A Nucleotide Switch in the *Escherichia coli* DnaA Protein Initiates Chromosomal Replication

EVIDENCE FROM A MUTANT DnaA PROTEIN DEFECTIVE IN REGULATORY ATP HYDROLYSIS IN VITRO AND IN VIVO*

Received for publication, August 28, 2001, and in revised form, December 19, 2001
Published, JBC Papers in Press, February 11, 2002, DOI 10.1074/jbc.M105393200

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The ATP-bound DnaA protein opens duplex DNA at the *Escherichia coli* origin of replication, leading to a series of initiation reactions in *vitro*. When loaded on DNA, the DNA polymerase III sliding clamp stimulates hydrolysis of DnaA-bound ATP in the presence of the IdaB/Hda protein, thereby yielding ADP-DnaA, which is inactive for initiation in *vitro*. This negative feedback regulation of DnaA activity is proposed to play a crucial role in the replication cycle. We here report that the mutant protein DnaA R334A is inert to hydrolysis of bound ATP, although its affinities for ATP and ADP remain unaffected. The ATP-bound DnaA R334A protein, but not the ADP form, initiates minichromosomal replication in *vitro* at a level similar to that seen for wild-type DnaA. When expressed at moderate levels in *vivo*, DnaA R334A is predominantly in the ATP-bound form, unlike the wild-type and DnaA E204Q proteins, which in *vitro* hydrolyze ATP in a sliding clamp- and IdaB/Hda-dependent manner. Furthermore, DnaA R334A, but not the wild-type or the DnaA E204Q proteins, promotes overinitiation of chromosomal replication. These *in vivo* data support a crucial role for bound nucleotides in regulating the activity of DnaA during replication. Based on a homology modeling analysis, we suggest that the Arg-334 residue closely interacts with bound nucleotides.

Chromosomal duplication occurs only once during the cell cycle and is regulated mainly by ingenious controls that act during the initiation of replication (1–3). The *Escherichia coli* initiator protein, DnaA, binds to the chromosomal origin of replication (oriC) and promotes a series of reactions leading to the formation of a replication fork (4, 5). This protein has high affinity for ATP and ADP, but only the ATP-bound form (ATP-DnaA) can initiate replication at oriC. ATP-DnaA (but not ADP-DnaA) causes local unwinding of the oriC DNA duplex, which creates a site of entry for the DnaB helicase. DnaB helicase loaded on the unwound site forms a complex with DnaG primase and expands the single-stranded region. DNA polymerase (pol) III holoenzyme is then loaded on the primed DNA and begins synthesis (6, 7). Several features of both DnaA and the oriC locus are important for the regulation of initiation.

Immediately after initiation, re-replication of oriC is restrained temporarily (8). *E. coli* DNA is modified at the adenine residue of the palindromic sequence GATC by DNA-adenine methyltransferase, but newly replicated DNA remains hemi-methylated until acted upon by DNA-adenine demethyltransferase (9). The hemimethylated oriC locus is bound by the protein SeqA, which likely inhibits the initiation of replication until full methylation is re-established (10–12). This eclipse time is speculated to persist for ~10 min under conditions in which the cellular doubling time is 30 min (9, 10).

Also after initiation, DnaA is likely inactivated by the hydrolysis of bound ATP to yield ADP-DnaA (3). In *vitro*, ATP hydrolysis is promoted by the pol III β subunit that is loaded onto DNA (the so-called sliding clamp) and by the protein IdaB (13–15). During processive DNA synthesis driven by the pol III holoenzyme, the ring-shaped pol III β subunit dimer encircles the post-replicated DNA duplex or the heteroduplex formed by an RNA primer on a DNA template (6, 7). The requirement of the DNA-bound form of the sliding clamp for DnaA-ATP hydrolysis ensures the timely coupling between DnaA inactivation and the replication cycle (14, 15). In previous studies, IdaB activity was detected in partially purified fractions (14) and more recently has been associated with a new protein, Hda (16). The inactivation of DnaA has been termed RIDA (for regulatory inactivation of DnaA) (14), which is characterized by a decrease in the level of ATP-DnaA following initiation (14, 17). In a synchronized culture, it takes ~15–20 min for ATP-DnaA levels (representing 80–90% of the total amount of ATP- and ADP-bound DnaA) to decrease to the basal level (~40% in the strain background examined). Thus, the sequestration of oriC by SeqA and the RIDA system presumably complement each other in defining the inter-initiation time of the cell cycle (3, 18, 19). In addition, DnaA is titrated by the datA locus, which is a site at 94.7 min that contains a DnaA box cluster,

* This work was supported in part by research grants from the Japan Society for the Promotion of Science and the Ministry of Education, Culture, Sports, Technology and Science of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: pol, DNA polymerase; RIDA, regulatory inactivation of DnaA; PEI, polyethyleneimine; IPTG, isopropyl-1-thio-β-D-galactopyranoside; SCR, structurally conserved region; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
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providing another means to repress DnaA activity and to prevent overinitiation (3, 20, 21).

