J. Biochem. **127**, 1–7 (2000)

Site-Directed Mutational Analysis of DnaA Protein, the Initiator of Chromosomal DNA Replication in *E. coli*

Tohru Mizushima[1]

Faculty of Pharmaceutical Sciences, Okayama University, and PRESTO, Japan Science and Technology Corporation, Okayama 700–8530

Received September 22, 1999; accepted October 7, 1999

DnaA protein, the initiator for chromosomal DNA replication in *Escherichia coli*, has various activities, such as oligomerization (DnaA–DnaA interaction), ATP-binding, ATPase activity and membrane-binding. Site-directed mutational analyses have revealed not only the amino acid residues that are essential for these activities but also the functions of these activities. Following is a summary of the functions and regulatory mechanisms of DnaA protein in the initiation of chromosomal DNA replication. ATP-bound DnaA protein, but not other forms of the protein binds to the origin of DNA replication and forms oligomers to open-up the duplex DNA. This oligomerization is mediated by a DnaA–DnaA interaction through the N-terminal region of the protein. After initiation of DNA replication, the ATPase activity of DnaA protein is stimulated and DnaA protein is inactivated to the ADP-bound form to suppress the re-initiation of DNA replication. DnaA protein binds to acidic phospholipids through an ionic interaction between basic amino acid residues of the protein and acidic residues of phospholipids. This interaction seems to be involved in the re-activation of DnaA protein (from the ADP-bound form to the ATP-bound form) to initiate DNA replication after the appropriate interval.

Key words: chromosomal DNA replication, DnaA protein, *Escherichia coli*, ORC, site-directed mutation.

Chromosomal DNA replication is a key step not only for genetic inheritance but also for the regulation of cell proliferation. Since DNA replication is regulated at the initiation step but not at the elongation step, regulatory mechanisms for the activities of initiator proteins play major roles in the regulation of DNA replication and thus, cell proliferation.

Among various initiator proteins for chromosomal DNA replication, DnaA protein, the initiator of chromosomal DNA replication in *Escherichia coli*, is the most thoroughly characterized protein (*1*). DnaA protein is conserved among all bacteria species (*1*). Establishment of *in vitro* DNA replication systems of *E. coli* (*2*) provided us a number of useful information about functions of DnaA protein. DnaA protein binds to four DnaA boxes located in *oriC* (the origin of chromosomal DNA replication in *E. coli*), forms oligomers that open-up the duplex DNA at *oriC*, and recruits DnaB protein (DNA helicase) (*1*). As for the mechanism that regulates the activity of the protein, adenine-nucleotides bound to the protein seem to play a major role. DnaA protein tightly binds to ATP and ADP (*3*). The protein bound with ATP is active, whereas the ADP-bound form is inactive in an *oriC* DNA replication system *in vitro* (*3, 4*). Acidic phospholipids, such as cardiolipin (CL), binds to DnaA protein to modulate its activity for adenine-nucleotide–binding (*5*).

[1]Correspondence: Tel/Fax: +81-86-251-7958, E-mail: mizushima@pheasant.pharm.okayama-u.ac.jp

As mentioned above, DnaA protein possesses several activities, such as *oriC*-binding, adenine-nucleotide–binding, acidic phospholipid-binding, ATPase activity, formation of oligomers by DnaA–DnaA interaction, and interaction with DnaB protein. Identification of the amino acid residues and domains that are essential for these activities is needed to understand the roles of these activities and thus, the total function of DnaA protein in the initiation of DNA replication in cells. Site-directed mutational analysis is very useful for this propose. In this article, I will review our recent studies on the identification of the functional domains of DnaA protein.

ATP-Binding Activity

In *oriC* DNA replication systems *in vitro*, re-constituted both from crude extracts and from purified proteins, the ATP-bound form of DnaA protein is active but the ADP-bound form is inactive (*3*). Since DnaA protein has Walker A and B motifs, which are important for the ATP-binding activities of various proteins, these motifs had been predicted to be the ATP-binding domains of DnaA protein. Some temperature-sensitive *dnaA* mutants had mutations in this region and mutant proteins were shown to lose the ATP-binding activity (*6, 7*). In order to identify the amino acid residues that are essential for the ATP-binding, to determine whether ATP-binding to DnaA protein is essential for DNA replication even *in vivo*, and to reveal the role of the ATP-binding activity of DnaA protein in DNA replication, we did site-directed mutational analyses for the Walker A and B motifs of DnaA protein. We constructed

