The Arg Fingers of Key DnaA Protomers Are Oriented Inward within the Replication Origin oriC and Stimulate DnaA Subcomplexes in the Initiation Complex*

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Background: ATP-DnaA molecules oligomerize and form two subcomplexes on the replication origin.

Results: The Arg fingers of DnaA bound at the outer edges of the DnaA complexes are oriented inward within the origin.

Conclusion: The Arg fingers, but not bound ATP, of the outer edge DnaA protomers promote construction of active initiation complexes.

Significance: An important mechanical basis in the initiation complex is revealed.

ATP-DnaA binds to multiple DnaA boxes in the *Escherichia coli* replication origin (oriC) and forms left-half and right-half subcomplexes that promote DNA unwinding and DnaB helicase loading. DnaA forms homo-oligomers in a head-to-tail manner via interactions between the bound ATP and Arg-285 of the adjacent protomer. DnaA boxes R1 and R4 reside at the outer edges of the DnaA-binding region and have opposite orientations. In this study, roles for the protomers bound at R1 and R4 were elucidated using chimeric DnaA molecules that had alternative DNA binding sequence specificity and chimeric oriC molecules bearing the alternative DnaA binding sequence binding site (Fig. 1A). This binding produces a highly ordered complex (i.e. an initiation complex) that stimulates local duplex unwinding (2–7). DnaB helicase is loaded onto the resultant single-stranded DNA via interaction with DnaA and DnaC helicase loader (3). The loaded DnaB triggers the formation of the sister replisomes needed for bidirectional DNA synthesis (8). ATP-DnaA is converted to ADP-DnaA, which is inactive for initiation, during replication (2, 7, 9, 10).

The *E. coli* oriC consists of an AT-rich duplex unwinding element (DUE) and a DnaA oligomerization region (DOR) that bears DnaA-binding sequences (EcoDnaA box) termed R1-2, R4, R5M, I1-3, r1-2, and C1-3 (2, 11–13) in addition to an IHF-binding site (Fig. 1A). The R1 and R4 boxes reside at the outer edges of the DOR and have a typical 9-mer consensus sequence of TTATNCACA (2). R1 and R4 have the highest affinities of the EcoDnaA boxes for both ATP-DnaA and ADP-DnaA (2, 3, 12, 14, 15) and are suggested to act as assembly cores for DnaA oligomerization (13, 16). The other EcoDnaA boxes reside between the R1 and R4 boxes and have moderate (R2) or low affinities (R5M, I1-3, r1-2, and C1-3) for DnaA. ATP-DnaA molecules form oligomers at these low affinity sites more readily than do ADP-DnaA molecules (11–14). IHF binds to a specific site between the R1 and r1 boxes (Fig. 1A) and stimulates ATP-DnaA assembly on oriC and unwinding of the DUE (4, 11, 17).

The DnaA-binding consensus sequence is asymmetric, and bound DnaA proteins would therefore also be expected to exhibit directionality. Consistent with this suggestion, a previous study demonstrated that cell growth was inhibited when the direction of the R1 box was inverted (18). However, the directionality of DnaA proteins bound at the oriC sequences, including R1 and R4, has not yet been determined.

Previously, we suggested that oriC can be subdivided into two structurally and functionally distinct subregions: the left-

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2 The abbreviations used are: DUE, duplex unwinding element; DOR, DnaA oligomerization region; ssDUE, single-stranded DUE; EcoDnaA, *E. coli* DnaA; TmaDnaA, *T. maritima* DnaA; chiDnaA, chimeric DnaA.
Role for Key DnaA Protomers in the Initiation Complex

DnaA contains four functional domains (Fig. 1B) (3, 20). Domain I interacts with DnaB and DiaA, a protein that stimulates assembly of DnaA molecules on oriC, and has a low affinity site for homodimerization (21–23). Domain II is a flexible linker (23, 24). Domain III includes AAA + family motifs (e.g. Walker A/B, Sensor 1/2, and Arg finger) in addition to B/H motifs and plays crucial roles in nucleotide binding, ATP hydrolysis, inter-DnaA interactions, and single-stranded DUE (ssDUE) binding (12, 19, 25–28). In particular, the Arg finger plays a predominant role in the recognition of ATP bound to DnaA (12). Domain IV contains a helix-turn-helix motif and is crucial for direct binding to DnaA boxes (Fig. 1B) (28–32).

ATP-DnaA molecules in initiation complexes form homo-oligomers that can adopt a spiral structure (12, 27, 31, 33). The Arg finger motif, Arg-285, of a DnaA protomer plays a crucial role in the cooperative binding of DnaA to the low affinity DnaA boxes of oriC (12). DnaA R285A retains the activities in Fig. 1A). DnaA R285A retains the activities of wild-type DnaA in that it is specifically impaired in binding to the low affinity DnaA boxes of oriC and would be expected to interact with adjacent protomers during assembly on each DnaA box sequence direction. (Fig. 1A). The R5 and R4 box sequence directions are opposite to one another. The right-half oriC sequences (R2, I3, and C1–3) are in the same direction as the R4 box sequence (Fig. 1A). The R5M and I1-2 sequences (left-half oriC) are in the same orientation as the R1 box sequence (Fig. 1A).

To distinguish between these two possibilities, we used a DnaA ortholog (TmaDnaA) and the cognate DnaA box (TmaDnaA box) from Thermotoga maritima, one of the most ancient hyperthermophile eubacteria (34). The consensus sequence for the EcoDnaA box (TTATNCACA) is highly conserved in many bacterial species (35); however, the consensus for the TmaDnaA box, AAACCTACCC, differs substantially (36). By contrast, TmaDnaA has 47% amino acid sequence similarity with E. coli (EcoDnaA), and the domain structures of TmaDnaA and the affinities of TmaDnaA for the TmaDnaA boxes are similar to those of EcoDnaA for the cognate consensus sequence (Fig. 1B). As with EcoDnaA, TmaDnaA has high affinity for ATP and ADP, and ATP-TmaDnaA, but not ADP-TmaDnaA, unwinds the cognate oriC in vitro (36). In this study, chimeric oriC sequences were constructed in which the R1 or R4 box of the E. coli oriC was substituted with the TmaDnaA box. Chimeric DnaA (chiDnaA) was also constructed, in which domain IV of EcoDnaA was substituted with that of TmaDnaA (Fig. 1B). These oriC and DnaA derivatives facilitated the analysis of DnaA protomer binding to the R1 and R4 boxes in vitro and in vivo. Experimental results were consistent with the AF-inward model (Fig. 1D).

