Cooperative DnaA Binding to the Negatively Supercoiled datA Locus Stimulates DnaA-ATP Hydrolysis*

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Timely initiation of replication in Escherichia coli requires functional regulation of the replication initiator, ATP-DnaA. The cellular level of ATP-DnaA increases just before initiation, after which its level decreases through hydrolysis of DnaA-bound ATP, yielding initiation-inactive ADP-DnaA. Previously, we reported a novel DnaA-ATP hydrolysis system involving the chromosomal locus datA and named it datA-dependent DnaA-ATP hydrolysis (DDAH). The datA locus contains a binding site for a nucleoid-associated factor integration host factor (IHF) and a cluster of three known DnaA-binding sites, which are important for DDAH. However, the mechanisms underlying the formation and regulation of the datA-IHF-DnaA complex remain unclear. We now demonstrate that a novel DnaA box within datA is essential for ATP-DnaA complex formation and DnaA-ATP hydrolysis. Specific DnaA residues, which are important for interaction with bound ATP and for head-to-tail inter-DnaA interaction, were also required for ATP-DnaA-specific oligomer formation on datA. Furthermore, we show that negative DNA supercoiling of datA stabilizes ATP-DnaA oligomers, and stimulates datA-IHF interaction and DnaA-ATP hydrolysis. Relaxation of DNA supercoiling by the addition of novobiocin, a DNA gyrase inhibitor, inhibits datA function in cells. On the basis of these results, we propose a mechanistic model of datA-IHF-DnaA complex formation and DNA supercoiling-dependent regulation for DDAH.

In Escherichia coli, the ATP-bound DnaA protein (ATP-DnaA) has an essential role in assembly of initiation complexes on the chromosomal replication origin oriC (1–5). oriC consists of an AT-rich duplex-unwinding element (DUE) and a DnaA oligomerization region that contains two subregions with oppositely oriented clusters of 9-bp DnaA-binding sites (DnaA boxes) (see Fig. 1A). DnaA boxes R1 and R4 at the outer edges of the DnaA oligomerization region are oriented oppositely and have the highest affinities for DnaA of the sequences in this region. Cooperative binding of ATP-DnaA molecules to lower affinity sites in each subregion of oriC results in formation of left-half and right-half DnaA subcomplexes (6–8). The left-half subcomplex binds to the region of oriC that contains the DUE and executes unwinding of DNA within the element (6, 9). IHF, a nucleoid-associated protein (NAP), binds to a specific IHF-binding site (IBS; see Fig. 1A) within the left-half subregion of oriC and bends DNA sharply (~180°), enhancing ATP-DnaA binding and duplex unwinding (9, 10). The DnaA subcomplexes also interact with a pair of DnaB helicases for loading of those to the single-stranded DNA (9, 11, 12).

DnaA consists of four domains (3). The N-terminal domain I has a DnaB-binding site (12). Domain II is a flexible linker (12). Domain III is the AAA+ domain that has motifs for binding and hydrolysis of ATP (Walker A and B and Sensor I and II) and for interaction between DnaA protomers (arginine-finger, Box VII, AID-1, and AID-2) (see Fig. 1B) (6, 13–16). DnaA arginine-finger Arg-285 has a key role in ATP-dependent head-to-tail interaction between DnaA protomers, which activates the formation of subcomplexes on oriC (7, 13). DnaA Box VII Arg-281 residue stabilizes the interaction (16). AID-1 Arg-227 and AID-2 Leu-290 residues stimulate protomer interactions in the left-half DnaA subcomplex (6). The C-terminal DnaA domain IV contains the helix-tern-helix motif bearing DnaA box binding activity (17).

Initiation of replication from oriC, and especially duplex unwinding, is regulated by negative DNA supercoiling (18), which is modulated by the actions of DNA topoisomerases such as DNA gyrase and by binding of NAPs. DNA supercoiling is involved in regulation of multiple cellular processes, including DNA replication, transcription, recombination, and nucleoid compaction (19–21). The supercoiling state of chromosomal DNA is altered dynamically according to the growth phase and extracellular stresses such as osmotic shock, heat, pH, and antibiotics to sustain cell growth (22–25). For example, in the osmotic transcriptional response in E. coli, DNA supercoiling regulates the association of RNA polymerase on DNA and expression of osmotic-response genes (23, 26). In Helicobacter pylori, DNA supercoiling affects the binding mode of the cognate DnaA and oriC (27). However, the importance
of supercoiling-dependent regulation for cellular DnaA activity remains unclear.

