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Negative control of DNA replication by hydrolysis of ATP bound to DnaA protein, the initiator of chromosomal DNA replication in *Escherichia coli*

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DnaA protein, the initiation factor for chromosomal DNA replication in *Escherichia coli*, is activated by ATP. ATP bound to DnaA protein is slowly hydrolyzed to ADP, but the physiological role of ATP hydrolysis is unclear. We constructed, by site-directed mutagenesis, mutated DnaA protein with lower ATPase activity, and we examined its function *in vitro* and *in vivo*. The ATPase activity of purified mutated DnaA protein (Glu204→Gln) decreased to one-third that of the wild-type DnaA protein. The mutation did not significantly affect the affinity of DnaA protein for ATP or ADP. The mutant *dnaA* gene showed lethality in wild-type cells but not in cells growing independently of the function of *oriC*. Induction of the mutated DnaA protein in wild-type cells caused an overinitiation of DNA replication. Our results lead to the thesis that the intrinsic ATPase activity of DnaA protein negatively regulates chromosomal DNA replication in *E.coli* cells.
Keywords: ATPase/DnaA/*Escherichia coli*/negative regulation/site-directed mutagenesis

Introduction

The initiation of DNA replication has to be strictly regulated so that it is coupled with cell division. However, the molecular mechanism underlying the control of initiation of DNA replication is unclear.

In *Escherichia coli*, chromosomal DNA replication has been studied genetically and biochemically. The functions of a number of replication factors have been elucidated (Kornberg and Baker, 1992). DnaA protein plays a crucial role in the initiation of chromosomal DNA replication (Hirota *et al.*, 1970; Kornberg and Baker, 1992). It specifically binds to DnaA boxes in the *oriC* region (Fuller and Kornberg, 1983; Fuller *et al.*, 1984), and opens up the double-stranded DNA for entrance of other replication proteins (Sekimizu *et al.*, 1987; Bramhill and Kornberg, 1988). DnaA protein has a high affinity for both ATP and ADP (Sekimizu *et al.*, 1987). Tightly bound ATP is slowly hydrolyzed to form the ADP-binding form of DnaA protein. In an *oriC* DNA replication system reconstituted with purified proteins, the ATP-binding form of DnaA protein is active, whereas the ADP-binding form is inactive (Sekimizu *et al.*, 1987). Expression of DnaAcos protein, which is not susceptible to inactivation by ADP binding, leads to overinitiation of chromosomal DNA replication in cells and in an *in vitro oriC* DNA replication system (Katayama, 1994; Katayama and Kornberg, 1994). We reported that synthesized organic compounds which were designed to block the ATP binding to DnaA protein specifically inhibited the *oriC* DNA replication *in vitro* (Mizushima *et al.*, 1996c). These results suggest that the activity of DnaA protein is regulated by its adenine nucleotide binding *in vivo*.

As DnaA protein bound to ATPγS or AMPPNP, non-hydrolyzable analogs of ATP, is active in DNA replication *in vitro* (Sekimizu *et al.*, 1987), the hydrolysis of ATP bound to DnaA may not be required for replication activity of the protein. Thus, the physiological role of the hydrolysis of ATP bound to DnaA protein remains to be elucidated. Since this hydrolysis resulted in formation of the ADP-binding form of DnaA protein, which is inactive in *oriC* DNA replication *in vitro* (Sekimizu *et al.*, 1987), we assumed that the ATPase activity of DnaA protein would be involved in its negative regulation of replication activity.

To test the above hypothesis, we constructed a mutant DnaA protein in which intrinsic ATPase activity decreases, and we examined its function in DNA replication *in vitro* and *in vivo*. For this, we constructed a mutant DnaA protein where the glutamic acid residue at position 204 was replaced by glutamine. This mutant DnaA protein maintained high affinities for ATP and ADP; however, the intrinsic ATPase activity decreased. We showed that induction of mutant DnaA protein in wild-type cells led to overinitiation of DNA replication, which means that the hydrolysis of ATP bound to DnaA protein probably negatively regulates chromosomal DNA replication.

Results

Construction and purification of mutant DnaA protein

It was reported that Glu203 of the F_1-ATPase β subunit is required for the hydrolysis of ATP (Ohtsubo *et al.*, 1987; Amano *et al.*, 1994). A comparison of amino acid sequences of other ATPases revealed that this glutamic acid residue (or asparatic acid residue) is highly conserved and, hence, is probably important for the activity of the enzyme (Yoshida and Amano, 1995). SecA protein is involved in protein translocation across inner membranes of *E.coli* and has a high affinity for ATP. ATP tightly bound to SecA protein is hydrolyzed by its intrinsic ATPase activity as is DnaA protein (Cunningham *et al.*, 1989; Akita *et al.*, 1990; Matsuyama *et al.*, 1990). Substitution of Asp133 of SecA protein with Asn was shown to decrease the intrinsic ATPase activity but did not affect its affinity for ATP (Sato *et al.*, 1996). Glu204 of the DnaA protein is predicted to be a residue required for the