Experimental Procedures

Strains—E. coli strains are listed in Table 1. A DNA fragment that included the tet gene was amplified from pBR322 using tet-oriC f and tet-oriC r primers (Table 2). This fragment was introduced into MG1655 using the λRed recombination system (38), resulting in strain SYM1 (MG1655 gidA::tet). For construction of strain NY20 (MG1655 asnA::kan), a DNA fragment including the frt-flanked kan and oriC regions was amplified from pRSoriC (see below) using primers pRS1 and pRS2 (Table 2). The resultant fragment was introduced into SYM1 cells using the λRed recombination system, and colonies that were resistant to kanamycin and sensitive to tetracycline were selected (39). A representative strain was named NY20. Strains NY21 (MG1655 oriCΔR4::TmaDnaA box asnA::kan), NY24 (MG1655 oriCΔR1::TmaDnaA box asnA::kan), and NY25 (MG1655 oriCΔR4::R4-inverted asnA::kan) were similarly constructed using DNA fragments amplified from the pRSoriC derivatives pRSR1 Tma, pRSR4 Tma, and pRSR4inv (see below), respectively. The altered oriC structures in these strains were confirmed by nucleotide sequencing. Strain NY26 was constructed by transduction using a P1 phage lysate of KP7364.

| TABLE 1 |
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| **Strain list** |
| | Strain | Relevant genotype | Reference |
| **MG1655** | Wild type | Laboratory stock |
| **SYM1** | MG1655 gidA::tet | This work |
| **NY20** | MG1655 asnA::kan | This work |
| **NY21** | MG1655 asnA::kan oriCΔR1::TmaDnaA box | This work |
| **NY24** | MG1655 asnA::kan oriCΔR4::TmaDnaA box | This work |
| **NY25** | MG1655 asnA::kan oriCΔR4::R4-inverted | This work |
| **NY26** | MG1655 Δasna::spec rnhA::kan | This work |
| **KH5402-1** | Δe·rilyΔH::tet(Am) tpr9829(Am) metE deo supF6(Ts) | Ref. 37 |
| **NA001** | Δhkc40-1 Δhka4C | Ref. 37 |
| **KA451** | H5402-1 Δasna ΔTma10rnhA::cat | Ref. 19 |
| **KP7364** | Δasna::spec rnhA::kan | Ref. 26 |

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Oligonucleotides and Plasmids—Oligonucleotides are listed in Table 2. Plasmids pKA384 and pTHMA-1 were used for overproduction of EcoDnaA and TmaDnaA, respectively, as described previously (19, 36). Plasmids pECTMA, pECTMAR285A, pECTMAD269A, and pECTMAdnaAcos were constructed and used for the expression of chiDnaA, chiDnaA R285A, chiDnaA D269A, and DnaAcos, respectively. To construct pECTMA, first a DNA fragment encoding DnaAcos was amplified by PCR using primers (R1tma12r, R1tma12rb, R1tma12rc, and R1tma12rd) and its flanking regions was amplified by PCR using MG1655-oriC and primers orir1tmafb and orir1tmars (Table 2). These three fragments were ligated by a site-directed mutagenesis using mutagenic primers as described previously (12, 26). To construct a plasmid bearing the TmaDnaA box at the site of the R4 box, a fragment was similarly amplified using primers R4tma12rb and R4tma12fb and self-ligated, resulting in pR4Tma12.

pRSoriC was a pBR322-derivative oriC plasmid that contained a region including gidA′-oriC-mioC-asnC-asnA′::kan. To construct pRSoriC, first a DNA fragment containing oriC and its flanking regions was amplified by PCR using MG1655-derived genomic DNA and primers pSA4 and pSA5. In addition, an frt-kan fragment was amplified by PCR using primers pSA4 and pSA5 (40). In addition, a pBR322 fragment containing the origin and bla regions was amplified by reverse PCR using pBR322 and primers pSA6 and pSA7. These three fragments were ligated by a sequence- and ligation-independent cloning system using T4 DNA polymerase, resulting in pRSoriC (41). pRSoriC derivatives bearing the TmaDnaA box at the site of the R1 or R4 box were constructed by PCR as for pR1Tma12 and pR4Tma12, resulting in pR5R1Tma and pR5R4Tma. A pRSoriC derivative bearing the inverted R4 box was constructed by site-directed mutagenesis using pRSoriC and mutagenic primers R4inv-f and R4inv-r, resulting in pR5R4inv. The inverted R4 box sequence was the same as that described previously (18).

DORAR1 was described previously as DARAR1 (4, 19). A right-half oriC fragment R2R4 and its derivatives, R2R4R4Tma and R2R4-R4inv were prepared by PCR using template DNA, pBRoriC, pR4Tma12, and pR5R4inv, respectively, as described previously (4, 19).

ssDUE-dsR1Tma was prepared by annealing radiolabeled ssDNA, ChDAB-M28-up, and ChDAB-M28-low (Table 2). ssDUE-dsR1 and ssDUE-dsNon were described previously (4).

Buffers—Buffer M contained 20 mM Tris-HCl (pH 7.5), 0.1 mg/ml bovine serum albumin, 8 mM dithiothreitol, 10 mM mag-
nesium acetate, 125 mM potassium glutamate, and 2 mM ATP. Buffer G’ contained 20 mM Hepes-KOH (pH 7.6), 1 mM EDTA, 4 mM dithiothreitol, 5 mM magnesium acetate, 10% (v/v) glycerol, 1 mM ATP, 0.1% Triton X-100, 0.1 mg/ml bovine serum albumin, 4 μg/ml poly(dA-dT)-(dA-dT), and 4 μg/ml poly(dI-dC)-(dI-dC). Other buffers were described previously (19, 36).

Purification of chiDnaA Proteins—chiDnaA and its derivative proteins were overproduced using KA451 (ΔdnaA ΔrnhA) (Table 1) cells bearing pECTMA or pECTMAR285A. Proteins were purified using a previously described method for EcoDnaA purification (12, 19).

EMSAs Using DNA with a Single DnaA Box—EMSAs was performed with 18-bp DNA fragments containing either the TmaDnaA box consensus or a nonsense sequence and with 15-bp DNA fragments containing either the EcoDnaA R1 box or a nonsense sequence, as described previously (36, 42). Briefly, ATP-EcoDnaA or ATP-chiDnaA was incubated in buffer for 10 min at 30 °C with the appropriate DNA fragment and αDNA (50 ng) as a competitor. This incubation was followed by electrophoresis on a 5% polyacrylamide gel (PAGE) and GelStar staining (Lonza).

oriC Plasmid Replication Assay—The oriC replication assay was performed as described previously (19). Briefly, EcoDnaA was incubated on ice in buffer M containing DnaB helicase, DnaC helicase loader, DnaG primase, histone-like protein HU, gyrase, SSB (single-stranded binding protein), DNA polymerase III holoenzyme, rNTPs, dNTPs including [α-32P]dATP, and a supercoiled form of pBRoriC or its derivative. Briefly, chiDnaA or EcoDnaA was added, and the reaction was incubated for 20 or 30 min at 30 °C prior to filtration using a GF/C glass filter and liquid scintillation counting.

DUE Unwinding (P1 Nuclease) Assay—This assay was performed as described previously (4, 19). Briefly, EcoDnaA was incubated on ice in buffer containing IHF (55 nm) and a supercoiled form of the oriC plasmid. Next, chiDnaA or EcoDnaA was added, and the reaction was incubated for 3 min at 38 °C, followed by incubation for 200 s at 38 °C in the presence of P1 nuclease (4 units; Yamasa Co.). Reactions were terminated by the addition of 0.5% SDS, and DNA was purified using a WIZARD spin column (Promega). A portion of the eluate was digested using the restriction enzyme AlwNI, and the digested DNA was subjected to electrophoresis using a 1% agarose gel and ethidium bromide staining. The oriC plasmids (4.7 kb) used had only a single site for AlwNI digestion. If the unwound DUE and the AlwNI site are cut, two fragments (2.5 and 2.2 kb) are yielded.

Form I* Assay—The form I* assay was performed as described previously (4, 22). Briefly, EcoDnaA was incubated on ice in buffer M containing SSB, IHF, DnaB, DnaC, gyrase, and the oriC plasmid. Next, chiDnaA or EcoDnaA was added, and the reaction was incubated at 30 °C for 15 min. Reactions were stopped by the addition of phenol and chloroform. DNA was precipitated in ethanol and resuspended in Tris-EDTA buffer, followed by 0.65% agarose gel electrophoresis and ethidium bromide staining. The relative amounts of form I* DNA were quantified using densitometry.