The cell cycle-coordinated initiation of chromosomal replication is sustained by multiple regulatory systems that affect cellular DnaA activity (1, 4, 28). After ATP-DnaA induces replication initiation, DnaA-ATP is hydrolyzed by the complex of the AAA+ domain-containing Hda protein and the DNA-loaded β-clamp subunit of the DNA polymerase III holoenzyme, yielding initiation-inactive ADP-DnaA (1, 29–31). This replication-coupled feedback system is called regulatory inactivation of DnaA (RIDA). Defects in RIDA result in over-initiation and the inhibition of cell growth (15, 32).

Previously, we demonstrated that a specific chromosomal locus (datA) promotes DnaA-ATP hydrolysis by a mechanism that we named datA-dependent DnaA-ATP hydrolysis (DDAH) (33). The datA locus (991 bp) includes a 262-bp region containing two known DnaA-box sequences (DnaA boxes 2 and 3) and an IBS, which is essential for repression of untimely initiation (Fig. 1A) (25–28). DNA-footprinting experiments suggest the presence of two further sites within the 262-bp datA region that bind DnaA (herein described as DnaA boxes 6 and 7) (Fig. 2A) (35). However, the roles of these boxes remain to be explored.

DnaA sensor II motif Arg-334 residue has a key role in DnaA-ATP hydrolysis in RIDA and DDAH (Fig. 1B) (15). DnaA arginine-finger Arg-285 and Box VII Arg-281 are also required for DDAH but not for RIDA (Fig. 1B) (13, 33). IHF temporarily binds to datA at the post-initiation stage and ensures once-per-cell-cycle initiation. However, the underlying mechanism of inter-DnaA interactions in DDAH and its regulation have not yet been determined.

We demonstrated that datA DnaA box 7, but not box 6, has an essential role in DDAH and that DnaA box 7 stimulates cooperative binding of ATP-DnaA on datA. Second, we showed that the DnaA AID-2 motif, but not AID-1, is required for DDAH. Third, we found that the DnaA arginine-finger, Box VII, and AID-2 motifs are required for ATP-DnaA-specific oligomer formation on datA. Fourth, we identified that negative DNA supercoiling stimulates DDAH. DNA supercoiling has little effect on the overall structure of datA-DnaA complexes but stabilizes ATP-DnaA oligomer formation and stimulates binding of IHF to datA. Consistent with these in vitro findings, activity of datA in vivo was impaired in the presence of novobiocin, an inhibitor of DNA gyrase. From these results, we propose a model of the mechanism of DDAH and its regulation by DNA supercoiling.

### Results

**Cooperative DnaA Binding to datA DnaA Boxes 2 and 7 Is Essential for DDAH**—We previously reconstituted the DDAH system in vitro with ATP-DnaA, IHF, and a DNA fragment containing a 991-bp datA sequence and found that DnaA boxes 2 and 3 and IBS were required for DDAH activity (33). Data from previous in vivo analyses are consistent with these in vitro results (35, 36). A datA region between DnaA boxes 1 and 2 might contain two additional DnaA boxes (boxes 6 and 7) with moderate homology with the consensus sequence (Fig. 2A).
To determine the involvement of DnaA boxes 6 and 7 in DDAH, we generated constructs with transversion mutations at these sites in bases that are identical to the DnaA-box consensus sequence. Inclusion of these mutant sequences in the reconstituted in vitro DDAH system indicated that DnaA box 7 was required for DDAH, whereas DnaA box 6 was dispensable (Fig. 2B).

To determine whether formation of DnaA oligomers at datA is inhibited by mutation of DnaA box 7, electrophoretic mobility shift assay (EMSA) was performed with the minimal datA DnaA boxes 6 and 7. A nucleotide sequence spanning a region from DnaA box 1 to DnaA box 2 is shown below. DnaA boxes 1 and 2 as well as potential DnaA boxes 6 and 7 are indicated by black and gray arrows, respectively. B, mutational analyses of DnaA boxes 6 and 7. [α-32P]ATP-DnaA (1 pmol) was incubated at 30 °C for 10 min in buffer containing IHF (0.5 pmol), and 50 or 100 fmol of PCR fragments harboring datA (991 bp) (wild type (WT) or containing substitutions in box 1 (subDnaAbox1), box 6 (subDnaAbox6), box 7 (subDnaAbox7), or box 2 (subDnaAbox2)) or oriC in the presence of 100 mM NaCl. The proportions of ADP-DnaA to the total ATP/ADP-DnaA are indicated as percentages (%).