two mutant DnaA proteins in which Lys178 (Walker A motif) was substituted with Ile or Asp235 (Walker B motif) was substituted with Asn, respectively (DnaA K178I and DnaA D235N) (*8*). Both of the mutant proteins were purified and were shown to lose their ability to binding ATP and ADP, indicating these two amino acid residues are essential for the ATP-binding activity of DnaA protein (*8*). We also showed these mutant proteins could not function in cells as an initiator protein for DNA replication by a plasmid complementation assay, suggesting that ATP-binding to DnaA protein is essential for DNA replication even *in vivo* (*8*). These mutant proteins were inactive in an *oriC* DNA replication system *in vitro* and thus we examined which step of DNA replication is defective. The duplex DNA opening activity of DnaA protein can be monitored by generation of sites at *oriC* that are sensitive to P1-nuclease. Assays with P1 nuclease revealed these mutant proteins have defects in duplex opening of DNA at *oriC* (*8*). The duplex DNA opening reaction at *oriC* by DnaA protein can be divided into two stages. In the first stage, DnaA protein binds to *oriC*. In the second stage, DnaA protein forms oligomers, which causes a torsional stress that helps to open-up the duplex DNA at *oriC*. Since both mutant DnaA proteins (DnaA K178I and DnaA D235N) could bind to *oriC* as strong as the wild-type protein, we concluded that only the ATP-bound form of DnaA protein can be oligomerized to open-up the duplex DNA at *oriC* (*8*). Another group also showed that Val184 in the Walker A motif is essential for the ATP-binding activity of DnaA protein (*7*). We also suggested ATP-binding to DnaA protein regulated various functions of DnaA protein other than DNA replication (*9–11*)

ATPase Activity

It has been known for over 10 years that DnaA protein has an intrinsic ATPase activity; ATP bound to DnaA protein is slowly hydrolyzed to ADP (*3*). Since this ATPase activity is not necessary for the DNA replication reaction *in vitro* and the ATPase activity is very weak (*3*), less attention had been paid to the ATPase activity of DnaA protein and thus, the function of the ATPase activity has been remained unknown. For the purpose of understanding the role of the ATPase activity in DNA replication in cells, a mutant DnaA protein that has ATP-binding activity but not ATPase activity (a mutant DnaA protein specific for ATPase activity) was needed. We tried to obtain such a mutant DnaA protein by site-directed mutagenesis. Yoshida and Amano compared amino acid sequences of various ATPases and predicted that Glu204 of DnaA protein is important for ATPase activity (*12*). Sato *et al.* showed that replacing Asp133 of SecA protein (corresponding to Glu204 of DnaA protein) with Asn decreased its ATPase activity but did not affect its affinity for ATP (*13*). Thus, we constructed a mutant DnaA protein, in which Glu204 is replaced with Gln (DnaA E204Q). Purified DnaA E204Q protein maintained high affinities for ATP and ADP, but its ATPase activity was decreased to one-third that of the wild-type protein (*14*). Thus, DnaA E204Q protein is a good tool for investigating the role of ATPase activity of DnaA protein in cells. Induction of DnaA E204Q protein in cells caused a dominant lethal phenotype in a manner depending on *oriC* function (*14–16*). *E. coli* cells with a mutation of the *rnhA* gene (the gene for RNaseH, which restricts the initiation site of DNA replication only to *oriC*) can replicate chromosomal DNA without the function of *oriC*. In that strain, induction of DnaA E204Q protein did not affect cell viability (*14*). These results suggest that the ATPase activity of DnaA protein plays an important role in the regulation of *oriC* DNA replication.

A common feature seen in DnaA protein and eukaryotic GTP-binding protein (G protein) is that purine-nucleotides and an intrinsic ATPase or GTPase activity regulate their activities. In the case of G protein, the intrinsic GTPase is activated by GTPase-stimulating factor (GAP) and this factor plays an important role in the regulation of the activity of G protein in signal transductions. Thus, we assumed the existence of factors that stimulate the DnaA ATPase and found such factors in crude extracts from *E. coli* cells (*17*). Interestingly, these factors were identical to the inactivation factors of DnaA protein (*17*), which had been shown to be involved in inactivation of DnaA protein (*18*). Based on these results, we concluded that the ATPase activity of DnaA protein negatively regulates the activity of this protein. In order to initiate DNA replication only once per cell cycle, the initiator protein should be inactivated soon after the initiation of DNA replication. Without this inactivation, over-initiation of DNA replication would occur and cells could not grow normally. The ATPase activity of DnaA protein seems to be involved in this inactivation by changing DnaA protein from the ATP-bound to the inactive ADP-bound form to suppress the re-replication from the newly synthesized origin. Recently, one of the stimulating factors for the DnaA ATPase was identified as the β subunit of DNA polymerase III (*19*). It was also shown that the DNA replication reaction itself stimulated the DnaA ATPase (*19*). These results also support the notion that the ATPase

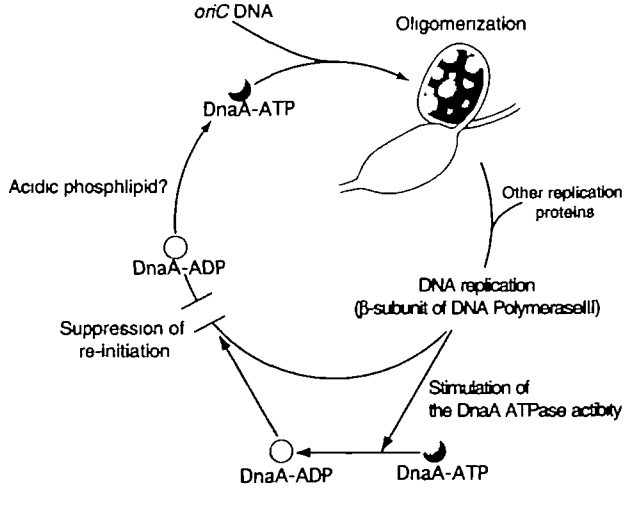

Fig. 1. **A proposed model for the regulation of the activity of DnaA protein.** ATP-bound DnaA protein binds to oriC and forms oligomers to open-up the duplex DNA. DnaB protein and other replication proteins are recruited to oriC and DNA replication is initiated. β-subunit of DNA polymerase III and the DNA replication reaction stimulate the ATPase activity to change DnaA protein to the inactive ADP-bound form. Re-initiation from the newly synthesized origin is suppressed by this inactivation for the appropriate period. Re-activation of the ADP-bound DnaA protein to the ATP-bound form seems to be at least partly mediated by acidic phospholipids in the membranes.