EMSAs of DnaA Oligomer Formation—This analysis was performed as described previously (19). Briefly, oriC DNA fragments were incubated at 30 °C for 10 min in buffer containing ATP-EcoDnaA and αDNA (200 ng) as a competitor. Reactions were analyzed at room temperature using 2% agarose gel electrophoresis followed by GelStar staining and densitometric scanning. For chiDnaA analysis, EcoDnaA was added on ice in the initial stage, and chiDnaA was added subsequently.

EMSAs of ssDUE Recruitment—This analysis was performed as described previously (4, 19). Briefly, 12.5 nM 32P-labeled DNA containing a 28-mer ssDUE was incubated with chiDnaA for 5 min on ice in buffer G’. EcoDnaA and a truncated oriC DNA, DORΔR1 (5 nM), were added to a portion of this mixture, and the reaction was incubated for 10 min at 30 °C in buffer G’. Reactions were analyzed using 4% PAGE at room temperature.

Results

Construction of Chimeric DnaA—To investigate the role of an individual DnaA molecule within the context of DnaA homo-oligomers at oriC, we constructed a chiDnaA consisting of EcoDnaA domains I–III and TmaDnaA domain IV (Fig. 1B). The consensus sequence of the EcoDnaA box (TTATNCACA) is widely conserved in eubacterial species, but the TmaDnaA box consensus differs substantially (AAAACTTACACC) (36). These distinct binding characteristics were used to develop the experimental approach. We reasoned that, when a specific DnaA box within oriC is substituted with a TmaDnaA box and both chiDnaA and EcoDnaA are co-incubated with the mutant oriC, chiDnaA could specifically bind to the substituted DnaA box, and, at the same time, EcoDnaA could bind to other intact DnaA boxes. This strategy appeared suitable to elucidate the role for an individual DnaA molecule within the DnaA homo-oligomers assembled on oriC.

EcoDnaA and TmaDnaA share a high degree of overall similarity in all protein regions except for domain II, which is a structurally flexible linker that varies in length and sequence between eubacterial species (Fig. 1B). The boundary between domains III and IV includes a short loop (i.e. a hinge) that connects the α helix of the domain III C terminus with that of the N terminus of domain IV (28, 31). The DNA-binding sites of domain IV reside in a helix-turn-helix motif in the middle region of this domain (29, 30). We therefore selected a site in the EcoDnaA domain IV N-terminal helix region for substitution with TmaDnaA domain IV (Fig. 1B). This resulted in the construction of chiDnaA, which consisted of amino acids 1–374 of EcoDnaA and amino acids 342–440 of TmaDnaA. Overproduction and purification of chiDnaA was performed as for EcoDnaA purification, and this resulted in a sample of >90% purity, as determined by SDS-PAGE.3

Nucleotide Binding Activity and DNA Binding Specificity of chiDnaA—To assess the basic activity of chiDnaA, the binding activities with adenine nucleotides and DnaA boxes were analyzed. A filter retention assay using EcoDnaA and chiDnaA revealed that the two purified proteins were similarly active in binding to both ATP and ADP (Table 3).

EMSA demonstrated that chiDnaA was able to bind specifically to DNA bearing the TmaDnaA box consensus sequence but was practically unable to bind to DNA bearing the EcoDnaA

3 Y. Noguchi and T. Katayama, unpublished observation.
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TABLE 3
Nucleotide binding of chiDnaA

| DnaA    | ATP  | ADP  | Stoichiometry |
|---------|------|------|---------------|
| EcoWT   | 76   | 19   | 0.45          |
| ChiWT   | 52   | 55   | 0.42          |
| ChiRA   | 77   | 35   | 0.53          |

R1 box sequence (Fig. 2, A and B). In this assay, chiDnaA exhibited TmaDnaA box binding activity, even at 200 nM; this was consistent with previous data for TmaDnaA binding to the TmaDnaA box (36). Similarly, EcoDnaA bound to the R1 box DNA (Fig. 2B), but no substantial binding of EcoDnaA to the TmaDnaA box was detected (Fig. 2A). Neither EcoDnaA nor TmaDnaA exhibited detectable affinity for the nonsense control sequence used in this assay. These results showed that chiDnaA and EcoDnaA bound with high specificity to the TmaDnaA and R1 boxes, respectively, and demonstrated the utility of chiDnaA for the functional categorization of individual DnaA molecules within an initiation complex.

Initiation Activity of chiDnaA—An oriC replication system reconstituted with purified proteins was used to evaluate the ability of chiDnaA to stimulate initiation in vitro. The in vitro system utilized a plasmid with oriC alongside DnaA, DnaB helicase, DnaG primase, and DNA polymerase III holoenzyme. In this experiment, we used chimeric oriC in which the DnaA box R1 was substituted with the TmaDnaA box. The R1 box is separated from the adjacent DUE by a 13-bp intervening region (Fig. 1A). The length of the intervening region is also important in initiation (4). Because the EcoDnaA box is a 9-mer and the TmaDnaA box is a 12-mer, we constructed four different chimeric oriC plasmids bearing the TmaDnaA box at positions shifted by 1 bp. The resultant plasmids were pR1Tma12 and its derivatives (Fig. 2C). To evaluate the initiation activity of chiDnaA and the chimeric oriC sequences, mixtures of EcoDnaA and various amounts of chiDnaA were included in the oriC replication-reconstituted systems (Fig. 2C). Plasmid pBRoriC (wild-type oriC) was replicated in a chiDnaA-independent manner. By contrast, pR1Tma12 (TmaDnaA box) exhibited substantial chiDnaA-dependent activity. A slight structural difference between EcoDnaA and chiDnaA might cause partial inhibition of DnaB helicase loading and replication when chiDnaA is used (see below), and this would explain the lower maximal DNA synthesis observed with pR1Tma12. Replication with pR1Tma12-derivative plasmids (i.e. pR1Tma12-b, -c, and -d) was severely inhibited even in the presence of chiDnaA (Fig. 2C). This confirmed the importance of the length of the intervening region between the DUE and the R1 box.

These results indicate that chiDnaA is fundamentally active in initiation when the TmaDnaA box is located at a proper position in oriC. The results also suggest that the right side of the EcoDnaA box corresponds to the right side of the Tma DnaA box in the construction of DnaA-DNA complexes (Fig. 2C).
To determine the orientation of DnaA protomers in an initiation complex, we used a chiDnaA R285A mutant protein to further investigate DnaA bound to the R1 box. The Arg-285 residue corresponds to the AAA+/H11001 Arg finger motif that is crucial for activation of an initiation complex (12). This residue is suggested to directly interact in a head-to-tail manner with ATP bound to the adjacent DnaA protomer (12, 27, 31). However, because the R1 DnaA box resides at the left edge of the DnaA assembly region of oriC, there are two functional possibilities for the Arg-285 residue of the DnaA protomer during initiation. First, if Arg-285 is oriented inward within oriC (AF-inward model in Fig. 1D) and interacts with ATP bound to the adjacent DnaA protomer, then this residue should be crucial for initiation. Second, if Arg-285 is oriented outward from oriC (AF-outward model in Fig. 1D), then it would not interact with the adjacent DnaA protomer and should therefore be irrelevant for initiation. In addition, if the AF-inward model is correct, the resultant initiation complexes should be active in initiation even when the R1 box-bound DnaA is in the ADP form. In contrast, initiation complexes should be inactive if the AF-outward model is correct, and the R1 box-bound DnaA is in the ADP form. We can thus infer using chiDnaA R285A and ADP-chiDnaA the orientation of the R1 box-bound DnaA protomer.