Some dnaA mutants that lack a tight affinity for adenine nucleotides exhibit overinitiation of replication (3). Based on an assumption that the control of DnaA activity by bound nucleotides plays a crucial role in controlling initiation, an explanation for overinitiation is that the mutant DnaA does not undergo inactivation by RIDA and continues to promote initiation. If this is the case, a prediction that follows is that overinitiation could also be caused by a mutant DnaA that has high affinity for adenine nucleotides but is defective for the hydrolysis of bound ATP by RIDA. Such a mutant, however, has not yet been reported.

Recently, we found that the arginine 334 residue of DnaA (Arg-334) is required for ATP hydrolysis, because substitution of arginine by histidine (the R334H mutation) inhibits RIDA and the intrinsic ATPase activity of DnaA (15). However, this mutant protein is unstable in its ability to promote initiation (15, 22, 23). In this study, we show that the R334A substitution also inhibits RIDA and the intrinsic ATPase activity of DnaA and that the expression of the DnaA R334A protein causes the overinitiation of chromosomal replication. These findings emphasize the importance of the Arg-334 residue in ATP hydrolysis and the significance of bound nucleotides in the control of DnaA activity by RIDA, and hence in the regulation of replication in E. coli. Arginine 334 is a conserved basic residue found in the Box VIII motif of members of the AAA+ protein family, which includes chaperone-like ATPases (24). Based on the motifs conserved among AAA+ proteins and the structure of one such protein (25), the archaeal Pyrobaculum aerophilum Cdc6 protein, we constructed a model of the tertiary structure of the DnaA ATP-binding domain (domain III) by the homology-modelling method. This model provides an explanation for the possible role of the Arg-334 residue in ATP hydrolysis.

Previously, the intrinsic ATPase activity of another DnaA mutant protein, DnaA E204Q, was reported to be only one-third that of the wild-type DnaA (26, 27). In this paper, we also investigated the in vitro and in vivo RIDA behaviors of this mutant protein and, in addition, re-examined several of the reported features in replicational initiation control in vivo.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media—E. coli K-12 derivatives were used. Relevant genotypes are shown in parentheses, as follows: KH5402-1 (thyA1) (14), WM453 (dnaA204) (28), and BW313 (ang-1) (29) have been previously described. YT411 (rnhA::cat), KA413 (dnaA46), KA429 (rnhA::cat soriC1071::Tn10), KA451 (rnhA::cat dnaA10), and KA450 (soriC1071::Tn10 dnaA17 (Am) rnhA199 (Am)) are derivatives of KH5402-1, as described (17, 23). KP7364 (DnaA::spec rnhA::kan) has also been described (14, 22).

The plasmid pH5284-2, a DnaA R334A overproducer, has the same structure as pGL04, a DnaA R334H overproducer (23), except for the dnaA29 (R334A) coding region. The dnaA29 (R334A) coding region was derived from an EcoRI-HindIII fragment from M13mp19 DNA carrying this dnaA allele. pmZ002-1, pmZ002-2, and pH5299-1 contain the dnaA00 (E204Q) wild-type dnaA, and dnaA29 (R334A) genes, respectively, downstream of the dnaA promoter carried on pmZ002 (26). pSN300 is a pmZ002 derivative bearing the lac promoter region between the EcoRI and BamHI sites instead of the dnaA promoter. This lac promoter region was obtained by PCR (polymerase chain reaction) using M13mp18 DNA and two primers, 5′-CGAATTCCGGCCCAAT-ACGCAAACCGCCT-3′ and 5′-GGCGGATCCCTGCTGGAATTGTATC-3′. pSN305, pSN306 (previously reported as pmZ003-2; Ref. 26), and pSN307 (previously reported as pMZ003-1; Ref. 26) are pSN300 derivatives containing the dnaA29 (R334A), wild-type dnaA, and dnaA00 (E204Q) coding regions, respectively, under the control of the lac promoter. The dnaA coding region was amplified by PCR using primers containing the BamHI (for the 5′ end) or HindIII (for the 3′ end) sites and ligated to M13mp19 DNA as described (22), and mutagenized as described below. BamHI-HindIII fragments isolated from mutagenized plasmids were used to construct expression plasmids.

PHSL99 is a pACYC184 derivative bearing the lacI′ gene. pK518 is a pUC18 derivative bearing a terC-derived 7-kb EcoRI fragment that was subcloned from pTER-L103 (30). M13E10 (31) and M13mpRE5 (32) are minichromosomes containing a minimal oriC region on the M13mp vector.

Tryptone medium contained 10 g/liter tryptone (Difco) and 5 g/liter NaCl (33). Supplemented TG medium was as described (17) except that 0.2% glyceral was used instead of glucose. For the selection of plasmids, tetracycline (15 μg/ml) and/or ampicillin (100 μg/ml) were included in these media unless otherwise noted. Other media and plasmids have been described (34).