activity of DnaA protein and its stimulation factors are involved in the inactivation of DnaA protein soon after the initiation of DNA replication. Figure 1 shows the current model for the regulation of the activity of DnaA protein in cell cycle.

Membrane-Binding Activity

Besides inactivation soon after the initiation of DNA replication, DnaA protein should be subjected to another regulation of its activity, that is, it should be activated upon initiation of DNA replication. How is ATP-bound DnaA protein supplied to initiate DNA replication? Two possibilities can be considered. One is that only newly synthesized DnaA protein, which may bind ATP quickly, functions as an initiator protein and the other is that ADP-bound DnaA is re-activated to the ATP-bound form by some mechanism. The former possibility is supported by the fact that inhibitors of protein synthesis immediately inhibit the initiation of DNA replication in cells. As for the latter possibility, membrane acidic phospholipids were shown to activate the inactive ADP-bound form of DnaA protein to the ATP-bound form *in vitro*. Acidic phospholipids, in particular, CL, decreased the affinity of DnaA protein for adenine-nucleotides and activated the ADP-bound form of DnaA protein to the ATP-bound form in the presence of high concentrations of ATP by stimulating the exchange reaction of ADP with ATP (*5, 20–23*). Some genetic lines of evidence support the idea that DnaA protein is activated by acidic phospholipids to initiate DNA replication *in vivo* (*24–28*). In order to better understand how DnaA protein interacts with acidic phospholipids and how it is involved in the regulation of DNA replication, identification of the amino acid residues of DnaA protein that are involved in membrane-binding is important.

A potential amphipathic helix region (from Asp357 to Val374) has been suggested to be involved in the membrane-binding of DnaA protein (*1, 29, 30*). Since, in general, protein-lipid interactions are mediated by hydrophobic interactions, hydrophobic amino acid residues were considered to be involved in the interaction between DnaA protein and acidic phospholipids. The potential amphipathic helix contains three hydrophobic amino acid residues (Leu363, Leu366, and Leu367), that are well conserved as hydrophobic amino acid residues in various species of bacteria. It had been reported that anionic residues in acidic phospholipids are indispensable for their interaction with DnaA protein (*5, 20–23*). Thus, there is also a possibility that the ionic interaction between anionic residues of acidic phospholipids and basic amino acid residues in DnaA protein is involved in the functional interaction. The potential amphipathic helix contains three basic amino acid residues (Arg360, Arg364, and Lys372), that are well conserved as basic amino acid residues in various species of bacteria. Thus, we introduced site-directed mutations at the positions of these hydrophobic or basic amino acids to construct DnaA430 or DnaA431 protein, respectively, both of which have triple mutations. Both mutant DnaA proteins maintained DNA replication and ATP-binding activities (*31*), suggesting these mutations did not affect the higher-order structure of DnaA protein. The functional interaction of DnaA protein with acidic phospholipids can be monitored by acidic phospholipid–dependent stimulation of the release of ATP (or ADP) from DnaA protein (*5*). The release of ATP bound to the mutant DnaA protein, in which three hydrophobic amino acid residues were mutated to hydrophilic ones (DnaA430) was stimulated by CL, as is the case with the wild-type protein (*31*). On the other hand, the release of ATP bound to another mutant DnaA protein, in which three basic amino acid residues were mutated to acidic ones (DnaA431) was not stimulated by CL (*31*). Among these three basic amino acid residues (Arg360, Arg364, and Lys372), Lys372 was shown to be the most important for the functional interaction of DnaA protein with CL by a site-directed mutational analysis using three mutant DnaA proteins with each single mutation (Makise *et al.*, *J. Biol. Chem.*, in press). These results suggest not only that the region (from Asp357 to Val374) is one of the membrane-binding domains of DnaA protein, but also that the ionic interaction between the basic amino acid residues of DnaA protein and the acidic residues of acidic phospholipids is involved in the functional interaction between DnaA protein and acidic phospholipids. We recently reported that another potential amphipathic helix (from Lys327 to Ile345), which is located very close to the amphipathic helix (from Asp357 to Val374) is also involved in membrane-binding (*32*). In conclusion, the interaction between DnaA protein and acidic phospholipids is mediated by ionic interactions between basic amino acid residues located in these two amphipathic helixes and acidic residues of acidic phospholipids. We proposed that a multisite electrostatic interaction between DnaA protein and acidic phospholipids might reduce the affinity of DnaA protein for adenine-nucleotides (Fig. 2).