Table I. Purification of DnaA E204Q protein

Fraction	Total protein (mg)	Total activity (units×10^{-3})	Specific activity (units×10^{-3}/mg)	Yield (%)
I Lysate	560	217	0.39	100
II Ammonium sulfate	42	45	1.1	21
III Dialysis pellet	24	33	1.4	15
IV Guanidine	15	24	1.6	11
V Superose gel	0.1	9	90	4

Strain KA450 transformed with pMZ001-1 was grown in 20 l of LB medium containing 25 μg/ml thymine at 37°C until the $OD_{595\ nm}$ reached 0.5, then arabinose was added to 1%. After 1 h of incubation, the cells were harvested by centrifugation, resuspended in buffer C (Sekimizu *et al.*, 1988b) containing 250 mM KCl to an $OD_{595\ nm}$ of 220 and stored at –80°C. The thawed cell suspension was diluted 2-fold with buffer C containing 250 mM KCl, and spermidine-HCl and egg white lysozyme were added to final concentrations of 20 mM and 400 μg/ml, respectively. After incubation at 0°C for 30 min and 37°C for 4 min, the suspension was centrifuged for 30 min in a Beckman 50.2 Ti rotor to collect the supernatant (fraction I, 35 ml). Solid ammonium sulfate (0.21 g/ml of fraction I) was added, with stirring, for 20 min and the suspension was centrifuged for 20 min at 20 000 r.p.m. (Beckman JA-20 rotor). The pellet was resuspended with buffer C (1/12 volume of fraction I) (fraction II, 4 ml) and dialyzed for 12 h against 4 l of buffer C. The precipitate which developed was collected by centrifugation for 30 min at 100 000 r.p.m. in a Beckman TLA100.3 rotor and resuspended by sonication in buffer C containing 0.6 M ammonium sulfate (fraction III, 4 ml). The suspension was washed twice with the same buffer and the pellet was finally resuspended in buffer C containing 4 M guanidine-HCl, 0.6 M ammonium sulfate and 10 mM magnesium acetate. Insoluble materials were removed by centrifugation at 100 000 r.p.m. in a Beckman TLA100.3 rotor. The supernatant (fraction IV) was gel-filtered on a Superose-12 column (Pharmacia fast protein liquid chromatography HR10/30) equilibrated with buffer D (Sekimizu *et al.*, 1988b) at a flow rate of 0.3 ml/min. Active fractions were pooled (fraction V). The activity of DnaA protein was measured by *oriC* complementation assay (Fuller *et al.*, 1981). One unit of replication activity promotes incorporation of 1 pmol of nucleotide/min at 30°C.

intrinsic ATPase activity (Yoshida and Amano, 1995), therefore, in the present study, we introduced a mutation in the *dnaA* gene to change Glu204 to Gln by site-directed mutagenesis (Kunkel, 1987). A coding region of the mutated *dnaA* gene (*dnaA400*) was conjugated with the arabinose operon to construct a plasmid for overproduction of the mutated DnaA protein (DnaA E204Q). To avoid contamination of wild-type DnaA protein in the fraction of DnaA E204Q, the plasmid was introduced into the KA450 strain [Δ*oriC1071*::Tn*10*, *rnhA199*(Am), *dnaA17*(Am)] carrying an amber mutation in the *dnaA* gene (Katayama, 1994). Addition of 1% arabinose caused overexpression of mutant DnaA protein, and its migration on SDS–PAGE was indistinguishable from that of the wild-type protein (data not shown). Purification of DnaA E204Q was performed by the method used for wild-type DnaA protein (Sekimizu *et al.*, 1988b), but with some modifications: ammonium sulfate precipitation was performed at 0.21 g/ml; the precipitate appeared after dialysis of the ammonium sulfate precipitation fraction has been applied directly to gel filtration column chromatography, following solubilization with guanidine–HCl. DnaA E204Q protein was purified to apparent homogeneity with a recovery of 4% (Table I). The purity of the final fraction (fraction V) exceeded 90%, as determined by SDS–PAGE (Figure 1).

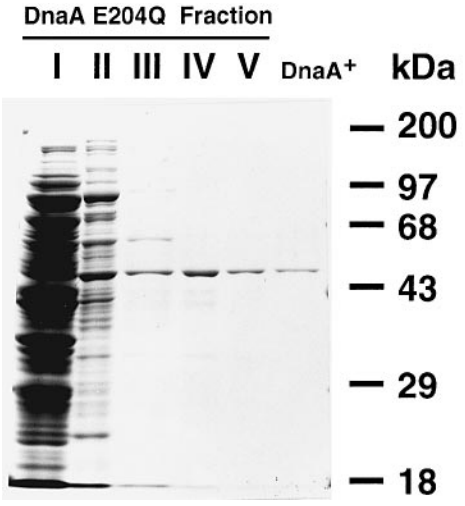

Fig. 1. Isolation of DnaA E204Q protein. Protein fractions from Table I were applied on an SDS–polyacrylamide (10%) gel and stained with Coomassie brilliant blue R-250.