Directed Mutagenesis of the dnaA Gene—Site-specific mutagenesis was performed using the method described (29). Briefly, using M13mp19 DNA that carries the dnaA-coding region flanked by the BamHI and HindIII sites at the 5′ and 3′ ends, respectively (22), uracil-containing single-stranded DNA was prepared with BW313 (ang-1) cells. For introduction of the R334A mutation, the resulting DNA was hybridized with a primer, 5′-GATCTAACGTCGACGTCATGGAAG-3′, the complementary DNA strand was synthesized in vitro, and the resulting double-stranded DNA was introduced into JM109 cells. The mutation was confirmed by sequencing. DNA fragments isolated by digestion with BamHI and HindIII were used for the plasmid constructions described above.

Purification of the DnaA R334A Protein—All procedures were essentially the same as those for purification of the DnaA R334H protein, as previously described by Takata et al. (23). Briefly, KA450 cells carrying pH5284-2 were grown in 15 liter of LB medium at 37 °C. Expression of the mutant dnaA gene was induced by adding 1% v-arabinose to logarithmic phase cells, and incubation was continued for 1.5 h. Ammonium sulfate at a final concentration of 0.22 g/ml was added to cleared lysates, and the resulting precipitate was collected by centrifugation, dissolved in buffer C, and dialyzed against the same buffer. Insoluble materials were collected by centrifugation, washed with 0.6 M ammonium sulfate, and dissolved with buffer C containing 0.6 M ammonium sulfate and 4 M guanidine HCl. A monomer fraction (fraction IV) of DnaA R334A protein was obtained by gel filtration chromatography using Superose 12 (Amersham Biosciences) equilibrated with buffer D (35).

In Vitro Minichromosomal Replication—A protein extract (fraction II) prepared from WM453 (dnaA204) was used to monitor in vitro DnaA-dependent minichromosomal replication, as described (28). The buffer consisted of 40 mM Hepes-KOH (pH 7.6), 40 mM phosphocreatine, 2 mM ATP, 0.5 mM each of GTP, CTP, and UTP, 10 mM magnesium acetate, 100 μg/ml creatine kinase, and 7% (w/v) polyvinyl alcohol (molecular weight 30,000–70,000). Reactions (25 μl) prepared in the above buffer contained 240 ng of fraction II, 200 ng (600 pmol) of minichromosome M13E10 RFI DNA, dNTPs at 0.1 mM each, and DnaA proteins as indicated. [α-32P]ATP (50–150 cpm/pmol) was included to enable the subsequent measurement of DNA synthesis by liquid scintillation counting of acid-insoluble materials.

ATP- and ADP-binding Assays—The ATP or ADP binding activity of DnaA proteins was determined by a nitrocellulose filter-retention assay (3). Proteins were incubated with [γ-32P]ATP (2 pmol) at 0 °C for 15 min in 40 μl of binding buffer (50 mM Tricine-KOH, pH 8.3, 0.5 mM magnesium acetate, 0.3 mM EDTA, 7 mM dithiothreitol, 20% (v/v) glycerol, 0.007% Triton X-100, and 0.25 mg/ml bovine serum albumin). Samples were passed through nitrocellulose membranes (Millipore HA, 0.45 μm). After washing, radioactivity retained on the filters was counted in a liquid scintillation counter as described (36).

In Vitro RIDA Systems—RIDA-active fractions II and III were prepared from WM433 as described (14). Briefly, fraction II was prepared by ammonium sulfate precipitation (0.24 g/ml) of fraction I proteins, and the activity was further concentrated as fraction III by the separation of proteins by DE52 column chromatography. These fractions were incubated with [γ-32P]ATP-bound DnaA protein and the oriC plasmid M13E10 in the same buffer as that used for in vitro replication with WM433 extracts. DnaA protein with bound nucleotides was isolated by immunoprecipitation, and radiolabeled nucleotides were separated by polyacrylamide gel electrophoresis and quantified as described (14).