Surprisingly, plasmid complementation analyses revealed that DnaA431 can function in cells as an initiator pro-

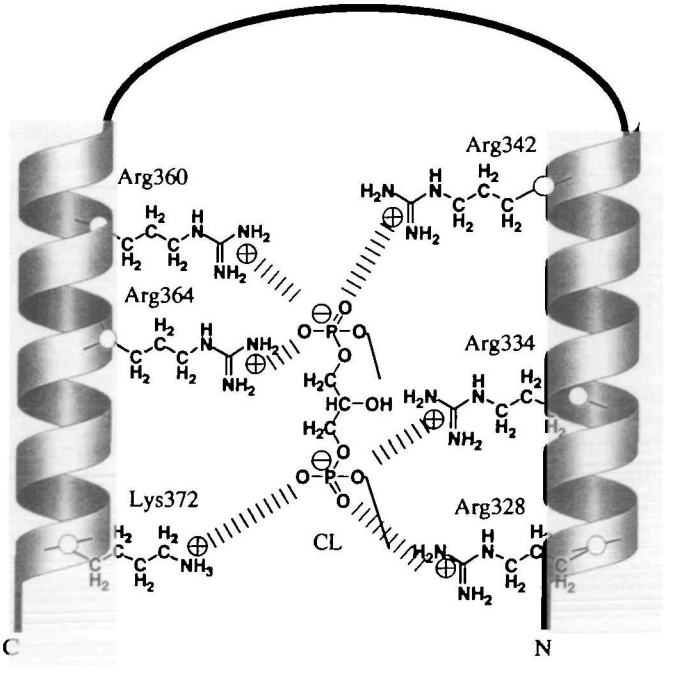

Fig. 2. **Schematic representation of a plausible electrostatic interaction between cationic residues on amphipathic helices in DnaA protein and a multi-anionic lipid** [Reproduced with permission from Author (*5*), 1999, *Biochemical Journal*, **340**, 433–438. © Biochemical Society] (*32*).

tein of DNA replication (Makise *et al.*, *J. Biol. Chem.*, in press). We interpret this result to mean that there is another pathway for re-activation of DnaA protein. Off course, we cannot deny the possibility that DnaA431 can functionally interact with acidic phospholipids *in vivo*. Establishment of an assay system for *in vivo* membrane-binding of DnaA protein is needed to address this possibility.

DnaA–DnaA Interaction

DnaA protein is involved in several interactions. It binds to *oriC*, adenine-nucleotide, acidic phospholipid, has ATPase activity, and interacts with DnaB protein and other DnaA proteins. The functional domains of all these activities except for the DnaA–DnaA interaction have been identified or suggested (see above and below). Genetic analyses and the predicted secondary structure suggested that the DnaA–DnaA interaction may be mediated by the N-terminal region of DnaA protein (*33, 34*). Using site-directed mutational analyses, we tried to identify amino acid residues that are located in the N-terminal region and that are essential for the replication activity of DnaA protein in order to examine the function of the N-terminal region of DnaA protein. We searched for amino acid residues in the N-terminal region of the DnaA protein that were conserved among DnaA proteins from various species and found two amino acid residues (Ile26 and Leu40). Interestingly, these two amino acid residues and Leu33 constitute a leucine-zipper–like structure that is generally thought to be involved in oligomerization of DNA-binding proteins. We constructed two mutant *dnaA* genes by replacing each of these hydrophobic amino acid residues with a hydrophilic amino acid, Ser. Plasmid complementation analyses revealed that these two amino acid residues are essential for the function of DnaA protein *in vivo* (*35*). Using an *oriC* complementation assay system, these two mutant DnaA proteins (DnaA I26S and DnaA L40S) were shown to be unable to induce DNA replication even *in vitro* (*35*). We further tested which stage of DNA replication is defective with these mutant DnaA proteins. We compared the duplex DNA-opening capability of these mutant DnaA proteins with the wild-type protein and found that neither of the mutant proteins could open the duplex DNA at *oriC*. As mentioned above, the duplex DNA-opening reaction at *oriC* by DnaA protein can be divided into two stages, that is, binding of DnaA protein to *oriC* and oligomerization of DnaA protein by the DnaA-DnaA interaction. The binding of the mutant DnaA proteins to *oriC* DNA was indistinguishable from that of the wild-type protein (*35*). Thus, it is most likely that these mutant proteins may be defective in oligomerization (DnaA-DnaA interaction). In other words, the N-terminal region of DnaA protein is involved in the DnaA–DnaA interaction. Recently, Sutton *et al.* reported evidence that the N-terminal region of DnaA protein is involved in the retention of DnaB in the replication complex (*36*).

oriC-Binding and DnaB-Binding Activities

Roth and Messer investigated the *oriC*-binding activity of DnaA protein using a solid-phase DNA-binding assay (*37*). By deletion analysis on DnaA protein, they found that the 94 C-terminal amino acid residues (from Val374 to Ser467) are required and sufficient for binding (*37*). Using spontaneous temperature-sensitive *dnaA* mutants, they identified three amino acid residues (Val383, Ile389, and Met411), that are located in this region and that are essential for both *oriC*-binding and DNA replication by DnaA protein.