Characterization of ATP and ADP binding and ATPase activity of DnaA E204Q protein

We first examined the binding of DnaA E204Q protein to ATP and ADP. DnaA protein and [α-^{32}P]ATP of various concentrations were incubated together, and the amount of ATP bound to the protein was determined by filter-binding assay (Sekimizu *et al.*, 1987). ATP bound to both DnaA E204Q and wild-type DnaA in a dose-dependent manner. Scatchard plot analysis revealed that the K_d value for the binding of ATP to DnaA E204Q was 46 nM (Figure 2A). For wild-type DnaA protein, the K_d was determined to be 48 nM, much the same value as reported previously (Sekimizu *et al.*, 1987). The numbers of ATP-binding sites per mutated and wild-type DnaA protein molecule were calculated to be 0.53 and 0.40, respectively. The affinity of DnaA E204Q for ADP was determined in the same manner. K_d values for the binding of ADP to DnaA E204Q and wild-type DnaA were 160 and 230 nM, respectively (Figure 2B). The value for wild-type DnaA protein is twice that reported previously (Sekimizu *et al.*, 1987). The numbers of ADP-binding sites per mutated and wild-type DnaA protein molecule were calculated to be 0.29 and 0.23, respectively. These results indicate that the mutation (Glu204 to Gln) probably does not change the affinity for ATP and ADP significantly.

Next, we compared the ATPase activity of DnaA E204Q with that of the wild-type protein. DnaA protein was pre-incubated with [α-^{32}P]ATP at 0°C and incubated further at 38°C in the presence of DNA. The complex of DnaA protein with ATP or ADP was recovered by immuno-precipitation, using anti-DnaA serum, and adenine nucleotide bound to DnaA protein was analyzed by thin layer

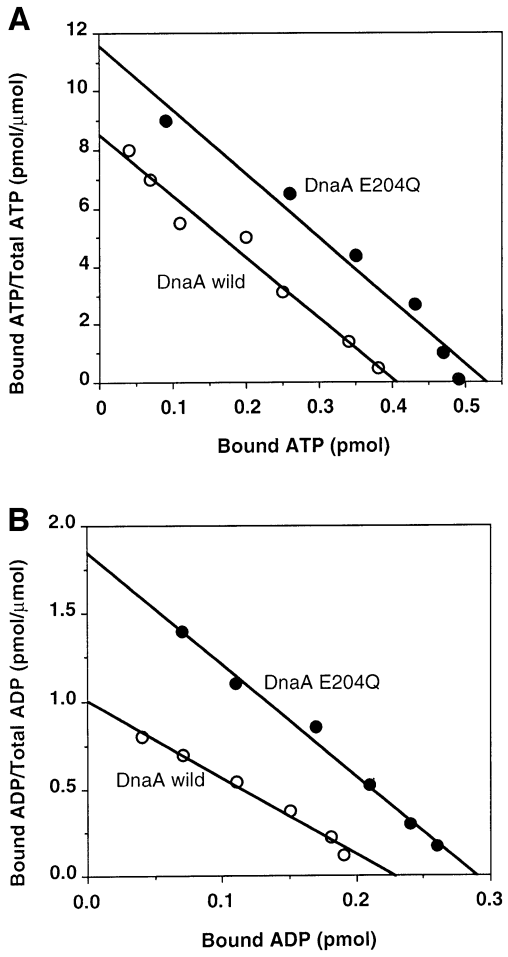

Fig. 2. ATP and ADP binding to DnaA E204Q protein. DnaA E204Q and wild-type DnaA protein (1 pmol) were incubated with various concentrations of [α-^{32}P]ATP (**A**) or [^{3}H]ADP (**B**) for 15 min at 0°C. The amount of bound ATP or ADP was determined by filter-binding assay, as described in Materials and methods, and a Scatchard plot analysis prepared. Linear repression was performed by the least-squares methods.

chromatography. The ATPase activity of DnaA E204Q decreased to about one-third that of wild-type protein (Figure 3), indicating that Glu204 is an important amino acid residue for the intrinsic ATPase activity of DnaA protein.

Replication activity in vitro of DnaA E204Q protein

We examined the replication activity of DnaA E204Q protein in an *oriC* complementation assay. As shown in Figure 4, DnaA E204Q protein was active in DNA replication. There are at least two differences between wild-type and mutated DnaA protein, with regard to DNA replication activities. One is that the specific activity (units/mg protein) of DnaA E204Q is about one-third that of the wild-type protein, the other is that the amount of synthesized DNA at a saturation level of DnaA E204Q protein is 200 pmol of nucleotides (template DNA, 600 pmol nucleotides), being about one-third that of the wild-type protein. The batch-to-batch variability in values (specific activity and DNA synthesis at a saturation level of DnaA protein) of the wild-type DnaA protein was <25%, thereby suggesting that differences in values between mutant and wild-type DnaA protein cannot be

