untimely initiations, since the duration of sequestration is only a portion of the interinitiation time of the chromosomal replication cycle. Cells having both the *dnaAcos* and Δ*seqA* mutations are temperature-sensitive at 42°C, a permissive temperature for the *dnaAcos* single mutant (Katayama et al., 1997); this suggests that the effects of the two defects are additive and perhaps enhance the occurrence of lethal extra initiations. Cumulatively, while these two means of regulating initiation (the β clamp–dependent inactivation of DnaA protein and the methylation control of *oriC*) appear to function in an independent manner, both seem necessary for precise control of the on-off switch for chromosomal replication. The methylation control acts to initially prevent reinitiations, and by the time *oriC* is no longer sequestered, presumably the initiation potential has dropped to levels too low to support initiation because of the inactivation of DnaA protein.

Importance of PCNA and the β Subunit in Cell Cycle Regulation

The *E. coli* β subunit is the counterpart of the eukaryotic PCNA (Herendeen and Kelly, 1996; Baker and Bell, 1998). Both proteins have similar tertiary structures and carry out similar functions during DNA synthesis; PCNA is the sliding clamp and interacts with RF-C, the clamp loader and the functional homolog of the γ complex. PCNA also associates with certain cell cycle–related proteins (Xiong et al. 1992). Direct interaction of PCNA with p21, whose expression is induced by DNA damage, inhibits synthesis of DNA by DNA polymerase δ (Waga et al. 1994). In this vein, while the activity of IdaB cannot be supplied by single proteins of the α or δ subunits of pol III, components known to interact with the β subunit (Stukenberg et al., 1991; Naktinis et al., 1995; Kim and

McHenry, 1996), IdaB protein may be a cell cycle regulatory protein that binds to β clamp (Figure 6A). Although the question of whether PCNA interacts with a protein(s) essential for initiating replication is still unanswered, the distinct dual roles of the β sliding clamp, as an essential component of the replicase and a participant in the negative regulation of DNA replication, may have been conserved during evolution.

Experimental Procedures

Bacterial Strains, Plasmids, Media, and Buffers

Strain WM433 [*dnaA204*(Ts)] is described by Tippe-Schindler et al. (1979). Strains KA473 [*dnaN*$^+$] and KA474 [*dnaN59*(Ts)] are derivatives of HC194 (Sakakibara and Mizukami, 1980) that contain *tna*A::Tn*10*. Plasmid pHB10S is a pBR322 derivative bearing the *dnaA* cistron; the ClaI-SalI fragment (3.7 kb) of pBR322 is ligated with a ClaI-XhoI fragment (1.9 kb) of the chromosome-derived *dnaA* region. M13*oriC*ΔLMR plasmid is an M13mp18 derivative bearing the HincII-PstI fragment (truncated *oriC* region) subcloned from pBS*oriC*ΔLMR (Bramhill and Kornberg, 1988) on the multicloning site. Strain KP7364 [Δ*dnaA*::*spec rnhA*::*kan*] is a derivative of KP245 (Miki et al, 1978); the complete *dnaA* gene, from the start codon to the termination codon, was replaced with the gene for spectinomycin resistance (T. Miki, unpublished data). Bacterial growth media used are described by Sambrook et al. (1989), except as indicated. Buffer A contains 25 mM HEPES·KOH (pH 7.6 at 1 M), 2 mM dithiothreitol, 0.1 mM EDTA, and 15% (v/v) glycerol. Buffer A" is the same as buffer A, except that 25 mM Bis-Tris·HCl (pH 6.0 at 1 M) replaces the 25 mM HEPES·KOH (pH 7.6 at 1 M). Buffer B is the same as buffer A, except that 2 mM ATP and 10 mM magnesium acetate are included. Buffer F is the same as buffer A, except that 10 mM magnesium acetate is included, and 20 mM Mes·NaOH (pH 6.0 at 1 M) replaces the 25 mM HEPES·KOH (pH 7.6 at 1 M). Buffer M contains 50 mM HEPES·KOH (pH 7.6 at 1 M), 100 mM NaCl, 30 mM ammonium sulfate, 5 mM magnesium acetate, 1 mM EDTA, 0.005% Triton X-100, 0.1 mM ATP, and 0.1 mM ADP. Buffer MB is the same as buffer M, except that ammonium sulfate is omitted. Buffer L is the same as buffer M, except that 5 mg/ml lysozyme is included. Buffer LA was the same as buffer L, except that 250 mM NaCl is included. Buffer LB is the same as buffer L, except for the inclusion of 500 mM NaCl.