Marszalek *et al.* investigated the DnaB-binding region of DnaA protein. They made a number of monoclonal antibodies against DnaA protein and determined their epitopes (*38*). One of the antibodies, which recognized the region form Pro111 to Glu148, inhibited the binding between DnaB and DnaA proteins (*38*). Thus, the domain of DnaA protein that is responsible for binding to DnaB protein may be located in this region, however, this hypothesis has not been proved by mutational analyses.

Similarities between Prokaryotic and Eukaryotic DNA Replication

Initiation of chromosomal DNA replication in prokaryotic and eukaryotic cells is very different. For example, the origin of prokaryotic DNA replication is unique but eukaryotic DNA replication is initiated from multiple origins. Furthermore, DNA replication and cell division are strictly separated in the eukaryotic cell cycle but these two events can simultaneously occur in prokaryotic cells growing rapidly. Thus, information concerning DNA replication in prokaryotic cells has rarely been applied to studies of DNA replication in eukaryotic cells. However, I think there must be

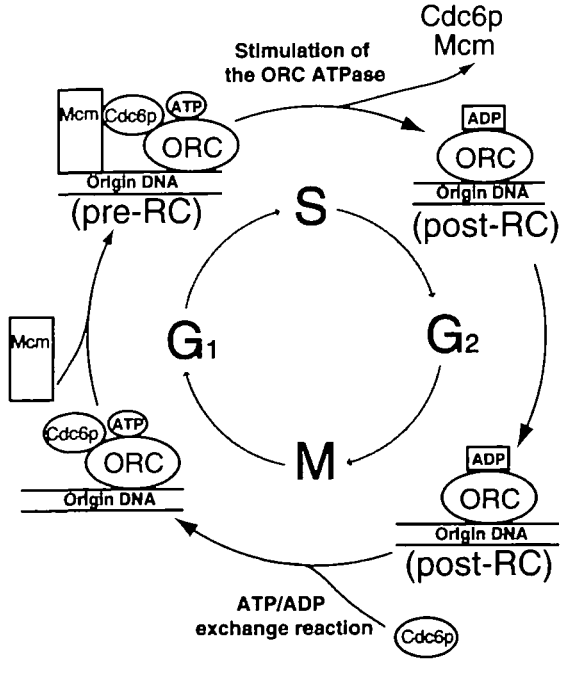

Fig. 3. **A proposed model for the function of ATPase activity of ORC.** At the first step of initiation of eukaryotic DNA replication, Cdc6p synthesized in the late M phase binds to ATP-bound ORC, which has already bound to the origin DNA. Cdc6p recruits Mcm proteins (DNA helicase) to form a pre-RC. Upon initiation of DNA replication, the ATPase activity of ORC (derived from Orc1p) may be activated to change Orc1p to the ADP-bound form. Since ORC containing the ADP-bound form of Orc1p cannot form the complex with Cdc6p, Cdc6p and thus, the Mcm proteins are released from the origin and a post-RC is constituted. An unknown ATP/ADP exchange reaction may convert ORC to the active ATP-bound form to initiate the next DNA replication.

similar regulatory mechanisms of initiation of DNA replication between prokaryotic and eukaryotic cells. Supporting this notion, the Origin Recognition Complex (ORC), the strongest candidate for a eukaryotic initiator of chromosomal DNA replication, was shown to require ATP for expression of its activity and to possess an intrinsic ATPase activity (*39, 40*) as is the case of DnaA protein. Thus, we wondered whether the mechanism regulating the activity of DnaA protein by its intrinsic ATPase activity (Fig. 1) can be applied to the ORC.

Initiation of chromosomal DNA replication in eukaryotic cells is achieved by the stepwise assembly of cell cycle–regulated protein complexes at the origins of DNA replication as is the case of prokaryotic cells. The mechanism regulating the assembly is well known for prokaryotic cells but not for eukaryotic cells. This is due to a lack of an *in vitro* DNA replication system for eukaryotic chromosomal DNA replication. However, genetic analyses of eukaryotic DNA replication have suggested the order of this assembly as follows. ORC is bound to the origins of chromosomal DNA replication throughout the cell cycle and is thought to function as a 'landing pad' for a series of cell cycle–regulated protein complexes (*41, 42*). During the late M phase, Cdc6p binds to ORC and then recruits the six minichromosome maintenance (Mcm) proteins (DNA helicase) to form a pre-replication complex (pre-RC) at the origin (*41, 42*). Thus, the first event in the initiation of DNA replication in eukaryotic cells is believed to be Cdc6p-binding to origin-bound ORC, but even for this first step of chromosomal DNA replication, an *in vitro* assay system has not been established.