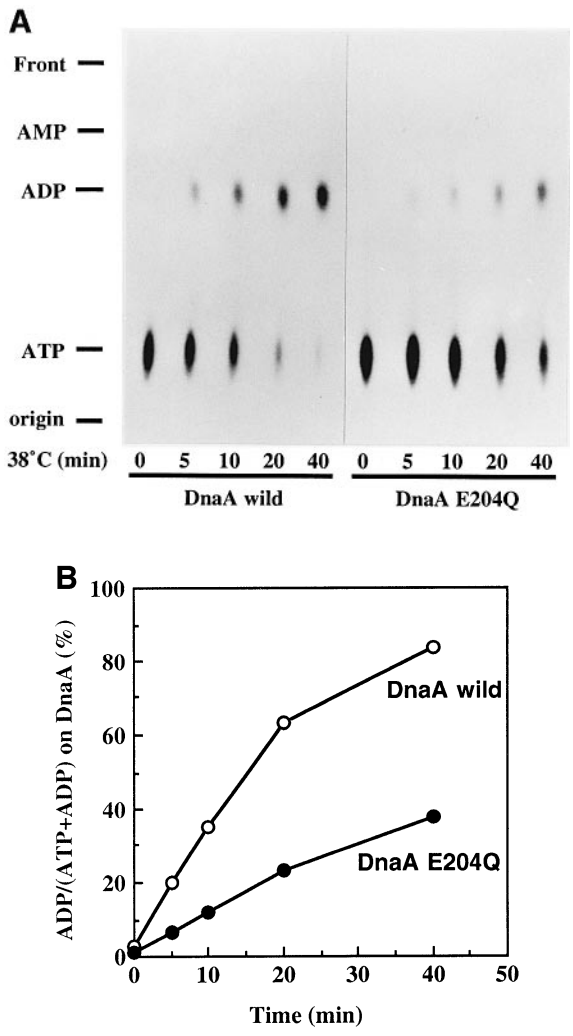

Fig. 3. Time course of hydrolysis of ATP bound to DnaA E204Q protein. Hydrolysis of ATP bound to DnaA E204Q or wild-type DnaA protein was monitored on thin layer PEI-cellulose plates (**A**), as described in Materials and methods. The autoradiograms were scanned by densitometry and the ratio ADP/(ADP and ATP) was determined (**B**).

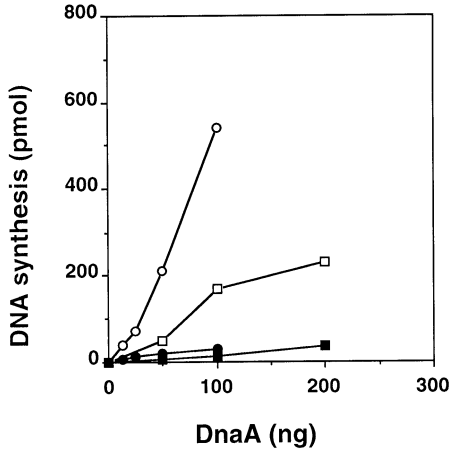

Fig. 4. Replication activity of DnaA E204Q protein in a crude extract. DnaA E204Q (□,■) and wild-type DnaA protein (○,●) were incubated with 1 μM of ATP (○,□) or ADP (●,■) for 15 min at 0°C. DNA replication in a crude extract was carried out as described in Materials and methods.

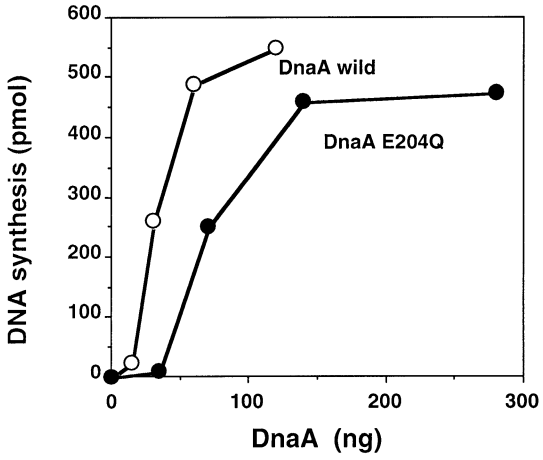

Fig. 5. Replication activity of DnaA E204Q protein in the *oriC* DNA replication system reconstituted with purified proteins. DNA replication in the *oriC* DNA replication system reconstituted with purified proteins was carried out as described in Materials and methods.

explained by this variability. Under our conditions, the wild-type DnaA protein purified using the present protocol showed a specific activity of 0.25×10^6 units/mg protein (Figure 4), which is one-fifth of the previously reported value (Sekimizu *et al.*, 1988b). The lower values are not caused by the modification of purification procedures, because wild-type DnaA protein purified according to the previously published protocol (Sekimizu *et al.*, 1988b) also showed low specific activity (data not shown). The lower value is also not due to denaturation of DnaA protein in our preparations, since the capacity of DnaA protein for ATP binding, which is a good criterion with which to assess the denaturation of DnaA protein, was much the same (Figure 2A) as the previous published data (Sekimizu *et al.*, 1987). We consider that the lower specific activity of wild-type DnaA protein is caused by assay conditions, because the specific activity of DnaA protein measured by mutant complementation assay varies according to the batch of the extracts and species of *oriC* template DNA.