Partial Purification of RIDA

WM433 cells were grown in LB medium (30°C; 200 l) which contained thymine (50 μg/ml), harvested, and stored at −80°C as described (Fuller et al., 1981). Frozen cell paste (600 g) was thawed on ice, resuspended in buffer A to an optical density (A_{595}) of 250, and a cleared lysate was obtained as described (Katayama and Crooke, 1995). To this supernatant (fraction I, 696 ml), ammonium sulfate (164 g, 0.235 g/ml of fraction I) was added slowly with stirring. After further stirring (20 min), the precipitate was collected by centrifugation (40,000 × g, 20 min, 2°C) and dissolved in buffer A" (13 ml). This sample (fraction II, 29.5 ml) was diluted in buffer A" to a conductivity equivalent to 50 mM NaCl and precipitated matter was removed by centrifugation (8,300 × g, 15 min, 2°C). The resulting supernatant was loaded (flow rate of 240 ml/min) onto a column (285 ml, 5.5 × 12 cm) of DE52 equilibrated with buffer A" that also contained 50 mM NaCl. The column was washed with the same buffer (5 column volumes), and bound proteins were eluted with a linear gradient of NaCl (10 column volumes; 50 to 250 mM) in buffer A". Active fractions were pooled (fraction III, 485 ml). To a portion (262 ml) of this fraction, ammonium sulfate (92 g, 0.35 g/ml of fraction III) was added with stirring. After additional stirring (30 min), precipitates were collected by centrifugation (18000 × g, 30 min, 2°C) and dissolved in buffer B (4 ml) that also contained 150 mM NaCl. Undissolved material was removed by centrifugation (12000 × g, 10 min, 2°C), and the supernatants were loaded (flow rate of 1.0 ml/min) onto a Superdex 200 HR16/60 column (FPLC, Pharmacia Biotech) equilibrated with buffer B that also contained 150 mM NaCl. Active fractions were pooled (fraction IV-150, 28.8 ml, 35 mg; fraction IV-500, 19.2 ml, 5.6 mg). Fraction IV-150 (6.5 ml) was dialyzed against chilled buffer F that contained 2 mM ATP (three changes of 300 ml each) and then centrifuged (12000 × g, 10 min, 2°C). The supernatant was loaded (flow rate of 0.2 ml/min) onto a MonoS HR5/5 column (FPLC, Pharmacia Biotech) equilibrated with buffer F that also contained 2 mM ATP. The column was washed with 4 column volumes of the same buffer, bound proteins were eluted with a linear gradient (10 column volumes; 0 to 0.4 M) of NaCl in buffer F, and active fractions were pooled (flowthrough IdaB fraction, 8.0 ml; fraction V, 1.4 ml). Fraction V (0.6 ml) was dialyzed against buffer F and centrifuged as above. The supernatant (0.5 ml) was loaded (flow rate of 50 μl/min) on MonoQ PC1.6/5 column (SMART, Pharmacia Biotech). The column was washed with 4 column volumes (0.4 ml) of the same buffer, bound proteins were eluted with a linear gradient (10 column volumes; 0 to 0.4 M) of NaCl in buffer F, and active fractions were pooled (fraction VI, 0.09 ml).

In Vitro DNA Replication Systems

Replication of an oriC *Plasmid in a Crude Protein Extract (In Vitro Complementation Reaction)*

An extract containing the proteins (except DnaA) necessary for replication of *oriC* plasmids was obtained by ammonium sulfate fractionation of a lysate prepared from WM433 [*dnaA204*] cells. DNA synthesis reactions (25 μl) were performed as described (Fuller et al. 1981; Crooke, 1995a), using M13E10 or M13mpRE85 as *oriC* plasmids (Smith et al., 1985; Nozaki et al, 1988). Incorporation of [α-^{32}P]-dTMP to acid-insoluble materials was measured by liquid scintillation counting.

Reconstitution of oriC *Plasmid Replication with Purified Proteins*

Conditions for *oriC* plasmid replication reaction (25 μl) were as described (Crooke, 1995a; Kubota et al. 1997). Briefly, the buffer conditions are 20 mM Tris·HCl (pH 7.5 at 1 M); 0.1 mg/ml bovine serum albumin; 8 mM dithiothreitol; 0.01% Brij-58; 8 mM magnesium acetate; 125 mM potassium glutamate; 2 mM ATP; 0.5 mM each of GTP, CTP, and UTP; and 100 μM each of dATP, dGTP, dCTP and [α-^{32}P]-dTTP (70–150 cpm/pmol). DNA replication proteins included are DnaB (150 ng), DnaC (92 ng), DnaG primase (45 ng), gyrase A subunit (185 ng), gyrase B subunit (360 ng), SSB (125 ng), HU (10 ng), pol III* (500 ng), and β subunit of pol III (80 ng; 1 pmol as dimer); M13E10*oriC* or M13*oriC*ΔLMR plasmid (200 ng; 600 pmol as nucleotide) was used. Mixtures were incubated (30°C, 30 min), and incorporation of [α-^{32}P]-dTMP into acid-insoluble materials was measured by liquid scintillation counting.

Inactivation of DnaA Protein In Vitro

DnaA protein was inactivated for replication of *oriC* plasmids as previously described (Katayama and Crooke, 1995). Briefly, DnaA protein (2.0 pmol) was incubated (30°C, 25 min) in buffer (10 μl) containing 40 mM HEPES·KOH (pH 7.6), 11 mM magnesium acetate, 2 mM ATP, 7% polyvinyl alcohol, 40 mM creatine phosphate, 0.1 mg/ml creatine kinase, 400 ng M13mp19 RFI DNA, and the indicated amounts of purified or partially purified proteins. At the end of the incubation, remaining DnaA activity in a portion (5 μl) of the mixture was measured in the in vitro complementation reaction (see above).