We established an assay system consisting of purified proteins for the interaction between ORC and Cdc6p from *Saccharomyces cerevisiae*. This interaction required DNA with a functional DNA replication origin (autonomously replicating sequence; ARS) (Mizushima *et al.*, submitted). ADP, but not ATP or ATP-γS, inhibited the origin-dependent interaction between ORC and Cdc6p (Mizushima *et al.*, submitted), suggesting that the ADP-bound forms of some proteins cannot induce the complex formation between ORC and Cdc6p. ORC has two subunits, Orc1p and Orc5p, that bind to ATP and the amino acid sequence of Cdc6p strongly suggests that Cdc6p is also an ATP-binding protein. Thus, we utilized mutant Orc1p, Orc5p, and Cdc6p proteins to determine which ATP-binding motifs were essential for the interaction. The results clearly showed that ATP-binding to Orc1p is required for the complex formation between ORC and Cdc6p. In other words, ORC containing the ADP-bound form of Orc1p cannot form the complex with Cdc6p, which is essential for the initiation of DNA replication (Mizushima *et al.*, submitted). Orc1p has an ATPase activity (*40*). Thus, these results suggested that the Orc1p ATPase activity in ORC may negatively regulate the interaction between ORC and Cdc6p and thus, inhibit the initiation of DNA replication to suppress the re-initiation. When the ATP bound to Orc1p is hydrolyzed, Cdc6p may be released from the origin (an event which occurs at the G1-S transition *in vivo*) and reformation of a new pre-RC at the origin would be inhibited (Fig. 3). This may contribute to ensuring a single round of DNA replication per S phase until Orc1p changes back to the ATP-bound form, an event which may occur during the M phase in cells (Fig. 3). It is interesting that prokaryotic and eukaryotic initiator proteins may share a common mechanism for regulation of their activities.

Conclusion and Future Perspective

The functional domains of DnaA protein, which I reviewed in this paper, are summarized in Fig. 4. Figure 4 also shows the important amino acid residues in each domain, which were determined by site-directed mutagenesis or random mutagenesis. Among the initiators of chromosomal DNA replication, DnaA protein is the one whose functional domains have been best characterized. Thus, determination of the higher-order structure of DnaA protein by X-ray crystallography, combined with information from domain-mapping experiments (Fig. 4), may provide enough information to completely understand the function and structure of DnaA protein. Since there have been no reports on the higher-order structure of initiator proteins of chromosomal DNA replication, such information may be very useful to understand the function and structure of other initiator proteins, such as ORC. Recently, highly efficient procedures for purifying DnaA protein have been established (*43*), which will make it easier to crystallize DnaA protein. We are currently trying to crystallize DnaA protein.

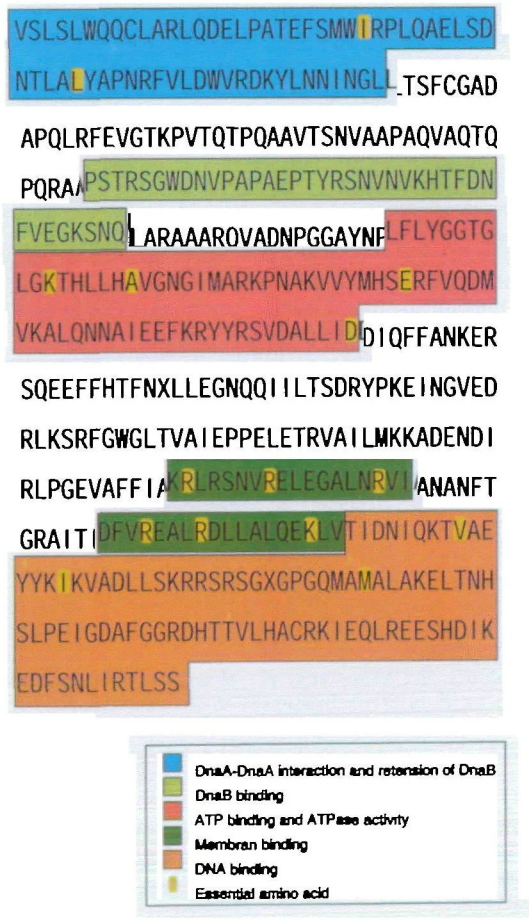

Fig. 4. **A summary of the functional domains of DnaA protein.** References: DnaA–DnaA interaction (*36*), retention of DnaB protein (*37*), DnaB-binding (*39*), ATP-binding (*7, 8*), ATPase activity (*14*), membrane-binding (*29, 31, 33*), DNA-binding (*38*). Amino acids that are essential for the function are indicated by yellow shadows.

It is interesting that there are a number of similarities in the function and regulatory mechanism of initiator proteins between prokaryotic and eukaryotic cells (DnaA protein and ORC). These results suggest that there are other common mechanisms of DNA replication between prokaryotic and eukaryotic cells. Since the mechanism of DNA replication is much better understood in prokaryotic cells than in eukaryotic cells, it is very useful if information obtained from studies of prokaryotic DNA replication can be applied eukaryotic DNA replication, as is the case of the ORC ATPase. Several projects of this type are currently being investigated in my laboratory.

I thank to Mr. Shinji Mima (Okayama University) for assistance in drawing figures.