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First, the filter retention assay confirmed that the purified chiDnaA protein retained nucleotide binding activity at levels similar to the wild-type form of chiDnaA (Table 3). In addition, EMSA confirmed that TmaDnaA box-specific binding activity was equivalent between chiDnaA and its derivatives (12, 27, 31). However, because the R1 DnaA box resides at the left edge of the DnaA assembly region of oriC, there are two functional possibilities for the Arg-285 residue of the DnaA protomer bound to the R1 box during initiation. First, if Arg-285 is oriented inward within oriC (AF-inward model in Fig. 1D) and interacts with ATP bound to the adjacent DnaA protomer, then this residue should be crucial for initiation. Second, if Arg-285 is oriented outward from oriC (AF-outward model in Fig. 1D), then it would not interact with the adjacent DnaA protomer and should therefore be irrelevant for initiation. In addition, if the AF-inward model is correct, the resultant initiation complexes should be active in initiation even when the R1 box-bound DnaA is in the ADP form. In contrast, initiation complexes should be inactive if the AF-outward model is correct, and the R1 box-bound DnaA is in the ADP form. We can thus infer using chiDnaA R285A and ADP-chiDnaA the orientation of the R1 box-bound DnaA protomer.
Quantification of P1 nuclease products indicated that the addition of the ATP form of chiDnaA (ATP-chiDnaA) promoted DUE unwinding of pR1Tma12 to a level similar to that of pBRoriC in the presence of ATP-EcoDnaA alone (Fig. 3, A and B), consistent with the initiation activity of chiDnaA (Fig. 2C). By contrast, unwinding of pR1Tma12 did not occur when ATP-EcoDnaA was added in the second stage. These data indicate that the EcoDnaA included in the first stage constituted a basal level that did not cause binding to the R1 box or unwinding of pR1Tma12 and that unwinding was dependent upon the subsequent addition of ATP-chiDnaA. These results underline the specificity of the assay, whereby additional chiDnaA specifically binds to the R1-TmaDnaA box on pR1Tma12 and stimulates initiation.

The addition of ADP-chiDnaA in the second stage of the assay also promoted DUE unwinding of pR1Tma12 at levels similar to that of ATP-chiDnaA (Fig. 3, A and B). By contrast, the ATP form of chiDnaA R285A (ATP-chiDnaA R285A) did not support unwinding (Fig. 3, A and B).
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These results suggest that the DnaA Arg finger, but not the bound ATP, of the R1 box-bound DnaA protomer is required for DUE unwinding. Thus, these results support the AF-inward model (Fig. 1D), in which the nucleotide-binding site of the R1 box-bound DnaA is oriented outward from oriC, and the Arg finger of the same DnaA molecule is oriented inward within oriC and is required for specific interaction with the adjacent DnaA protomer.

The DnaA Arg Finger of the R1 Box-bound DnaA Is Required for Initiation—During initiation, DUE unwinding is followed by loading of DnaB helicase onto the resultant ssDNA region. Loading of DnaB is dependent on the temporal interaction with DnaC helicase loader and the DnaA complex on oriC. The activity of the R1 box-bound chiDnaA in DnaB loading was assessed using a form I assay. This assay uses a supercoiled form (form I) of oriC plasmid with DnaA, DnaB, DnaC, and DNA gyrase. DnaB is loaded on the ssDNA and, in the presence of DNA gyrase, migrates along the ssDNA by unwinding the duplex DNA. This unwinding results in the production of a highly negative supercoiled form of DNA, form I* (4, 22). The presence of form I* DNA therefore acts as an indicator of DnaB loading. Form I and form I* are distinguished using agarose gel electrophoresis and fluorescent staining. In the present experiments, mixtures of pR1 Tma12, DnaB, DnaC, DNA gyrase, and a low amount of EcoDnaA were prepared. Various amounts of EcoDnaA or chiDnaA were subsequently added, and reactions were incubated at 30 °C.

Quantification of the relative levels of form I pR1 Tma12 DNA demonstrated that ADP-chiDnaA and ATP-chiDnaA promoted DnaB loading at similar levels (Fig. 3, C and D). DnaB loading was minimal in the presence of ATP-chiDnaA R285A or ATP-EcoDnaA. The inactivity of ATP-EcoDnaA in this assay supports the specific requirement for DnaA binding to the R1-TmaDnaA box. These results are consistent with those of the DUE unwinding assay (Fig. 3, C and D) and support the idea that the Arg finger, but not the bound ATP, of the R1 box-bound DnaA is required for constructing DnaA complexes active in DnaB loading as well as DUE unwinding.

Replication activity was also assessed using an in vitro system reconstituted with purified protein. In the present experiments, pR1 Tma12 was incubated with replicative proteins and DnaA. To exclude the possibility that chiDnaA could be non-specifically involved in DnaA complexes during the cooperative binding process, EcoDnaA was first added at a level insufficient to initiate replication. EcoDnaA and chiDnaA were added subsequently, and reactions were incubated at 30 °C (Fig. 3E). DNA synthesis was active with ATP-chiDnaA and ADP-chiDNA but not with ATP-chiDnaA R285A. Slight activity was detected with ATP-EcoDnaA. This activity was more pronounced at higher protein concentrations and might have occurred as a result of nonspecific binding to the R1-TmaDnaA box. Substantially similar results were exhibited even when EcoDnaA and chiDnaA were mixed at various ratios and these mixtures were added to replication reactions, including pR1 Tma12.3 These results, which are consistent with those of the oriC unwinding and DnaB loading assays, indicate that the Arg finger, but not ATP, of the R1 box-bound DnaA is required for replication initiation in vitro.

Role of the DnaA Arg Finger of the R1 Box-bound DnaA in ssDUE Binding—To investigate the specific roles of the Arg finger of the R1 box-bound DnaA in initiation reactions, we analyzed the interaction of oriC-DnaA complexes with ssDUE. We previously proposed a model suggesting that after DUE unwinding in an initiation complex, the resultant upper strand of ssDUE binds to an ATP-DnaA homo-oligomer constructed on the left-half oriC (4, 19). This reaction is dependent on the DnaA complex constructed on the ATP-DnaA-preferential low affinity boxes (i.e. R5M, I1-2, and rI-2) and is stimulated by DnaA bound to the R1 box (Fig. 4A). This stimulation is explained by a probable interaction between the R1 box-bound DnaA and the DnaA cluster bound to the low affinity boxes. This interaction is thought to assist recruitment of ssDUE to the DnaA cluster (4, 19) (Fig. 4A).

To analyze the interaction of ssDUE with the DnaA complex, we performed EMSA using a truncated oriC fragment (DORAR1) and [32P]ssDUE ligated to oligo-dsDNA (ssDUE-dsDNA) (Fig. 4A) using our previously described method (4, 19). DORAR1 contained an oriC region but lacked a region containing DUE, the R1 box, and the IHF binding site (Fig. 4A). [32P]ssDUE-dsDNA carried the 28-mer ssDUE upper strand and dsDNA bearing the DnaA R1 box, TmaDnaA box, or nonsense sequence (ssDUE-dsR1, ssDUE-dsR1Tma, or ssDUE-dsNon, respectively) (Fig. 4, A and B). In these experiments, ssDUE-dsDNA was first incubated in the presence or absence of ATP-chiDnaA. Second, a portion of each initial reaction was added to buffer containing ATP-ChiDnaA. DnaB, DnaC, DNA gyrase, and a low amount of EcoDnaA were prepared. Various amounts of EcoDnaA or chiDnaA were subsequently added, and reactions were incubated at 30 °C. These results suggest that the DnaA Arg finger, but not the bound ATP, of the R1 box-bound DnaA protomer is required for DUE unwinding. Thus, these results support the AF-inward model (Fig. 1D), in which the nucleotide-binding site of the R1 box-bound DnaA is oriented outward from oriC, and the Arg finger of the same DnaA molecule is oriented inward within oriC and is required for specific interaction with the adjacent DnaA protomer.