[1] PI Nuclease Assay—This assay was done as described (23, 36). Briefly, buffer (50 μl) containing 60 ng Hphl-KOH (pH 7.6), 5 mM magnesium acetate, 30% (v/v) glycerol, 400 ng of the pBS oriC plasmid, 3.7 kb, 0.32 mg/ml bovine serum albumin, and 5 μM ATP was preincubated at 38 or 28 °C for 1 min. ATP-bound DnaA protein was then added and incubation was continued for 3 min at 38 °C or 6 min at 28 °C, followed by incubation with PI nuclease (10 units; Yamasa Co.) for 25 s at the same temperature. After the reaction was stopped by addition of 0.3% SDS, DNA was extracted with phenol/chloroform,
precipitated with ethanol, digested in buffer with SacI, and analyzed by agarose (1%) gel electrophoresis. DNA fragments of 2.1 and 1.6 kb are generated by this restriction enzyme when open complexes are formed, and thus the oriC site is digested with P1 nuclease (23). The extent of open complex is indicated by the percentage of these fragments. Total DNA was prepared by the method described by Atlung and Hansen (37). Briefly, cell pellets stored at -80°C were resuspended in 270 μl of 10 mM Tris-HCl (pH 8.0), 10 mM NaCl, 10 mM EDTA (pH 8.0), 1% Triton X-100, and incubated for 2 h at 37°C with 150 μg of lysozyme and 150 μg of proteinase K. The lysate was heated at 65°C for 2 h, and nucleic acids were precipitated with ethanol and resuspended in 100 μl of TE buffer. DNA was digested with EcoRI and PstI, and the fragments were separated by electrophoresis (50 V) on a 20-cm 0.7% agarose gel. The gel was soaked in 0.5 μg/ml ethidium bromide for 40 min, photographed, soaked in 0.37% HCl for 10 min, washed twice in deionized water, and soaked for 40 min in 0.5 M NaOH, 1.5 M NaCl solution and for 60 min in 0.5 M Tris-HCl (pH 7.2), 3 M NaCl solution. DNA was then transferred to a GeneScreen Plus filter (DuPont/NEN, Boston, MA) by the capillary method (34). After transfer, the gel was stained with ethidium bromide and photographed again to verify a complete transfer of the DNA. The filter was hybridized with [α-32P]dCTP-labeled probes for the oriC and terC regions. After washing, the filter was exposed on an imaging plate (Fuji Film, Japan) and the intensity of the bands was quantified by using BAS2500 (Fuji Film). Each gel also included DNA samples prepared from KA413 (dnaA46) and pKA58 (HindIII). Restriction fragments (0.5 kb from KA413 and 1.0 kb from pKA58) were isolated from 0.7% agarose with the QIAEX II gel extraction kit (Qiagen, Valencia, CA) and resuspended in TE buffer. Approximately 25 ng of oriC and terC fragments were labeled with the Megaprime DNA labeling system (Amersham Biosciences). Total counts of 10^5 cpm were obtained for both probes.

Measurement of DNA Synthesis—Cells were cultured in tryptone medium with 3 μCi of [3H]thymine (Moravek Biochemicals, Brea, CA) at a concentration of 25 μg/ml (38). Acid-insoluble materials were precipitated with chilled 5% trichloroacetic acid, and radioactivity was measured with a scintillation counter.

Determination of Cellular Levels of the Adenine Nucleotide Forms of DnaA Protein—Cells were labeled by growth in supplemented SG medium containing 0.4 mCi/ml [32P]orthophosphate as described (17). The coordinates in the initial DnaA domain III structure were energy-minimized using the consistent-valence force field implemented in DISCOVER, with an 8-A cutoff for nonbonded interactions. During energy minimization, template forcing was used to reorient the main chain at the SCRs in the initial DnaA domain III structure by using a harmonic potential with a force constant, K = 100 kcal/mol Å². In step 2, the loop regions in the initial DnaA domain III structure were similarly energy-minimized. For step 3, the molecular dynamics began with the energy-minimized structure at 300K, 10,000 steps. In step 4, the resulting model was further energy-minimized by using 100 steps of the steepest descent and 1000 steps of the conjugate gradient. The orientations of ADP bound to DnaA domain III were initially determined using those for ADP in the Cdc6-ADP complex as a structural template (25). The molecular dynamics of the initial ADP-DnaA domain III complex structure was computed using the consistent-valence force field implemented in DISCOVER at 300K, 10,000 steps. During simulations, template forcing was used to restrain the main chain at the initial ADP-DnaA domain III structure. Thereafter, the resulting model was further energy-minimized by using 100 steps of the steepest descent and 1000 steps of the conjugate gradient. The domain III structural images shown here were generated using the MOLSCRIPT program (39).

RESULTS

Initiation Activity of the DnaA R334A Protein—Assuming that ATP-DnaA is the active form that initiates chromosomal replication in vivo, DnaA mutants that are defective in hydrolysis of the bound ATP may cause overinitiation because the ATP-bound form of DnaA accumulates. We previously found that the DnaA R334H protein is defective in ATP hydrolysis in vitro, but its ability to promote initiation was unstable at 30°C and defective at 20°C in vivo and in vitro (15, 22, 23). In this study, we substituted the DnaA arginine 334 with alanine (see "Materials and Methods"). Using methods similar to those used for the preparation of wild-type DnaA and the DnaA R334H protein (23), we overproduced the DnaA R334A protein in a dnaA-null host strain and purified it to near homogeneity (Fig. 1A).