C–F, requirement for DnaA box 7 for DnaA oligomerization on datA. C, the black bar indicates the datA del5 fragment (262 bp) and datA del7 fragment (43 bp) used in EMSA. D, the indicated amounts of ATP-DnaA or ADP-DnaA were incubated with datA del5 or datA del5 with the subDnaAbox7 mutation (150 fmol) along with IHF (6 pmol) and 150 ng of λDNA (as a competitor) at 15 °C for 5 min followed by EMSA with a 2% agarose gel and ethidium bromide staining. The gel images are shown in black and white inverted mode with ordinary contrast (upper) and higher contrast for better visibility of topoisomers (lower). IHF indicates IHF-bound datA, C1–C4 indicates the lower datA-IHF complexes with 1–4 DnaA molecules, and ≥C5 indicates higher datA-IHF complexes with ≥5 DnaA molecules. The proportions of higher (●; ≥C5; △, △) and lower complexes (○; C1–C4; ▲, ▲) of ATP-DnaA (○, ▲) or ADP-DnaA (●, △, △) were determined using the data shown in C and are plotted as percentages. G, cooperative DnaA binding to DnaA boxes 6 and 7 with neighboring DnaA box 2. The indicated amounts of ATP-DnaA were incubated with derivatives of datA 672 fragment (WT or with the subDnaAbox6, subDnaAbox7, or subDnaAbox2 mutations; 43 bp, 500 fmol) at 30 °C for 5 min followed by EMSA with 8% PAGE.

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Mechanism of DnaA-ATP Hydrolysis by Supercoiled datA

region that is competent for DDAH (datA del5; Fig. 2C) in the presence of IHF and either ATP-DnaA or ADP-DnaA. ATP-DnaA formed large complexes (≥5 with at least 5 DnaA molecules per substrate molecule of datA del5 when the input molar ratio was 13–20 DnaA molecules per datA (Fig. 2, D and E). By contrast, ADP-DnaA mainly formed smaller complexes (C1-C4) with one to four DnaA molecules per substrate, consistent with previous results (33). Both ATP-DnaA and ADP-DnaA formed mainly smaller complexes on datA del5 with a DnaA box 7 mutation (subDnaAbox7; Fig. 2, D and F).

EMSA was also conducted with short dsDNA fragment (datA 672 fragment; 43 bp) containing DnaA boxes 6, 7, and 2. Mutation of individual DnaA-box sequences demonstrated that binding of DnaA to box 7 was dependent on box 2 (Fig. 2G). These results indicate that cooperative DnaA binding involving box 7 and box 2, but not box 6, is essential for formation of ATP-DnaA oligomers that are required to activate DDAH.

Inter-DnaA Interaction Motifs Are Required for ATP-DnaA-specific Oligomerization on datA—Previously, we revealed that DnaA AAA+ motifs, arginine-finger Arg-285, and Box VII Arg-281 stimulated successive interactions of ATP-DnaA boxes 6, 7, or 2, elevating the catalytic reaction rate of DDAH. By contrast, DnaA R285A and R281A datA interaction via AID-2 destabilizes ADP-DnaA oligomers on DDAH. Another possibility is that DnaA L290A forms oligomerization on datA, which is required for ATP-DnaA specific oligomerization on datA (Fig. 3, H–J).

dataA DnaA Box 7 Is Required to Repress Untimely Initiation in Vivo—To investigate the in vivo function of datA DnaA box 7, flow cytometry analysis was performed on MG1655 cells with plasmids containing dataA (pKK40) or its mutant derivatives, which were cultivated at 37°C in LB medium (Fig. 4A). To determine the number of oriC copies, growing cells were incubated in the presence of rifampicin and cephalaxin (inhibitors of replication initiation and cell division, respectively) until chromosomal replication was complete. The number of chromosomes per cell determined by flow cytometry then corresponds to the number of oriC copies per cell at the time of addition of the antibiotics (37–39). In rapidly growing cells, depending on growth conditions, a single cell contains 2, 4, or 8 sister oriC copies before replication initiation, and initiation occurs simultaneously on each oriC copy, whereas the previous round of replication is ongoing, resulting in 4, 8, or 16 copies of oriC in a cell (38).