These results, which are consistent with those of the oriC unwinding and DnaB loading assays, indicate that the Arg finger, but not ATP, of the R1 box-bound DnaA is required for replication initiation in vitro. These results suggest that the DnaA Arg finger, but not the bound ATP, of the R1 box-bound DnaA protomer is required for DUE unwinding. Thus, these results support the AF-inward model (Fig. 1D), in which the nucleotide-binding site of the R1 box-bound DnaA is oriented outward from oriC, and the Arg finger of the same DnaA molecule is oriented inward within oriC and is required for specific interaction with the adjacent DnaA protomer.

These results suggest that the DnaA Arg finger, but not the bound ATP, of the R1 box-bound DnaA protomer is required for DUE unwinding. Thus, these results support the AF-inward model (Fig. 1D), in which the nucleotide-binding site of the R1 box-bound DnaA is oriented outward from oriC, and the Arg finger of the same DnaA molecule is oriented inward within oriC and is required for specific interaction with the adjacent DnaA protomer.
FIGURE 4. Role of the Arg finger of the R1 box-bound DnaA in ssDUE binding. A, a schematic of ssDUE recruitment assay. $[^{32}P]$ssDUE ligated to dsDNA bearing the DnaA box is incubated with DnaA, followed by further incubation in the presence of ATP-EcoDNA oligomers complexed with DOR$R_1$. Resultant complexes are analyzed by EMSA. DnaA domains and oriC sequences are illustrated as in Fig. 1. B and C, ssDUE recruitment assay using EcoDNA and chiDNAA. In the first stage, ATP-chiDNAA (100 nM) was incubated on ice for 5 min with $[^{32}P]$ssDUE ligated to dsDNA bearing the R1 box (ssDUE-dsR1), TmaDNA box (ssDUE-dsR1Tma), or nonsense sequence (ssDUE-dsNon) (12.5 nM). In the second stage, one-fifth portions of these mixtures were incubated at 30 °C for 5 min in buffer containing DOR$R_1$ (5 nM) and the indicated amounts of ATP-EcoDNAA. EMSA was performed using 4% PAGE and BAS2500 image analysis (B). The amounts of ssDUE-dsDNA complexed with EcoDNAA-DOR$R_1$ were quantified, and the levels relative to the input ssDUE-dsDNA are shown as Complex (%) (C). Two independent experiments were done, and both data and mean values are shown. D and E, ssDUE recruitment assay using chiDNAA R285A. In the first stage, ATP-chiDNAA (ATP), ADP-chiDNAA (ADP), or ATP-chiDNAA R285A (RA) (100 nM) was incubated on ice for 5 min with ssDUE-dsR1Tma or ssDUE-dsNon (12.5 nM). In the second stage, one-fifth portions of these mixtures were incubated with DOR$R_1$ and ATP-EcoDNAA as described above, followed by EMSA using 4% PAGE and BAS2500 image analysis (D). The amounts of ssDUE-dsDNA complexed with EcoDNAA-DOR$R_1$ at 60 nM EcoDNAA were quantified, and the levels relative to the input ssDUE-dsDNA were shown as Complex (%) (E). Two independent experiments were done, and both data and mean values are shown.
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In the presence of chiDnaA, binding of ssDUE-dsR1Tma was substantially higher than binding of ssDUE-dsR2Tma (Fig. 4, B (lanes 11 and 12 and lanes 17 and 18) and C), supporting the specific binding of chiDnaA. The minimal binding of ssDUE-dsNon in the absence of chiDnaA is consistent with our previous results as explained above (Fig. 4B, lanes 13–15) (4, 19). Binding of ssDUE-dsNon was slightly higher when chiDnaA was present. This might also have been due to basal, nonspecific binding of chiDnaA (Fig. 4B, lanes 16–18).

Based on the results above, next we investigated the requirement for ATP and the Arg finger of chiDnaA in the stimulation of ssDUE-dsR1Tma binding (Fig. 4D). Binding of ssDUE-dsR1Tma was stimulated at similar levels with ADP-chiDnaA and ATP-chiDnaA; however, binding was not stimulated in the presence of ATP-chiDnaA/pR4 (Fig. 4, D (lanes 1–12) and E). Binding of ssDUE-dsNon was inefficient, as described above; however, ATP-chiDnaA and ADP-chiDnaA, but not chiDnaA R285A, could enhance binding slightly. This stimulation may have been due to the basal, nonspecific binding of chiDnaA to ssDUE-dsNon (Fig. 4, D (lanes 13–20) and E). These results are consistent with those shown in Fig. 3 and support the idea that the Arg finger, but not the bound ATP, of DnaA at the R1 box is crucial for the interactions with the DnaA complexes formed on DORAR1 and hence the stimulation of ssDUE binding.

Role of the R4 Box-DnaA in DnaB Loading—Next, using similar methods as those used to assess the R1 box-bound DnaA (Fig. 3), we investigated the orientation of DnaA bound to R4, the high affinity binding site in the right-half oriC (Fig. 1A). DnaA binding at R4 is thought to trigger cooperative binding of DnaA molecules to the neighboring low affinity sites. DnaA boxes in the right-half oriC generally have the opposite orientation to those in the left-half oriC (Fig. 1A). Unlike the left-half oriC, the right-half oriC is dispensable for DUE unwinding (4).

Loading of DnaB helicase onto the unwound region is stimulated by ATP-chiDnaA promoted DnaB loading onto pR4Tma12 at a level comparable with DnaB loading on pBRoriC stimulated by ATP-EcoDnaA (Fig. 5, D and E). ADP-chiDnaA was also able to stimulate DnaB loading onto pR4Tma12, albeit at a lower level than ATP-chiDnaA. These results indicate that both the ATP and ADP forms of R4 box-bound chiDnaA can activate DnaB loading. ATP-DnaA might interact more effectively with DnaB than ADP-DnaA (see below), and this would explain the reduced stimulation of DnaB loading with the ADP form. By contrast, ATP-chiDnaA R285A did not stimulate DnaB loading activity. ATP-EcoDnaA also stimulated DnaB loading on pR4Tma12 but at a lower level than ATP-chiDnaA/pR4Tma12 or ATP-EcoDnaA/pBRoriC. ATP-EcoDnaA would not bind to the TmaDnaA box at the R4 position; the DnaB loading observed with the ATP-EcoDnaA/pR4Tma12 combination might have occurred as a result of stimulation of unwinding and DnaB loading using the left-half subcomplex in addition to possible DnaB binding at the low affinity boxes in the right-half oriC. No substantial DnaB loading was observed when ATP-EcoDnaA R285A was used (Fig. 5, D and E).

Taken together, these results support a crucial role for the Arg finger of DnaA bound to the R4 box in stimulating DnaB loading. This is consistent with the idea that the DnaA Arg finger on the R4 box is oriented inward within oriC and is involved in interacting with DnaA bound to the C1 site in a head-to-tail manner (AF-inward model; Fig. 1D). The ATP of the R4-bound DnaA might stimulate interaction with DnaB; a specific site in domain III of DnaA is suggested to interact with DnaB during the process of DnaB loading (44, 45).