DnaA R334A protein can initiate minichromosomal replication in vitro and can bind adenine nucleotides (Figs. 1 and 2). When assessed at 30°C in a minichromosomal replication-competent crude extract (28), the initiation activity of DnaA R334A was found to be comparable with that of the wild-type protein (Fig. 1B). Moreover, ADP-bound DnaA R334A is unable to initiate replication, similar to what is observed for the wild-type protein (Fig. 1C), which indicates that bound nucleotides also control the activity of the mutant protein. The respective binding affinities for ATP and ADP are comparable for the mutant and the wild-type DnaA proteins (Fig. 2); the dissociation constants (K_d) of DnaA R334A for ATP and ADP are 19 and 18 nM, respectively, and those of wild-type DnaA are 14 and 26 nM, respectively, consistent with previous data (36). Similar results regarding nucleotide affinities and initiation activity were obtained for DnaA R334H (15, 23).

Another DnaA mutant, the DnaAcos protein, overinitiates chromosomal replication at 30°C (2, 38). When this protein is incubated in replication-competent crude extracts at 30°C, minichromosomal replication continues for over 45 min, whereas replication initiated by the wild-type DnaA ceases within 20 min (40). This is explained by observations that DnaAcos, but not the wild-type DnaA, is resistant to DnaA-specific inactivation by components of this extract, and thus the initiation activity of DnaAcos is more stable than that of wild-type DnaA (15, 40). This DnaA-inactivating system is now termed RIDA (14, 16, 17). When the activity of the DnaA R334A protein was assessed under similar conditions, the results obtained were basically similar to those seen for the DnaAcos protein (Fig. 1D and E).

The initiation activity of the DnaA R334H protein is cold-sensitive, in that open complex formation at oriC, which can be detected by P1 nuclease assay, is significantly inhibited at 28°C but not at 38°C (the optimal assay temperature), when compared with what is observed for wild-type DnaA (23). The ability of the DnaA R334A protein to promote open complex formation, as assessed by the P1 nuclease assay, is not significantly inhibited at 38°C nor even at 28°C, similar to what
was shown for wild-type DnaA at each temperature (Fig. 1, F and G).

The DnaA R334A Protein Is Insensitive to RIDA—We next asked whether DnaA R334A-bound ATP is hydrolyzed in a RIDA-dependent manner in vitro (Fig. 3) to determine whether the DnaA R334A protein is less sensitive to RIDA, as was shown for DnaA R334H (15). In these experiments, we used partially purified protein fractions (fractions II and III (14, 15) containing the DNA polymerase III holoenzyme and the Ida/B Hda protein, which are required for RIDA. The wild-type DnaA-bound ATP in these fractions is efficiently hydrolyzed to yield ADP-form molecules, whereas DnaA R334A-bound ATP is not (Fig. 3, A and B). Although a slight residual activity (30–40% hydrolysis) was seen for DnaA R334A, the distinction between DnaA R334A and wild-type activity is evident.

Similar experiments were performed with DnaA E204Q. Although the intrinsic ATPase activity of this protein was reported to be approximately one-third that of the wild-type protein (26), its sensitivity to RIDA had not been examined. As shown in Fig. 3C, the hydrolysis of DnaA E204Q-bound ATP is efficient in the RIDA reaction system, similar to what was seen for the wild-type protein.

Intrinsic ATPase Activity of the DnaA R334A Protein—To shed further light on the mechanism of the RIDA insensitivity of DnaA R334A, we assessed its intrinsic ATPase activity (36). After incubation in reaction buffer, DnaA protein was isolated by immunoprecipitation, and the recovered nucleotides were separated by PEI-cellulose thin layer chromatography, as was done for the RIDA assay (above). The results indicate that the intrinsic ATPase activity of DnaA R334A is minimal at 38 °C (Fig. 4). Similar results were obtained when the protein was incubated at 30 °C (data not shown). As DnaA R334A is competent for the initiation of replication and exhibits affinity for adenine nucleotides, and as its activity is controlled by bound nucleotides, these results indicate that the R334A mutation specifically affects hydrolysis of the bound ATP.

In Vivo DnaA-ATP Hydrolysis—We next assessed the in vivo relative proportions of ATP- and ADP-bound mutant DnaA proteins. Cells were grown in a synthetic medium containing [32P]orthophosphate, DnaA protein was isolated by immunoprecipitation, and the bound nucleotides were analyzed by thin layer chromatography (14). In normally growing cells, the ATP-bound form of DnaA represents 10–20% of the total ATP- and ADP-DnaA molecules (14, 17).