Next, we examined the effect on replication activity of pre-incubation of DnaA E204Q protein with ADP. DnaA protein was pre-incubated with 1 μM of ADP, and the DNA replication reaction was performed in the *oriC* complementation assay system. As shown in Figure 4, the activity of DnaA E204Q protein was diminished by pre-incubation with ADP, as found in the case of the wild-type DnaA. This result suggests that the ADP-binding form of DnaA E204Q protein is inactive, like wild-type DnaA protein, i.e. the mutant protein is susceptible to inactivation by ADP binding.

We next asked whether DnaA E204Q protein is active in the *oriC* DNA replication system reconstituted with purified proteins. DnaA E204Q protein did show DNA replication activity in the system, in a dose-dependent manner (Figure 5). The specific activity of DnaA E204Q protein was half that of wild-type DnaA protein, and the amount of synthesized DNA at a saturation level with DnaA E204Q protein was much the same as that with the wild-type protein.

Table II. *oriC*-dependent lethality of *dnaA400*

Strain	JM109 (wild-type)	KA450 (Δ*oriC*)
pMZ002-1	$<5.0\times10^3$	1.4×10^6
pMZ002-2	1.0×10^7	2.0×10^6
pBR322	3.6×10^7	1.4×10^6

oriC-dependent lethality by the dnaA400 gene encoding DnaA E204Q protein

The results from biochemical studies of DnaA E204Q protein described above showed that the intrinsic ATPase activity of DnaA E204Q protein decreased without loss of affinity for ATP and ADP. To determine the physiological role of the intrinsic ATPase activity of DnaA protein, we examined the influence on chromosome DNA replication of the *dnaA400* mutation encoding the DnaA E204Q protein. We assumed that the ATPase activity is involved in negative regulation of *oriC* DNA replication. If such is indeed the case, the introduction of the *dnaA400* gene into wild-type *E.coli* cells should lead to an overinitiation of chromosomal DNA replication, in an *oriC*-dependent manner, resulting in a lethal phenotype of the mutant. The coding regions of the wild-type *dnaA* gene and the *dnaA400* gene were conjugated with the *dnaA* promoter, and resultant plasmids were introduced into a wild-type strain (JM109). As shown in Table II, the transformation efficiency of pMZ002-1 (*dnaA400*) was $<1/10^3$ of that of pMZ002-2 (wild-type *dnaA*). In other words, the *dnaA400* gene showed a lethal phenotype in wild-type cells. When the KA450 strain (Δ*oriC*) was used as a recipient for transformation, the transformation efficiency of pMZ002-1 (*dnaA400*) was much the same as that of pMZ002-2 (wild-type *dnaA*) (Table II), indicating that lethality by the *dnaA400* gene required functions of the *oriC* sequence. These results suggest that DnaA E204Q protein does affect initiation of DNA replication from the *oriC* region, the result being the lethal phenotype. However, the possibility that genotype differences between strains (JM109 and KA450) influence the outcome of results in Table II cannot be ruled out at this stage.

Overinitiation of DNA replication by induction of DnaA E204Q protein in cells

We next examined the mechanism of lethality by the *dnaA400* mutation. A coding region of the *dnaA400* gene was introduced into the 3′ downstream region of the *lac* promoter to construct a system where the expression of DnaA E204Q protein is under the control of the *lac* promoter (pMZ003-1). JM109 cells harboring pMZ003-1 were labeled continuously with [^{3}H]thymine in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG; 0.1 mM), and incorporation of thymine into trichloroacetic acid (TCA)-insoluble fractions was measured. The rate of thymine incorporation into JM109 cells harboring pMZ003-1 (*dnaA400*) was 3-fold more than that into JM109 cells harboring pMZ003-2 (wild-type *dnaA*) (Figure 6). In other words, induction of DnaA E204Q protein caused an overinitiation of DNA replication in cells. After addition of IPTG, the growth rate of cells harboring pMZ003-1 (*dnaA400*) was slower than that of cells harboring pMZ003-2 (wild-type *dnaA*) (data not shown). Therefore, the higher incorporation of [^{3}H]thym-

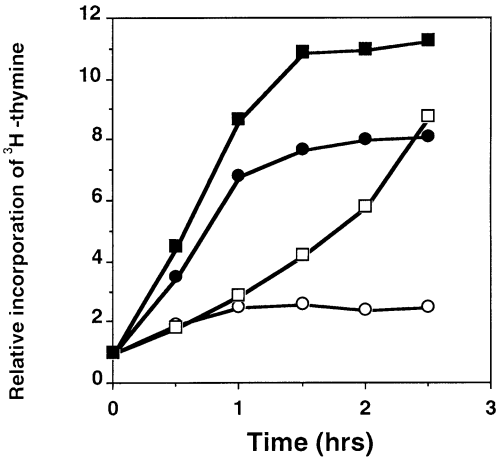

Fig. 6. DNA synthesis in *E.coli* cells transformed with an expression plasmid of DnaA E204Q or wild-type DnaA protein. JM109 cells carrying pMZ003-1 (*lac-dnaA400*) (●,■) or pMZ003-2 (*lac-dnaA*$^+$) (○,□) plasmid grew in LB medium containing [^{3}H]thymine until the $OD_{600\ nm}$ reached 0.1. IPTG (final concentration, 0.1 mM) was added (time, –30) and incubation was continued for 30 min. Then, chloramphenicol was added (final concentration, 200 μg/ml) (time, 0) (○,●). Portions (200 μl) were withdrawn at intervals and the amount of DNA synthesized was calculated, as described in Materials and methods.