Using methods similar to those for R1 box analysis (Fig. 3E), replication initiation activity was assessed using an in vitro reconstituted system and pR4Tma12 (Fig. 5F). Basal ATP-EcoDnaA was added, followed by the addition of EcoDnaA or chiDnaA and incubation at 30°C. Replication of the plasmid was stimulated by the addition of ATP-chiDnaA, ATP-EcoDnaA, or ADP-chiDnaA but not by the addition of ATP-chiDnaA R285A. As above, moderate stimulation occurred with ADP-chiDnaA and ATP-EcoDnaA. Substantially similar results were exhibited even when mixtures of EcoDnaA and chiDnaA in various ratios were added to reactions including pR4Tma12.3 These data are consistent with those of the form I’ assay and support the AF-inward model.

Role of the Arg Finger of the R4 Box-bound DnaA in DnaA Assembly—To analyze the role that R4-bound DnaA plays in DnaA assembly on oriC, EMSA was performed using right-half oriC DNA (R2R4), which encompasses the R2–R4 region. A small amount of ATP-EcoDnaA or ADP-EcoDnaA was preincubated on ice with pBRoriC or pR4Tma12, and this was followed by incubation at 38°C in the presence of additional EcoDnaA or chiDnaA (Fig. 5, B and C). Quantification of the P1 nuclease products showed that a moderate level of unwinding occurred in pBRoriC or pR4Tma12 without the second addition of DnaA. This is consistent with the dispensability of the right-half oriC for DUE unwinding. Additional ATP-EcoDnaA stimulated further unwinding of both pBRoriC and pR4Tma12. By contrast, the addition of chiDnaA did not stimulate further unwinding in either pBRoriC or pR4Tma12 (Fig. 5, B and C). These results are consistent with the above data and with the specificity of chiDnaA binding to the TmaDnaA box at the R4 position. When ADP-DnaA was used, unwinding did not take place, supporting the specificity of ATP-DnaA in this assay (Fig. 5, B and C).

Next, DnaB loading activity was assessed using the form I’ assay, as performed for R1 box analysis (Fig. 3, C and D). Reaction mixtures containing pR4Tma12, a low level of ATP-EcoDnaA, DnaB, and other required proteins were incubated at 30°C in the presence of additional EcoDnaA or chiDnaA. The addition of ATP-chiDnaA promoted DnaB loading onto pR4Tma12 at a level comparable with DnaB loading on pBRoriC stimulated by ATP-EcoDnaA (Fig. 5, D and E). ADP-chiDnaA was also able to stimulate DnaB loading onto pR4Tma12, albeit at a lower level than ATP-chiDnaA. These results indicate that both the ATP and ADP forms of R4 box-bound chiDnaA can activate DnaB loading. ATP-DnaA might interact more effectively with DnaB than ADP-DnaA (see below), and this would explain the reduced stimulation of DnaB loading with the ADP form. By contrast, ATP-chiDnaA R285A did not stimulate DnaB loading activity. ATP-EcoDnaA also stimulated DnaB loading on pR4Tma12 but at a lower level than ATP-chiDnaA/pR4Tma12 or ATP-EcoDnaA/pBRoriC. ATP-EcoDnaA would not bind to the TmaDnaA box at the R4 position; the DnaB loading observed with the ATP-EcoDnaA/pR4Tma12 combination might have occurred as a result of stimulation of unwinding and DnaB loading using the left-half subcomplex in addition to possible DnaB binding at the low affinity boxes in the right-half oriC. No substantial DnaB loading was observed when ATP-EcoDnaA R285A was used (Fig. 5, D and E).

Taken together, these results support a crucial role for the Arg finger of DnaA bound to the R4 box in stimulating DnaB loading. This is consistent with the idea that the DnaA Arg finger on the R4 box is oriented inward within oriC and is involved in interacting with DnaA bound to the C1 site in a head-to-tail manner (AF-inward model; Fig. 1D). The ATP of the R4-bound DnaA might stimulate interaction with DnaB; a specific site in domain III of DnaA is suggested to interact with DnaB during the process of DnaB loading (44, 45).

Using methods similar to those for R1 box analysis (Fig. 3E), replication initiation activity was assessed using an in vitro reconstituted system and pR4Tma12 (Fig. 5F). Basal ATP-EcoDnaA was added, followed by the addition of EcoDnaA or chiDnaA and incubation at 30°C. Replication of the plasmid was stimulated by the addition of ATP-chiDnaA, ATP-EcoDnaA, or ADP-chiDnaA but not by the addition of ATP-chiDnaA R285A. As above, moderate stimulation occurred with ADP-chiDnaA and ATP-EcoDnaA. Substantially similar results were exhibited even when mixtures of EcoDnaA and chiDnaA in various ratios were added to reactions including pR4Tma12.3 These data are consistent with those of the form I’ assay and support the AF-inward model.

Role of the Arg Finger of the R4 Box-bound DnaA in DnaA Assembly—To analyze the role that R4-bound DnaA plays in DnaA assembly on oriC, EMSA was performed using right-half oriC DNA (R2R4), which encompasses the R2–R4 region. A derivative of R2R4 in which the R4 box was substituted with the TmaDnaA box (R2R4–R4Tma), was also used (Fig. 6A). DnaA complexes were formed without detectable levels of binding intermediates when ATP-EcoDnaA was co-incubated with R2R4 DNA (Fig. 6, B and C). This is consistent with previous research (19) and suggests that DnaA binds cooperatively in this region. In other previous papers that analyzed binding intermediates (12, 46, 47), competitor DNA was not used for stabilizing the intermediates.

DnaA assembly was severely inhibited when R2R4–R4Tma DNA was used (Fig. 6, B and C). This is consistent with previous
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research (43) and indicated that DnaA binding to the R4 box is crucial for DnaA assembly on the right-half oriC.

Next, a similar assay was performed using EcoDnaA, chiDnaA, and R2R4-R4Tma DNA (Fig. 6, D and E). ATP-chiDnaA and ADP-chiDnaA promoted DnaA assembly in the presence of ATP-EcoDnaA at similar levels, but DnaA assembly with ATP-chiDnaA R285A was impaired (Fig. 6, D and E). These results suggest that, when the TmaDnaA box sequence is present at the R4 position, ATP-chiDnaA and ADP-chiDnaA can promote cooperative binding of DnaA molecules to the low affinity DnaA boxes but that ATP-chiDnaA R285A is impaired in this ability. This is consistent with the idea that the DnaA Arg finger on the R4 box-bound DnaA is oriented toward the DnaA box C1 (AF-inward model). Moderate activity of ATP-chiDnaA R285A in DnaA assembly might be due to Arg finger-independent inter-DnaA interactions at other sites (48).

In addition, to investigate the importance of orientation of the R4 box-bound DnaA in DnaA assembly, EMSA was performed using a derivative of R2R4 in which the R4 box sequence was inverted (R2R4-R4inv). DnaA assembly was moderately impaired when R2R4-R4inv DNA was used (Fig. 6, F and G). This indicates that the orientation of the R4 box-bound DnaA is relevant to DnaA assembly on the right-half oriC, consistent with the above results and previous in vitro research (43). Resid-
ual activity of R2R4-R4inv in DnaA assembly was also observed, which might be due to Arg finger-independent inter-DnaA interactions, such as inter-domain I interactions (3).

In Vivo Examination of the Arg Finger of DnaA at the R1 and R4 Boxes—

The orientation of DnaA bound to the R4 and R1 boxes was assessed in vivo using modified strains. MG1655-derivative mutant strains were constructed that had the Tma-DnaA box sequence at the chromosomal R1 box (strain NY24) or R4 box (strain NY21) (Table 1). Strain construction details are provided under “Experimental Procedures.” NY24 and NY21 cells grew at the same rate (generation time of 20–23 min) as the parental strain containing wild-type oriC (NY20); however, regulation of initiation was impaired (see below). This is consistent with previous descriptions of strains with mutations in the R1 or R4 box (43).