Microsequencing of Protein

Proteins were separated by SDS-polyacrylamide (10%) gel electrophoresis and blotted onto a PVDF membrane (Millipore). A 40 kDa protein on a strip of the membrane was sequenced by an Edman degradation method with a Protein Sequencer Model 492 (Applied Biosystems). Twelve amino-terminal residues were obtained with yields greater than 6 pmols.

Assessing Hydrolysis of ATP Bound to DnaA Protein by Cell-Free Extracts or Purified Proteins

DnaA protein typically was incubated with 1.5 μM [α-^{32}P]-ATP (10^5 cpm/pmol) on ice according to the method by Sekimizu et al. (1987). As previously described (Kurokawa et al., 1998), a portion (1 μl), which contained 1 pmol of DnaA protein, was incubated (30°C) for the indicated time in a mixture (25 μl) that contained 40 mM HEPES·KOH (pH 7.6); 10 mM magnesium acetate; 2 mM ATP; 0.5 mM each of GTP, CTP, and UTP; 0.1 mM each of dATP, dGTP,

dCTP, and dTTP; 7% polyvinyl alcohol (MW 30000–70000); 40 mM creatine phosphate; 0.1 mg/ml creatine kinase; 200 ng DNA; and the indicated amounts of proteins. The reaction was stopped by chilling on ice and incubated (2°C, 30 min with rotation) in buffer L (0.75 ml), which contained anti-DnaA antiserum (5 μl) and protein A-Sepharose (60 μl, 50% slurry, Pharmacia Biotech). Immunoprecipitated material was collected by centrifugation (1500 × g, 1 min, 2°C), washed in buffer L and then in buffer M, and protein-bound nucleotides were extracted with 1 M HCOOH (40 μl) that contained ATP, ADP, and AMP (5 mM each). A portion (0.5 μl) was applied to a sheet of PEI cellulose (Merck), and nucleotides were separated by thin layer chromatography (TLC) using 1 M HCOOH and 0.5 M LiCl as a solvent. Levels of the different nucleotides were determined with a Bioimage analyzer BAS2000 (Fujix). In this assay, ca. 50% of input DnaA was recovered in the final fraction.

Determination of the Cellular Levels of the Adenine Nucleotide Forms DnaA Protein

Labeling of cells with ^{32}P-orthophosphate was followed by the method of Echols et al. (1961). Cells were grown to saturation in TG medium (30°C) (Echols et al., 1961), which was modified with 100 mM Tris·HCl (pH 7.2), 25 μg/ml thymine, 40 μg/ml for each of twenty amino acids, and 640 μM K_2HPO_4. This culture was inoculated to an optical density (A_{660}) of 0.01 in the same medium (4 ml) except 320 μM K_2HPO_4 and ^{32}P-orthophosphate (0.1 mCi/ml) were included, growth was continued until the fresh culture reached an optical density (A_{660}) of 0.2, and then a portion was shifted to 42°C. Cells in portions (2 ml) of the cultures (30°C and 42°C) were collected by a brief centrifugation, washed in a solution of NaCl (1 ml, 0.9%), lysed in buffer LA (0.8 ml) by incubation (0°C, 15 min) and rapid freezing in liquid nitrogen. Lysates were thawed on ice, clarified by centrifugation (12000 × g, 5 min, 2°C), and mixed with buffer MB (60 μl), which contained protein A-Sepharose (50% slurry; Pharmacia Biotech). Anti-DnaA antiserum or preimmune serum (5 μl) was added, and the contents of the tubes were mixed by inverting five times followed by continual mixing by rotation (2°C, 30 min). Immunoprecipitates were isolated by centrifugation (1500 × g, 1 min) and washed twice with buffer LB (1.0 ml) and three times with buffer MB (1.0 ml), inverting the tubes 20 times for each wash. After removal of the final wash solution, the immunoprecipitated material was extracted with 1 M HCOOH (20 μl), which contained ATP, ADP, and AMP (5 mM each). Radiolabled nucleotides in the extract were separated by TLC and their levels measured as described above.

Acknowledgments

We are grateful to Dr. Hisaji Maki, Dr. Satoko Maki, and Dr. Mike O'Donnell for providing highly purified pol III*, its subassemblies, and valuable suggestions. We are also indebted to Dr. Yoshimasa Sakakibara and Dr. Deog Su Hwang for the *dnaN* mutant and pBS*oriC*ΔLMR, respectively. Large scale culture was done by Kyowa Hakko Kogyo Co. T. Kubota was a recipient of a predoctoral fellowship from the Japan Society for the Promotion of Science. This work was in part supported by Grants-in-aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan and by grants from the National Institutes of Health (GM49700) and the National Science Foundation (MCB9408830).

Received March 23, 1998; revised June 8, 1998.

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