ine into the cells harboring pMZ003-1 cannot be explained by differences in growth rates. When we examined the effect of the induction of DnaA E204Q protein on DNA synthesis in cells growing at 28°C, at which the generation time was more than twice that at 37°C, we observed much the same stimulation of DNA synthesis upon induction (data not shown). Therefore, the overinitiation caused by induction of DnaA E204Q protein is probably independent of the growth rate.

The initiation but not elongation of *oriC* DNA replication in wild-type cells is sensitive to inhibitors of protein synthesis, such as chloramphenicol (Lark and Renger, 1969). We thus examined the effect of chloramphenicol on DNA synthesis induced by expression of DnaA E204Q protein. Chloramphenicol (final, 200 μg/ml) was added 30 min after the addition of IPTG (0.1 mM), and the level of DNA synthesis was monitored by the incorporation of [^{3}H]thymine into TCA-insoluble fractions. DNA synthesis in cells inducing wild-type DnaA protein continued for 1 h after the addition of chloramphenicol (Figure 6). When DnaA E204Q protein was induced, a much higher level of DNA synthesis continued until 1.5 h after the addition of chloramphenicol (Figure 6). These observations suggest that more replication forks existed in DnaA E204Q protein-inducing cells than in the wild-type protein-inducing cells when chloramphenicol was added to the culture.

Discussion

In the present study, we tested our hypothesis that the intrinsic ATPase of DnaA protein negatively regulates the initiation of chromosomal DNA replication in cells. We designed mutant DnaA protein (DnaA E204Q), whose intrinsic ATPase activity is lower than that of wild-type DnaA protein, and observed the influence of expression of the mutant protein on DNA synthesis in cells. Transformation of wild-type cells with a plasmid carrying the mutant *dnaA* gene, encoding the DnaA E204Q protein, caused a lethal phenotype, and expression of the mutant DnaA protein stimulated DNA synthesis. Therefore, the lethal phenotype is apparently caused by overinitiation of chromosomal DNA replication, as a result of a lack of negative regulation of the initiation of DNA replication by the intrinsic ATPase activity of DnaA protein.

DnaA E204Q did show activity in *oriC* replication systems; however, the specific activity was lower than that of the wild-type protein. Since DnaA protein bound to non-hydrolyzable analogs of ATP was seen to be active in DNA replication *in vitro* (Sekimizu *et al.*, 1987), hydrolysis of ATP bound to DnaA protein does not contribute to the replication reaction. Therefore, the lower specific activity of DnaA E204Q protein cannot be caused by the lower ATPase activity. A conformational change of DnaA protein by the mutation (Glu204→Gln) may decrease its activity in DNA replication or render it sensitive to denaturation during the course of purification. The ADP-binding form of DnaA E204Q, like wild-type protein, was inactive in DNA replication. Therefore, the overinitiation of DNA replication induced by expression of DnaA E204Q protein in cells is not due to the fact that the mutant protein is insusceptible to inactivation by ADP binding. Thus, the character of the DnaA E204Q protein differs from that of the DnaAcos protein, which is not susceptible to inactivation by ADP binding.

An inactivation factor specific for DnaA protein (IdaA protein) has been identified (Katayama and Crooke, 1995). The inactivation activity of IdaA protein required ATP and DNA (Katayama and Crooke, 1995), which are necessary for the intrinsic ATPase activity of DnaA protein (Sekimizu *et al.*, 1987). Recently, we identified a stimulatory activity for DnaA ATPase in a crude extract from *E.coli* cells (unpublished results). The stimulatory activity co-migrates with the inactivation activity of DnaA protein, under several chromatographic conditions (unpublished results). These results suggest that IdaA protein inhibits *oriC* replication *in vitro* by enhancing the intrinsic ATPase activity of DnaA protein. Together with our present data, we propose that IdaA protein contributes to the negative regulation of initiation of chromosomal DNA replication *in vivo* by its stimulatory activity for DnaA ATPase.

DnaAcos protein is not inactivated by IdaA protein (Katayama, 1994). The resistance of DnaAcos protein to IdaA protein may be due to the fact that this protein loses its affinity for ATP and ADP (Katayama, 1994) and thus cannot take on the ADP-binding form, which is inactive in *oriC* replication. DnaAcos protein in the absence of ATP probably take on a conformation similar to the ATP-binding form of wild-type DnaA protein (Katayama *et al.*, 1995).