NY20, NY24, and NY21 cells were transformed with pING1-derivative plasmids carrying genes encoding chiDnaA (pChiWT), chiDnaA R285A (pChiR285A), chiDnaA D269A (pChiD269A), or chiDnaAcos (pChiDnaAcos). The pING1 plasmid is derived from pBR322 and carries the arabinose promoter and the araC repressor gene. Plasmid dnaA expression occurred mainly as a result of leaky expression, which occurred at a similar level in all cells (Fig. 7, A and B). The total amounts of DnaA-DNA complex relative to the total DNA were quantified and used as a measure of complex formation (%) (C). Two independent experiments were done, and both data and mean values are shown. D and E, EMSA using EcoDnaA and chiDnaA. ATP-EcoDnaA (80 nM) was incubated in buffer containing R2R4-R4Tma (35 nM). The indicated amounts of ATP-chiDnaA (ATP-ChiWT), ADP-chiDnaA (ADP-ChiWT), or ATP-chiDnaA R285A (ATP-ChiRA) were added, and reactions were incubated at 30 °C for 10 min. EMSA was performed as described above (D). The relative amounts of DnaA-DNA complexes were quantified. Error bars, S.D. from three independent experiments (E). F and G, EMSA using the right-half oriC fragments. The indicated amounts of ATP-EcoDnaA were incubated at 30 °C for 10 min with the right-half oriC (35 nM) bearing the wild-type sequence (R2R4), the R4 box replaced with the TmaDnaA box sequence (R2R4-R4Tma), or the inverted R4 box (R2R4-R4inv). EMSA was performed as described above (F). The relative amounts of DnaA-DNA complexes were quantified. Error bars, S.D. from three independent experiments (G).
FIGURE 7. Role for the Arg finger of DnaA bound to the R1 and R4 boxes in initiation in vivo. A and B, amounts of cellular chiDnaA were determined by Western blotting. Cells of strain KP7364 (ΔdnaA::spec rnhA::kan) (A) or NY20 (B) carrying the indicated plasmid were grown in LB medium at 37 °C to an absorbance (A660) of 0.1. A portion (500 μl) of each culture was analyzed by Western blotting using anti-EcoDnaA antibody as described previously (26). Arrows, bands corresponding to chiDnaA or to a mixture of chiDnaA and EcoDnaA. For a quantitative standard, the indicated amounts of purified EcoDnaA were mixed with whole cell extract of NY26 (ΔdnaA::spec rnhA::kan). The amounts of cellular DnaA were quantified in two independent experiments. Disruption of rnhA activates alternative origins independent of DnaA (49). chiDnaA has a molecular mass similar to EcoDnaA. C, in vivo analysis of R1 and R4 box-bound DnaA. Cells of strains NY20 (wild-type oriC, WT), NY24 (chromosomal R1 box substituted with TmaDnaA box; R1Tma), and NY21 (chromosomal R4 box substituted with TmaDnaA box; R4Tma) carrying pNG (vector), pChiWT (chiDnaA), pChiR285A (chiDnaA R285A), or pChiD269A (chiDnaA R269A) were grown at 37 °C in LB medium containing ampicillin. When the absorbance A660 reached 0.1, culture portions were withdrawn and used for quantification of cell size (cell mass) using flow cytometry. Additional culture portions were further incubated for 4 h in the presence of rifampicin and cephalaxin. DNA contents were quantified using flow cytometry. Equivalent chromosome numbers are shown. Cell mass relative to that of NY20 cells bearing pNG1 (1.0) is indicated at the top right of each panel. Two independent experiments were performed, and indistinguishable results were exhibited. D, in vivo analysis of cells bearing the inverted R4 box. Cells of strains NY20, NY21, and NY25 (chromosomal R4 box inversion; R4inv) were grown and analyzed using flow cytometry as described above. Cell mass relative to that of NY20 cells (1.0) is indicated at the top right of each panel. For NY25 cells, three independent transductants were analyzed, and similar results were obtained for each; representative data are shown. E, analysis of NY20, NY21, and NY25 cells grown in a minimum medium. Flow cytometry analysis was performed as described above except that minimum medium described previously (18) was used. For NY25 cells, three independent transductants were analyzed, and similar results were obtained for each; representative data are shown.
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fold lower (26). It is therefore conceivable that, in cells where ATP is abundant (millimolar levels) and concentrations are 10-fold higher than ADP levels, DnaA D269A accelerates the dissociation of ADP and binds primarily with ATP. Consistent with this, we previously demonstrated that overinitiation of replication occurred in cells bearing DnaA D269A (26). The DnaAcos protein, which bears mutations in domain III, maintains initiation activity at 30 °C but is severely impaired in ATP and ADP binding. This results in a severe overinitiation of replication that leads to inhibition of cell growth (50–52).

Flow cytometry analysis was performed using the constructed cell lines (Fig. 7C). Cells were rapidly grown in LB medium at 37 °C. Cephalixin and rifampicin were then added to the culture to stop cell division and replication initiation while permitting progression of established replisomes. Incubation was continued to allow run-out replication of the chromosomes. The resulting cells therefore contained the same number of chromosomes as there were copies of oriC at the time of antibiotic addition (53). Flow cytometry of NY20 cells bearing pING1 revealed a major peak at eight chromosomes and a minor peak at 16 chromosomes. This indicated that a newborn cell contained eight sister chromosomes. The data indicating that NY24 cells bearing pING1 (Fig. 7C) were similar to that of NY24 cells bearing pChiWT are also consistent with the above results. A moderate level of asynchronous initiations was observed even in minimum medium (Fig. 7E). Data from a previous research indicated that cells with the inverted R4 box had a slightly elevated level of asynchronous initiations in the same minimum medium (18) (see “Discussion”). Replication initiation in NY21 cells was asynchronous in the minimum medium (Fig. 7E), which was consistent with the above results and with the previous reports (18, 43).

Discussion

Discovering the functional structure of DnaA homo-oligomers complexed with oriC is crucial for understanding the regulation and mechanism of replication initiation. However, attribution of specific roles to individual DnaA protomers in the complexes has remained elusive. In this study, we performed detailed analysis using chimeric DnaA molecules and chimeric oriC sequences. Chimeric DnaA molecules bound specifically to a typical TmaDnaA box rather than to a typical EcoDnaA box. Chimeric oriC sequences harbored the TmaDnaA box at the R1 or R4 box positions. Experimental data indicated that the Arg finger of DnaA bound at the R1 or R4 box is crucial for accurate replication initiation both in vitro and in vivo. Further results showed that replication initiation was active even when ADP-DnaA rather than ATP-DnaA was bound at the R1 or R4 box. For the R1 box-bound DnaA, the Arg finger-dependent interaction would be crucial for the construction of a specific DnaA complex conformation with DUE unwinding activity (Figs. 3 and 4). For the R4 box-bound DnaA, the Arg finger-dependent interaction would be crucial for the construction of a specific DnaA complex with DnaB helicase loading activity (Fig. 5) and would also be important for cooperative DnaA binding at low affinity sites (Fig. 6). Flow cytometry data from cells with chimeric DnaA molecules and chimeric oriC sequences were consistent with the in vitro data (Fig. 7C). Taken together, we propose that the Arg fingers, but not the bound nucleotide, of the R1- or R4-bound DnaA are oriented inward within oriC and are used for functional interactions with DnaB bound to the flanking low affinity site (AF-inward model in Figs. 1D and 8A). The concept of the Arg fingers of DnaA bound to the R1 and R4 facing inward is a two-dimensional description of what is a three-dimensional situation.
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These conclusions are consistent with a previous in vivo study indicating the specific roles of the R1 and R4 boxes (18, 43) and with in vivo footprint experiments of DnaA binding showing that binding to the R1, R2, and R4 boxes was maintained stably during the cell cycle (54). ATP-DnaA is much more abundant than ATP-DnaA during the cell cycle, except at the time of replication initiation (10, 55). According to our results (Figs. 3 and 4), replication initiation is not inhibited even if ATP-DnaA binds to the R1 box. Rather, stable DnaA binding to the R1 box would allow efficient activation of initiation complexes. Supporting this is the observation that the addition of ATP-DnaA stimulates initiation of oriC replication when ATP-DnaA is present only at basal levels (56).