As constitutive expression of the dnaA29 (R334A) gene carried on pBR322 inhibits the growth of normal host cells (see below), constitutive expression of this and other dnaA-alleles was achieved by placing them downstream of the lac promoter on a pBR322-derivative in lac+ cells. When dnaA29 (R334A) expression is induced by the addition of 1 mM IPTG, the proportion of ATP-bound DnaA proteins increases from 10% to 65% (Figs. 5A and 6). Upon induction, the overall amount of ATP-bound molecules significantly increases (Fig. 6, A–C); a slight increase in the level of ADP-bound molecules is also observed, which may be attributed to a slight residual sensitivity to RIDA.
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**Fig. 3. The DnaA R334A protein is resistant to RIDA in vitro.** [α-32P]ATP-bound, wild-type DnaA, DnaA R334A, and DnaA E204Q proteins (1.0 pmol) were incubated for 20 min at 30°C in buffer (25 μl) with 2 mM ATP, M13E10 RF I DNA (200 ng) and the indicated amounts of RIDA-active fraction II (A) or fraction III (B, C) proteins (1.0 pmol) were incubated for 20 min at 30°C. After incubation, DnaA with tightly bound nucleotides was recovered, and the ratio of DnaA-bound ATP and ADP was assessed by PEI-cellulose thin layer chromatography. The relative intensities of ATP and ADP were quantified with a BAS2500 image analyzer (Fuji Film). WT, wild-type DnaA; R334A, DnaA R334A; E204Q, DnaA E204Q.

**Fig. 4. Intrinsic ATPase activity.** [α-32P]ATP-bound, wild-type DnaA (WT) and DnaA R334A (R334A; ○) proteins (2.3 pmol) were incubated at 38°C for the indicated time in buffer (40 μl) with M13E10 RF I DNA (200 ng) as described (36). Nucleotide-bound DnaA proteins were recovered by immunoprecipitation and the nucleotides were analyzed by PEI-cellulose thin layer chromatography. The relative intensities of ATP and ADP were quantified with a BAS2500 image analyzer (Fuji Film). The ratio of ADP-DnaA to total ATP- and ADP-bound DnaA is shown as a percentage.

In contrast, induction of the wild-type protein is not associated with an increase in the level of ATP-DnaA (Fig. 5A), consistent with our previous data (14). Instead, the level of ADP-bound molecules dramatically increases, suggesting that DnaA-bound ATP is rapidly hydrolyzed (Fig. 6, B and E). The data shown in Fig. 6 (B and E) also suggest that the total levels of nucleotide-bound DnaA are comparable between the two strains before and after induction, especially from 0 to 130 min. Thus, we used the same conditions to assess the frequency of initiation, as described below.

When DnaA E204Q protein is similarly expressed, ATP-form molecules do not accumulate to significant levels (Fig. 5A). To eliminate background caused by the presence of the host wild-type DnaA, dnaA (E204Q) was expressed from a pBR322 derivative in a dnaA-null rnhA double mutant. The absence of the rnhA function allows chromosomal replication from alternative origins to take place (41). In the dnaA-null mutant, a significant increase in the level of ATP-bound DnaA E204Q protein was not seen (Fig. 5B). These results are consistent with the sensitivity of the DnaA E204Q protein to RIDA (Fig. 3C).

**Fig. 5. In vivo nucleotide-bound forms of DnaA.** Cells were grown in supplemented TG medium containing [32P]orthophosphate, and nucleotide-bound DnaA proteins were recovered by immunoprecipitation. The results of PEI-cellulose thin layer chromatography to identify DnaA-bound nucleotides are shown. The origin and positions to which ATP and ADP migrate are indicated by arrows. Anti-DnaA antiserum (A) and preimmune serum (P) were used. The ratio of ATP-DnaA to total ATP- and ADP-bound DnaA was shown as a percentage. A, pHSL9 (lacI)-bearing KH5402-1 (wild-type dnaA) was used as the host strain for the plasmids pSN306 (wild-type dnaA) (WT), pSN305 (dnaA29 (R334A)) (R334A), pSN307 (dnaA400 (E204Q)) (E204Q) and the vector pSN300 (none). Cells were grown at 37°C until the optical density (A660) of the culture reached 0.2, and were further incubated for 90 min at the same temperature in the presence (+) or absence (-) of 1 mM IPTG. For cells bearing pSN307 (dnaA400(E204Q)) incubated at 42°C, results similar to those shown above were obtained (data not shown). B, KA451 (dnaA::Tn10 rnhA::cat) was used as the host for the following pBR322 derivatives bearing dnaA alleles: pMZ002-1 (E204Q), pMZ002-2 (WT), and pMZ002 (none). Cells were grown at 37°C until the optical density (A660) of the culture reached 0.3. NA, not available.

Upon induction of DnaA R334A, the oriC/terC ratio increases significantly, whereas induction of the wild-type DnaA or DnaA E204Q proteins results in only a slight increase or no change (Table 1). After induction of DnaA R334A, the oriC/terC ratio increases gradually over 120 min, to a maximal level of ~5.5 times the basal level (Fig. 7). In contrast, induction of the wild-type DnaA increases the ratio by at most 2.4-fold (Fig. 7), which is consistent with previous observations (37, 42). There is no significant difference in the overall level of total DnaA before induction between the wild-type and DnaA R334A-expressing strains, as assessed by immunoblotting (38); after
induction, the DNA content increases to 3–3.5 times the uninduced level in both these strains (data not shown). These results indicate that expression of DNA A R334A causes overinitiation of chromosomal replication in vivo.