A common feature seen in DnaA protein and GTP-binding protein (G protein), an important factor for the signal transduction system in eucaryotic cells, is that their activities are regulated by forms of purine nucleotide bound to proteins. GTP bound to G protein is slowly hydrolyzed to GDP by the intrinsic GTPase activity of G protein to form the inactive, GDP-binding form (Gilman, 1987). This reaction corresponds to hydrolysis of the ATP bound to DnaA protein by its intrinsic ATPase activity. The GTPase activity of G protein is stimulated by GTPase-

activating protein (GAP), which negatively regulates signal transduction (McCormick, 1989). We consider that IdaA protein of *E.coli* corresponds to GAP in eucaryotic cells (Katayama and Crooke, 1995). Coupled with binding of the ligand to the receptor, G protein bound to GDP became active, i.e. the GTP-binding form was produced, by the exchange reaction of GDP with GTP. We propose that acidic phospholipids in membranes of *E.coli* give signals to DnaA protein corresponding to the role of the receptor in eucaryotic signal transduction, because acidic phospholipids, such as cardiolipin and phosphatidylglycerol, stimulate the exchange reaction of ADP bound to DnaA protein with ATP by reducing the affinity of DnaA protein for ADP (Sekimizu and Kornberg, 1988; Yung and Kornberg, 1988; Castuma *et al.*, 1993; Mizushima *et al.*, 1996a), and that acidic phospholipids are required for *oriC* DNA replication *in vivo* (Xia and Dowhan, 1995).

Materials and methods

Bacterial strains

Strains JM109 (*recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1,* Δ(*lac-proAB*)/F′[*traD36, proAB*$^{+}$, *lacI*^{q}, *lacZ*Δ*M15*]), BW313 (HfrKL16PO/45[*lys(61-62)/dut1, ung1, thi-1, relA1*]) and KA450 [Δ*oriC1071*::Tn*10, rnhA199*(Am), *dnaA17*(Am), *trpE9829*(Am), *tyrA*(Am), *thr, ilv, thyA*] were from Katayama (1994) and WM433 (*dnaA204, leu19, pro19, trp25, his47, thyA59, arg28, met55, deoB23, lac11, strA56, sul1, hsdS*^{K12}) was from our laboratory stock.

Site-directed mutagenesis of the dnaA gene

Site-specific mutation was performed according to the methods of Kunkel (1987). Phage M13mp19, which contains an antisense strand of the coding region of the *dnaA* gene at the polylinker site (*Bam*HI–*Hin*dIII) (Guo, Miki and Sekimizu, unpublished), was introduced into *E.coli* BW313 (*ung1*) and the uracil-containing single-stranded DNA was isolated. An oligonucleotide primer, 5′-ATATGCACTCC**C**AGCCG-CTTTGTTC-3′, was used as a mismatch primer for replacement of Glu204 by Gln in DnaA (the changed base is indicated in bold). The complementary DNA strand was synthesized *in vitro* with the uracil-containing single-stranded DNA and the primer. The resultant double-stranded DNA was introduced into JM109. According to the results of DNA sequencing, two of three independent phages contain the mutation. Double-stranded DNA (pMZ000-1 with the mutation, pMZ000-2 without the mutation) was prepared and used for the construction of plasmids.

Construction of plasmids

We used the plasmid pKA231, which is a derivative of pKA233-5 (Katayama, 1994) and contains the wild-type *dnaA* gene under the control of the arabinose operon, to construct the plasmid for overproduction of the DnaA E204Q protein. *Hin*dIII linker (Takara) was ligated into the *Aat*II site of pKA231, which was filled-in in advance with the DNA polymerase I large fragment (pMZ001). The *Eco*RI–*Hin*dIII region of pMZ001 was replaced with the *Eco*RI–*Hin*dIII region of pMZ000-1 which contains the coding region of the *dnaA400* gene. The resultant plasmid was named pMZ001-1 and was used for overproduction of DnaA E204Q protein.

For analysis of the dominant lethality of the *dnaA400* gene, we introduced the coding region of the *dnaA400* gene into the downstream region of the *dnaA* promoter. The *Eco*RI–*Stu*I fragment of plasmid pKP2204, which is a miniR plasmid containing a promoter of the *dnaA* gene and the spectinomycin-resistant gene (Guo, Miki and Sekimizu, unpublished), was ligated into the *Eco*RI–*Nru*I site of pBR322 (pMZ002). The *Bam*HI–*Hin*dIII fragment of pMZ000-1 or pMZ000-2 was ligated into pMZ002 to construct pMZ002-1 or pMZ002-2, respectively.

To acquire a controllable expression system for DnaA E204Q protein, we constructed a plasmid in which the *dnaA400* gene is located under the *lac* promoter. We amplified the *lac* promoter by PCR with M13mp18RFI and two appropriate primers (primers 1 and 2). As these primers contained the *Eco*RI or *Bam*HI recognition site in their 5′ or 3′ end, respectively, the PCR product contains *Eco*RI and *Bam*HI recognition sites in the 5′ and 3′ ends, respectively. The PCR product was digested with *Eco*RI and *Bam*HI and ligated to pMZ002 to construct pMZ003. The *Bam*HI–*Hin*dIII fragment of pMZ000-1 or pMZ000-2 was ligated into pMZ003 to construct pMZ003-1 or pMZ003-2, respectively.