Consistent with the previous results mentioned above, our results also indicate that initiation remains active even when ATP-DnaA binds to the R4 box (Fig. 5, D–F). A moderate inhibition of DnaB loading was observed with ATP-chiDnaA and oriC with the TmaDnaA box at the R4 position (Fig. 5, D and E). This inhibition could be as a result of slight structural differences in domain IV between EcoDnaA and TmaDnaA. Otherwise, because domain III of DnaA bears a possible DnaB-binding site (44, 45), it is possible that ATP binding promotes a conformational change at domain III that stimulates interaction with DnaB at this site. Although DnaB loading activity was reduced when ADP-DnaA was bound to the R4 box, the remaining activity as assessed in vitro would be sufficient to support timely initiation under the in vivo conditions.

The Arg finger of the R1 box-bound DnaA was crucial for DUE unwinding and ssDUE binding (Figs. 3 and 4). These results are consistent with the idea that the Arg finger of the R1 box-bound DnaA interacts with the ATP bound to DnaA residing on the neighboring low affinity region (AF-inward model in Fig. 1D). This interaction would promote the recruitment of ssDUE to DnaA complexes formed on the low affinity region of the left-half oriC via IHF binding-dependent DNA bending (Fig. 8A) (4, 19). Our results indicated that the ATP on the R1 box-bound DnaA was not important for initiation (Figs. 3 and 4). This is consistent with the idea that the R1 box-bound DnaA does not bind another DnaA on the DUE side. In addition, previous results of footprint experiments did not detect binding of DnaA molecules in the region between the DUE and the R1 box (14, 19, 27, 46). These observations also support ssDUE recruitment as described above rather than the model of a continuous ATP-DnaA filament at the DUE region formed from ATP-DnaA bound to the R1 box, as suggested previously (5, 15).

In the right-half oriC region, the presence of an intact R2 box was insufficient for DnaA complex formation at the right-half oriC in the absence of the R4 box (Fig. 6B). Moderate DnaA complex formation activity was maintained even when chi-DnaA R285A was bound to the TmaDnaA at position R4 or when the R4 box was inverted (Fig. 6, D–G). This is consistent with the possibility that other domain III residues or domain I of the R4 box-bound DnaA might assist in inter-DnaA interactions in the right-half oriC. We therefore suggest that the Arg finger of the R4 box-bound DnaA is a stimulator, but not a prerequisite, in the formation of DnaA homo-oligomers on the right-half oriC.

Our data are consistent with the idea that the Arg finger of the R4 box-bound DnaA plays a crucial role in the loading of DnaB helicase (Fig. 5, D and E). Previous studies suggested that formation of stable DnaA complexes on oriC was required for loading of DnaB on unwound DNA (48). It is plausible that the Arg finger of the R4 box-bound DnaA supports the formation of stable DnaA complexes, although DnaA complexes can be formed with moderate efficiency in the absence of this finger, as shown in the EMSA data (Fig. 6, D and E) and discussed above. The in vivo analysis confirmed the importance of the Arg finger of the R4 box-bound DnaA in replication initiation (Fig. 7C). Consistently, when the R4 box was inverted, DnaA assembly on the right-half oriC and replication initiation were impaired (Figs. 6F and G and 7D). In a previous study, cells with an oriC construct similar in the inversion of the R4 box were grown in a minimal medium and analyzed using flow cytometry (18). The asynchrony index indicating levels of asynchronous initiations was 8.1 for those cells. This value was intermediate between the index value (5.7) of a wild-type synchronous control and the index value (11.0) of moderately asynchronous cells with the R2 box mutation (18). Here, the R4 box-inverted cells showed
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moderate asynchrony even in minimal medium (Fig. 7E). The subtle difference in the level of asynchrony between the present and previous data might have been due to differences in genetic elements of the strains, growth conditions (e.g. aeration or the timing of harvest), or flow cytometry equipment. The right-half oriC was reported to be more important for stimulating initiation in cells growing in rich medium than in cells in poor medium (57), consistent with the present results (Fig. 7, D and E) and with the specific role for the right-half subcomplex in stimulation of DnaB loading (4).

Previous results from pull-down experiments with various oriC fragments suggest that a pair of DnaB helicases bind to an initiation complex (4). The data suggest that the first DnaB-binding site is located in DnaA domain I, which contains the Glu-21 and Phe-46 residues, and that the second binding site is found at the N terminus of domain III (22, 23, 44, 45). In this study, we propose a model in which the DnaA subcomplexes constructed on each half of the oriC region are oriented in opposite directions (AF-inward model; Figs. 1D and 8A). This symmetric direction of DnaA subcomplexes could be significant for the probable symmetric binding of a pair of DnaB helicases to the two DnaA subcomplexes. It would be reasonable to infer that the interaction between DnaA domain III and DnaB can contribute to DnaB orientation and that this binding mode of DnaB-DnaA subcomplexes can be a stimulating factor in efficient bidirectional and synchronous loading of a pair of DnaB helicases onto the single-stranded oriC region. Examination of DnaB helicase orientation when bound to an initiation complex is important for future studies.

Sequence similarity analysis suggests that multiple DnaA boxes are found within the predicted oriC in many bacterial species; however, sequence analysis did not successfully predict the E. coli oriC low affinity sites (58). Experimental analysis of DnaA boxes has been performed only in a few species. In Caulobacter crescentus and Mycobacterium tuberculosis, DnaA boxes, including some with low affinity, were identified experimentally (59, 60) (Fig. 8B). The orientation of the oriC-DnaA boxes in these species resembled the arrangement in E. coli, whereby the direction of DnaA boxes in the left half of the DnaA box cluster region opposed the direction in the right-half region (Fig. 8B). Two oriC subregions, oriC1 and oriC2, were recently identified flanking the dnaA gene in Helicobacter pylori. The DnaA box sequences of oriC1 and oriC2 are oriented in the same direction, and it is suggested that the DnaA-oriC1 subcomplex interacts with the DnaA-oriC2 subcomplex via DNA looping of the dnaA gene region (61, 62). In the resultant complex, the directions of the DnaA complexes formed at oriC1 and oriC2 would then be opposite (Fig. 8C). The structural feature of two subregions bearing DnaA box clusters in opposite orientations might therefore be a common feature in eubacteria and could be important for bidirectional loading of replisome helicases.

Formation of specific DnaA complexes is required also at important sites other than oriC. The DnaA-reactivating sequences and datA, which each contain at least three DnaA boxes, require construction of specific DnaA homo-oligomers for the promotion of reactivation and inactivation of DnaA, respectively (10, 63–65). The datA locus promotes the hydrolysis of ATP bound to DnaA for repression of untimely initiations (10). Exchange of ADP to ATP is stimulated when ADP-DnaA interacts with DnaA-reactivating sequences, resulting in reactivation of DnaA (64, 65). These complexes have indispensable roles in the regulation of the replication cycle (2, 7, 9). The chimeric DnaA resources that were developed in this study will be invaluable in investigating the roles of these additional complexes.

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