Chromosomal Replication—There are two types of overinitiation with respect to the extent of replication (3, 43). In one type, when additional replisomes are present the whole genome is replicated, resulting in chromosomal overreplication. The overinitiation that occurs in the dnaAcos mutant, the GroE-overexpressing dnaA46 mutant, and the DnaA46-overexpressing dnaA46 mutant results in this "inertia" mode of replication. In the other type, only oriC and nearby regions are replicated by additional replisomes. The overinitiation that occurs in wild-type DNA A-overexpressing cells and DNA A A184V protein-overexpressing cells results in this "attenuation" mode of replication.

To distinguish which type of replication takes place in DNA A R334A-bearing cells, we measured overall DNA synthesis by monitoring the incorporation of [3H]thymine in thyA-defective host cells (Fig. 8). In all cases in which the wild-type DNA A, DNA A R334A, and DNA A E204Q proteins are induced, significant overreplication of the whole chromosome is not observed. Similar results have been reported for cells in which the wild-type DNA A is overexpressed (37, 42). Thus, additionally formed replisomes in DNA A R334A cells are likely restricted to the vicinity of oriC. In contrast to what has been previously reported (26, 27), we never observed overreplication promoted by DNA A E204Q; in those previous experiments, JM109 host cells with an intact thyA gene were used to measure the incorporation of [3H]thymine (see "Discussion").

Inhibition of Cell Growth by the DNA A R334A Protein—Overinitiation inhibits the growth of some dnaA mutants (3). In the dnaAcos mutant, cell division is inhibited in an sfb-independent manner, resulting in cellular filamentation and the inhibition of cell proliferation (44). An alternative oriC-independent replication system operates in the absence of RNase H1, and cells lacking oriC can grow even in the presence of overinitiating dnaA mutant alleles such as dnaAcos (3, 16, 38). We found that the introduction of the dnaA29 (R334A) allele on a pBR322 derivative (pHS299-1) inhibits colony formation when the host cells are employing the normal replication system, but does not inhibit colony formation when the oriC-independent system is operating (Table II). These results suggest that the DNA A R334A protein specifically affects initiation at oriC and are consistent with the data suggesting that DNA A R334A promotes overinitiation.

The dnaA400 (E204Q) allele (borne on the pBR322 derivative pMZ002-1) was previously reported to cause a similar inhibition, although the host strain used in this study was JM109, which has a genetic background entirely different from that of KH5402-1, the parental strain of KA450 (ΔoriC dnaA(Am) rnaA(Am)), which was used as the positive control in that report (26). When KH5402-1 (wild-type dnaA) is used as
a host, the dnaA400 (E204Q) allele does not inhibit cell growth at 37 °C (Table II) or at 42 °C (data not shown). Immunoblotting analysis indicated that the relative amounts of DnaA in KA450 cells bearing pHS299-1 (dnaA29 (R334A)) and pMZ002-1 (dnaA400 (E204Q)) are ~0.84 and 2.4, respectively, if the level of wild-type dnaA in KA450 cells bearing pMZ002-2 (wild-type dnaA) is defined as 1 (data not shown). Thus, the inhibition conferred by pHS299-1 likely is not the result of overexpression of the mutant DnaA protein.

Homology Modeling of DnaA Domain III—To determine how the Arg-334 residue functions in ATP hydrolysis, we constructed a model of DnaA domain III, the ATP-binding domain (5, 45). This domain contains AAA+ motifs, including the Walker-type NTP binding motifs and AAA+–specific motifs (24). We found that the primary amino acid sequence and the predicted secondary structure of DnaA domain III have significant homology with a region from the archaeal P. aerophilum Cde6 protein (domains I and II) that includes AAA+ motifs (25) (Fig. 9A). The structure of the ADP-bound form of this protein has been solved by x-ray crystal diffraction analysis (25). Specifically using the AAA+ motif homology, we carried out a homology-modeling analysis of DnaA domain III, as described under “Materials and Methods” (Fig. 9B). In the predicted structure, the α-helix that includes the DnaA Box VIII motif is located near the bound ADP. The Arg-334 side chain is close to the ADP β-phosphate (<4 Å), which suggests that this residue has an important role in mediating interactions with ATP. In contrast, when the Arg-334 residue is replaced with His or Ala, the side chains of these residues are further (>5.5 Å) from the phosphate bond (Fig. 10). Similarly, the Glu-204 residue is located at a distance that precludes a close interaction with ATP (Fig. 9B).