REFERENCES

1. Skarstad, K. and Boye, E. (1994) The initiator protein DnaA: evolution, properties and function. *Biochim. Biophys. Acta* **1217**, 111–130
2. Fuller, R.S., Kaguni, J.M., and Kornberg, A. (1981) Enzymatic replication of the origin of the *Escherichia coli* chromosome. *Proc. Natl. Acad. Sci. USA* **78**, 7370–7374
3. Sekimizu, K., Bramhill, D., and Kornberg, A. (1987) ATP activates dnaA protein in initiating replication of plasmids bearing the origin of the *E. coli* chromosome. *Cell* **50**, 259–265
4. Mizushima, T., Sasaki, S., Ohishi, H., Kobayashi, M., Katayama, T., Miki, T., Maeda, M., and Sekimizu, K. (1996) Molecular design of inhibitors of *in vitro oriC* DNA replication based on the potential to block the ATP binding of DnaA protein. *J. Biol. Chem.* **271**, 25178–25183
5. Sekimizu, K. and Kornberg, A. (1988) Cardiolipin activation of dnaA protein, the initiation protein of replication in *Escherichia coli*. *J. Biol. Chem.* **263**, 7131–7135
6. Hansen F.G., Koefoed, S., and Atlung, T. (1992) Cloning and nucleotide sequence determination of twelve mutant *dnaA* genes of *Escherichia coli*. *Mol. Gen. Genet.* **234**, 14–21
7. Carr, K.M. and Kaguni, J.M. (1996) The A184V missense mutation of the *dnaA5* and *dnaA46* alleles confers a defect in ATP binding and thermolability in initiation of *Escherichia coli* DNA replication. *Mol. Microbiol.* **20**, 1307–1318
8. Mizushima, T., Takaki, T., Kubota, T., Tsuchiya, T., Miki, T., Katayama, T., and Sekimizu, K. (1998) Site-directed mutational analysis for the ATP binding of DnaA protein. Functions of two conserved amino acids (Lys-178 and Asp-235) located in the ATP-binding domain of DnaA protein *in vitro* and *in vivo*. *J. Biol. Chem.* **273**, 20847–20851
9. Mizushima, T., Tomura, A., Shinpuku, S., Miki, T., and Sekimizu, K. (1994) Loss of flagellation in *dnaA* mutants of *Escherichia coli*. *J. Bacteriol.* **176**, 5544–5546
10. Mizushima, T., Katayama, T., and Sekimizu, K. (1996) Effect on DNA topology by DnaA protein, the initiation factor of chromosomal DNA replication in *Escherichia coli*. *Biochemistry* **35**, 11512–11516
11. Mizushima, T., Yokoyama, K., Mima, S., Tsuchiya, T., and Sekimizu, K. (1997) Inhibition of thymidine transport in *dnaA* mutants of *Escherichia coli*. *J. Biol. Chem.* **272**, 21195–21200
12. Yoshida, M. and Amano, T. (1995) A common topology of proteins catalyzing ATP-triggered reactions. *FEBS. Lett.* **359**, 1–5
13. Sato, K., Mori, H., Yoshida, M., and Mizushima, S. (1996) Characterization of a potential catalytic residue, Asp-133, in the high affinity ATP-binding site of *Escherichia coli* SecA, translocation ATPase. *J. Biol. Chem.* **271**, 17439–17444
14. Mizushima, T., Nishida, S., Kurokawa, K., Katayama, T., Miki, T., and Sekimizu, K. (1997) Negative control of DNA replication by hydrolysis of ATP bound to DnaA protein, the initiator of chromosomal DNA replication in *Escherichia coli*. *EMBO J.* **16**, 3724–3730
15. Sakamoto, K., Makise, M., Tsuchiya, T., and Mizushima, T. (1998) Effect of environmental factors on the dominant lethality caused by expression of a mutated DnaA Protein with decreased intrinsic ATPase activity. *FEMS. Micro. Lett.* **159**, 93–97
16. Makise, M., Sakamoto, K., Tsuchiya, T., and Mizushima, T. (1999) Identification of high-copy-number plasmid suppressor of a lethal phenotype caused by mutant DnaA protein that has a decreased intrinsic ATPase activity. *Biol. Pharm. Bull.* **22**, 904–909
17. Kurokawa, K., Mizushima, T., Kubota, T., Tsuchiya, T., Katayama, T., and Sekimizu, K. (1998) A stimulation factor for the hydrolysis of ATP bound to DnaA protein, the initiator of chromosomal DNA. *Biochem. Biophys. Res. Commun.* **243**, 90–95
18. Katayama, T. and Crooke, E. (1995) DnaA protein is sensitive to a soluble factor and is specifically inactivated for initiation of *in vitro* replication of the *Escherichia coli* minichromosome. *J. Biol. Chem.* **270**, 9265–9271
19. Katayama, T., Kubota, T., Kurokawa, K., Crooke, E., and Sekimizu K. (1998) The initiator function of DnaA protein is negatively regulated by the sliding clamp of the *E. coli* chromosomal replicase. *Cell* **10**, 61–71
20. Yung, B.Y. and Kornberg, A. (1988) Membrane attachment activates dnaA protein, the initiation protein of chromosome replication in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **85**, 7202–7205
21. Castuma, C.E., Crooke, E., and Kornberg, A. (1993) Fluid membranes with acidic domains activate DnaA, the initiator protein of replication in *Escherichia coli*. *J. Biol. Chem.* **268**, 24665–24668
22. Hase, M., Ishikawa, Y., Sekimizu, K., Tsuchiya, T., and Mizushima, T. (1998) Effect of glycerol on the affinity of DnaA protein for ATP in the presence of cardiolipin. *J. Biochem.* **123**, 680–683
23. Mizushima, T., Ishikawa, Y., Obana, E., Hase, M., Kubota, T., Katayama, T., Kunitake, T., Watanabe, E., and Sekimizu, K. (1996) Influence of cluster formation of acidic phospholipids on decrease in the affinity for ATP of DnaA protein. *J. Biol. Chem.* **271**, 3633–3638
24. Xia, W. and Dowhan, W. (1995) *In vivo* evidence for the involvement of anionic phospholipids in initiation of DNA replication in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **92**, 783–787
25. Shinpuku, T., Mizushima, T., Guo, L., Miki, T., and Sekimizu, K. (1995) Phenotypes of *dnaA* mutants of *Escherichia coli* sensitive to detergents and organic solvents. *Biochem. Biophys. Res. Commun.* **212**, 84–89
26. Mizushima, T., Shinpuku, T., Katayama, H., Kataoka, K., Guo, L., Miki, T., and Sekimizu, K. (1996) Phenotypes of *dnaA* mutants of *Escherichia coli* sensitive to phenothiazine derivatives. *Mol. Gen. Genet.* **252**, 212–215
27. Suzuki, E., Kondo, T., Makise, M., Mima, M., Sakamoto, K., Tsuchiya, T., and Mizushima, T. (1998) Alteration in the contents of unsaturated fatty acids in *dnaA* mutants of *Escherichia coli*. *Mol. Microbiol.* **28**, 95–102
28. Suzuki, E., Kondo, T., Makise, M., Mima, M., Sakamoto, K., Tsuchiya, T., and Mizushima, T. (1988) Alteration in contents of unsaturated fatty acids in mutants defective in DNA replication. *Biol. Pharm. Bull.* **21**, 657–661
29. Garner, J. and Crooke, E. (1996) Membrane regulation of the chromosomal replication activity of *E. coli* DnaA requires a discrete site on the protein. *EMBO J.* **15**, 3477–3485
30. Garner, J., Durrer, P., Kitchen, J., Brunner, J., and Crooke, E. (1998) Membrane-mediated release of nucleotide from an initiator of chromosomal replication, *Escherichia coli* DnaA, occurs with insertion of a distinct region of the protein into the lipid bilayer. *J. Biol. Chem.* **273**, 5167–7513
31. Hase, M., Yoshimi, T., Ishikawa, Y., Ohba, A., Guo, L., Mima, S., Makise, M., Yamaguchi, Y., Tsuchiya, T., and Mizushima, T. (1998) Site-directed mutational analysis for the membrane binding of DnaA protein. Identification of amino acids involved in the functional interaction between DnaA protein and acidic phospholipids. *J. Biol. Chem.* **273**, 28651–28656