Preparation of wild-type DnaA protein

Wild-type DnaA protein was purified by the method described elsewhere (Sekimizu *et al.*, 1988b), except that a newly constructed overproducer was used (Kubota *et al.*, 1997). The specific activity of the protein was 0.3×10^{6} units/mg. The purity of the fraction used exceeded 90%, as determined by SDS–10% PAGE.

ATP- or ADP-binding assay

The ATP- or ADP-binding activity of DnaA protein was determined by filter-binding assay (Sekimizu *et al.*, 1987). DnaA protein (1 pmol) was incubated with [α-^{32}P]ATP or [^{3}H]ADP at 0°C for 15 min in 40 μl of buffer G [50 mM HEPES/KOH, pH 8.0, 0.5 mM magnesium acetate, 0.3 mM EDTA, 5 mM dithiothreitol (DTT), 10 mM ammonium sulfate, 17% (v/v) glycerol and 0.005% Triton X-100]. Samples were passed through nitrocellulose membranes (Millipore HA 0.45 μm). The radioactivity remaining on the filters was counted in a liquid scintillation counter.

Measurement of hydrolysis of ATP bound to DnaA protein

The ATPase activity of DnaA protein was measured by immunoprecipitation and thin layer chromatography. DnaA protein (1 pmol) was incubated with 1.5 μM [α-^{32}P]ATP (10^{5} c.p.m./pmol) at 0°C for 15 min and further incubated at 38°C in a reaction mixture containing 40 mM HEPES/KOH, pH 7.6, 11 mM magnesium acetate, 0.5 mM GTP, CTP and UTP, 2 mM ATP, 100 μM dNTP, 7% polyvinyl alcohol, 40 mM creatine phosphate, 100 μg/ml creatine kinase and 200 ng of M13E10. Anti-DnaA serum and protein A–Sepharose in buffer L (50 mM HEPES/KOH, pH 7.6, 5 mM magnesium acetate, 1 mM EDTA, 5 mM ammonium sulfate, 0.005% Triton X-100, 100 mM NaCl, 0.1 mM ATP, 0.1 mM ADP and 5 mg/ml lysozyme) was added to the mixture, which was then incubated at 4°C for 30 min, with rotation. The mixtures were centrifuged at 5000 r.p.m. for 1 min and washed with buffer L and buffer M (buffer L without lysozyme). Precipitates were extracted with 40 μl of 1 M HCOOH and 5 mM each of ATP, ADP and AMP. Samples (0.5 μl) were spotted on PEI-cellulose plates (Merck). Chromatography was performed with 1 M HCOOH, 0.5 M LiCl and the plates were autoradiographed.

oriC DNA replication in vitro in a crude extract

Replication of minichromosomes in a crude extract (fraction II) was assayed as described (Fuller *et al.*, 1981). Template DNA (200 ng, 600 pmol of nucleotides), M13E10, 240 μg of fraction II from WM433 (*dnaA204*) and DnaA protein were mixed with reaction cocktails (Fuller *et al.*, 1981) and incubated for replication at 30°C for 20 min. The reaction was terminated by chilling on ice and adding 10% TCA. Samples were passed through Whatman GF/C glass-fiber filters. The amount of radioactivity on the filter was measured in a liquid scintillation counter, and the amount of DNA synthesized (pmol of nucleotides) was calculated.

oriC replication system in vitro reconstituted from purified proteins

Reaction cocktails (Sekimizu *et al.*, 1988a) (25 μl) contained 20 mM Tris–HCl (pH 7.4), 0.1 mg/ml bovine serum albumin, 8 mM DTT, 0.01% Briji 58, 8 mM magnesium acetate, 125 mM potassium glutamate, 2 mM ATP, 0.5 mM each of GTP, CTP and UTP, 200 ng of pBS*oriC* (600 pmol of nucleotide), 400 ng of SSB, 40 ng of β subunit of DNA polymerase III, 10 ng of HU, 180 ng of GyrA, 360 ng of GyrB, 150 ng of DnaB, 90 ng of DnaC, 10 ng of primase, various amounts of DnaA, 450 ng of DNA polymerase III* and 0.1 mM each of dATP, dGTP, dCTP and [α-^{32}P]TTP (50–150 c.p.m./pmol). The reaction was terminated by chilling on ice and adding 10% TCA. Samples were passed through Whatman GF/C glass-fiber filters and the amount of radioactivity on the filter was measured in a liquid scintillation counter, while the amount of DNA synthesized (pmol of nucleotides) was calculated.

Measurement of chromosomal DNA replication in vivo

Cells were grown to full growth in LB medium containing [^{3}H]thymine (Sigma) (25 μg/ml, 55 Ci/mmol) for 12 h. A portion of this culture was diluted 50-fold with the same medium and cultured at 37°C. Portions (200 μl) were withdrawn at appropriate intervals and mixed with TCA (10%, final concentration). Samples were passed through Whatman GF/C glass-fiber filters. The amount of radioactivity on the filter was measured in a liquid scintillation counter.

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