**DISCUSSION**

We found that the DnaA R334A protein is defective in the hydrolysis of bound ATP in vitro and in vivo but that it retains the ability to promote initiation in a bound nucleotide-dependent manner. As a moderate level of this protein, but not of the wild-type protein, causes overinitiation in vivo, we speculate that the ATP-bound form, but not the ADP-DnaA form, of DnaA is active for initiation in vivo. These results suggest that the nucleotide bound to DnaA plays a crucial role as a molecular switch for initiating chromosomal replication. Furthermore, our data support the notion that RIDA plays an indispensable role in the in vivo regulation of this initiation switch. Recently, a newly identified protein, Hda, was found to be required for RIDA in vitro and in vivo (16). This protein exhibits IdaB activity, which was proposed to be involved in the RIDA reaction. Inactivation of the hda gene results in an increase in cellular ATP-DnaA content, up to ~70% of total ATP- and ADP-DnaA molecules, and in the overinitiation of chromosomal replication. Our present data are highly consistent with these results.

Other findings highlighting the nucleotide-directed control of DnaA and of other initiation proteins in eukaryotic systems have recently emerged. One report demonstrates that ATP-DnaA, but not ADP-DnaA, binds to a specific sequence called the ATP-DnaA box (46), which is a 6-mer motif that lies adjacent to the 9-mer DnaA box. This sequence is present in the dnaA promoter, and ATP-DnaA represses the transcription of the dnaA gene more tightly than does ADP-DnaA. An interaction between the ATP-DnaA and ATP-DnaA boxes found at oriC is suggested to be required to open duplex DNA (47). In S. cerevisiae, the origin recognition complex, a protein assembly, must be bound by ATP to bind yeast origins of replication (autonomous replicating sequences). Further, autonomous replicating sequence singled-stranded DNA stimulates ATP hydrolysis of the origin recognition complex subunit Orc1, which may play an important role in the inhibition of untimely initiations (2, 48, 49). The nucleotide-dependent control of replication-initiating proteins may be ubiquitous in prokaryotic and eukaryotic replisomes. As ATP-dependent initiation is a feature of the DnaA R334A and DnaA R334H proteins (Fig. 1) (15), Arg-334 is unlikely to be the residue responsible for the allosteric change that triggers the molecular switch.

**Table II**

| Strain       | Genotype | Transformation efficiency |
|--------------|----------|--------------------------|
|              | rnhA     | dnaA | oriC | pHS299-1 | pMZ002-1 | pMZ002-2 | pMZ002 |
| KH5402-1     | +        | +    | +    | <0.003   | 2.7      | 2.5      | 2.5    |
| YT411        | ▶cat     | +    | +    | <0.003   | 0.01     | 0.94     | 0.94   |
| KA451        | ▶cat     | ▶Tn10 | +    | <0.003   | 0.73     | 0.63     | 1.4    |
| KA429        | ▶cat     | +    | △    | 1.2      | 1.3      | 0.62     | 0.62   |
| KA450        | Am       | Am   | △    | 1.2      | 1.7      | 1.3      | 0.63   |

After incubation for 24 h at 37 °C on LB plus thymine and antibiotics (as described under “Materials and Methods”), colonies with a diameter of >~0.5 mm were counted. Am, amber mutation; △, del-1017.
**Fig. 9.** **AAA⁺ modules and a structural model of DnaA domain III.**

**A**, homology between the AAA⁺ module domains of DnaA and *P. aerophilum* Cdc6. The upper sequence is DnaA domain III and the lower is *P. aerophilum* Cdc6 domains I and II. The extents of identity and similarity (including identity) between the two amino acid sequences are 18 and 31%, respectively. The identical and similar amino acids are indicated with yellow and green boxes, respectively. Amino acid positions are indicated and Arg-334 is shown by a bold font. The defined AAA⁺ motifs indicated are boxed. α-helices and β-strands in *P. aerophilum* Cdc6 that were used for structural modeling are indicated by red and blue bars, respectively. **B**, ribbon drawing of the structure of DnaA domain III. A structural model of the ADP-bound DnaA domain III was constructed by a homology modeling method (see "Materials and Methods"). ADP and the side chains of the Glu-204, Arg-334 and Leu-367 residues are shown as a ball-and-stick model. The Leu-367 residue defines the C terminus of this domain, and the N terminus is shown as N-term.
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Fig. 10. The environment around the Arg-334 residue in DnaA domain III. ADP and the side chains of the indicated residues are shown as ball-and-stick models. A, wild-type DnaA; B, DnaA R334A; C, DnaA R334H.

...isms regarding this process remain to be elucidated.

The initiation of replication is regulated by multiple pathways during the E. coli cell cycle (3, 18, 19). Upon induction of the DnaA R334A protein, the ATP-DnaA proportion of total nucleotide-bound DnaA molecules increases rapidly (Fig. 6), whereas the increase in the oriC/CterC ratio is slower than the increase in the level of ATP-DnaA (Fig. 7). This discrepancy may be caused by RIDA-independent regulatory systems that operate under these conditions. The SeqA protein binds the hemimethylated oriC DNA that is transiently present after replication, which temporarily blocks re-initiation (10). Whether the Arg-334 residue in DnaA domain III is also required for ATP hydrolysis in this complex.

The structural model (Fig. 9B), the distance between the side chain of the Arg-334 residue and the ADP and the side chains of the indi...
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