32. Yamaguchi, Y., Hase, M., Makise, M., Mima, S., Yoshimi, T., Ishikawa, Y., Tsuchiya, T., and Mizushima, T. (1999) Involvement of Arg 328, Arg 334, and Arg 342 of DnaA protein in its functional interaction to acidic phospholipids. *Biochem. J.* **340**, 433–438
33. Sutton, M.D. and Kaguni, J.M. (1997) The *Escherichia coli dnaA* gene: four functional domains. *J. Mol. Biol.* **274**, 546–561
34. Schaper, S. and Messer, W. (1997) Prediction of the structure of the replication initiator protein DnaA. *Proteins* **28**, 1–9
35. Mima, S., Yamagachi, Y., Kondo, T., Tsuchiya, T., and Mizushima, T. (1999) Role of the amino-terminal region of the DnaA protein in opening of the duplex DNA at the *oriC* region. *FEMS. Micro. Lett.* **176**, 163–167
36. Sutton, M.D., Carr, K.M., Vicente, M., and Kaguni, J.M. (1998) *Escherichia coli* DnaA protein. The N-terminal domain and loading of DnaB helicase at the *E. coli* chromosomal origin. *J. Biol. Chem.* **273**, 34255–34262
37. Roth, A. and Messer, W. (1995) The DNA binding domain of the initiator protein DnaA. *EMBO J.* **14**, 2106–2111
38. Marszalek, J., Zhang, W., Hupp, T.R., Margulies, C., Carr, K.M., Cherry, S., and Kaguni, J.M. (1996) Domains of DnaA protein involved in interaction with DnaB protein, and in unwinding the *Escherichia coli* chromosomal origin. *J. Biol. Chem.* **271**, 18535–18542
39. Bell, S.P. and Stillman B. (1992) ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. *Nature* **357**, 128–134
40. Klemm, R.D., Austin, R.J., and Bell, S.P. (1997) Coordinate binding of ATP and origin DNA regulates the ATPase activity of the origin recognition complex. *Cell* **88**, 493–502
41. Stillman, B. (1996) Cell cycle control of DNA replication. *Science* **274**, 1659–1664
42. Dutta, A. and Bell, S.P. (1997) Initiation of DNA replication in eukaryotic cells. *Annu. Rev. Cell. Dev. Biol.* **13**, 293–332
43. Kubota, T., Katayama, T., Ito, Y., Mizushima, T., and Sekimizu, K. (1997) Conformational transition of DnaA protein by ATP: structural analysis of DnaA protein, the initiator of *Escherichia coli* chromosome replication. *Biochem. Biophys. Res. Commun.* **232**, 130–135