The results of flow cytometry showed that whereas MG1655 cells with the pACYC177 plasmid (which does not contain dataA) had 8 or 16 replication origins per cell, those with pKK40 contained 4 or 8 origins, and some cells had 6 origins as a result of asynchronous initiation (Fig. 4A). The asynchronous index (AI), which evaluates the level of improper timing of initiation (38, 40–42), was increased in cells with pKK40 compared with pACYC177 (46% versus 4.8%). In cells with pKK40, the origin number to mean cell mass (ori/mass), an indicator of initiation activity, was reduced to 0.67 relative to the reference value of 1.0 in cells with pACYC177. These results were consistent with those of previous studies (43, 44) and indicated that oversupply of dataA moderately inhibited initiation. In addition, introduction of pKK40 caused moderate reduction in cell growth relative to pACYC177 (doubling time 38 min versus 26 min) along with delay in the timing of cell division (cell mass with pKK40 1.3 x cell mass with pACYC177). By contrast, initiation parameters and growth rates were not substantially affected by oversupply of the dataA substitution mutants subDnaAbox7 (pHT28) or subDnaAbox2 (pKK41), whereas subDnaAbox6 (pKK120) inhibited initiations and cell growth like the WT dataA (pKK40) (Fig. 4A), all of which is consistent with our in vitro data (Fig. 2B).

Ori/mass ratios and AI were also analyzed in cells with chromosomal dataA mutations (Fig. 4, B and C). In ΔdataA cells, synchronous initiation was severely inhibited (AI = 90%), and the number of oriC copies per cell increased compared with WT cells, but initiation frequency and cell growth were hardly affected (Fig. 4B), as reported previously (34, 35, 45). Cells with dataA mutations had growth rates comparable with those of WT or ΔdataA cells (Fig. 4, B and C). In LB medium, chromosomal dataA subDnaAbox7 or subDnaAbox2 mutants exhibited severe asynchronous initiation (AI = 114% or 127%, respectively), whereas a subDnaAbox6 mutant did not (AI = 9.2%; Fig. 4B), further indicating that DnaA box 7 is essential for dataA function in vivo. Similar results were obtained in cells cultivated in supplemented M9 medium (Fig. 4C) except for an unexpected increase in cell mass in the presence of dataA mutations. The delay in cell division implies that DnaA boxes 6, 7, or 2 might have supportive roles in the regulation of cell division by dataA, as suggested previously (46). Also, in dataA subDnaAbox6
mutant cells, initiation frequency was slightly decreased (ori/mass = 0.83 or 0.85 in LB or supplemented M9 medium, respectively) compared with that in WT cells, suggesting the possibility that datA DnaA box 6 might repressively regulate DDAH in vivo at a limited level.

Negative DNA Supercoiling Promotes DDAH Efficiency—To assess whether DNA supercoiling affects DDAH activity, ATP-DnaA and IHF were incubated with a supercoiled or PstI-digested linear form of the datA-containing plasmid pKX40 (Fig. 5A). ATP-DnaA molecules were efficiently converted into ADP-DnaA at 30 °C for 10 min in buffer containing 100 mM NaCl and either the supercoiled or linear form of pKX40 (Fig. 5, B and C), consistent with previous results in buffer containing 100 mM potassium glutamate (33). The parental vector, pACYC177, did not substantially show DDAH activity; only a slight activity would be caused by feeble DnaA-intrinsic ATPase (47). To address a question of whether DNA supercoiling stabilizes nucleoprotein complexes on datA, we examined resistance of DDAH activity against higher salt concentrations (i.e. 150 or 200 mM NaCl). Notably, whereas DDAH activity of the supercoiled form of pKX40 was resistant to 150 mM and 200 mM NaCl, that of the linear form was moderately inhibited at 150 mM NaCl, and the activity was further aggravated at 200 mM (Fig. 5, B and C).

In the presence of 150 mM NaCl, DDAH activities of the supercoiled and linear forms were further analyzed. Both DNA titration and time-course experiments indicated that DDAH catalyzed by datA in a supercoiled form was more efficient than

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FIGURE 3. DnaA motifs were required for formation of DDAH-active ATP-DnaA-specific oligomers on datA. A, [α-32P]ATP-DnaA (wild type (WT), R227A, or L290A; 1 pmol) was incubated at 30 °C for 10 min in buffer containing IHF (0.5 pmol) and 100 fmol of PCR fragments harboring datA wild type as described in Fig. 2B. B–J, oligomerization of DnaA mutants on datA. B, the indicated amounts of ATP-DnaA or ADP-DnaA (WT or L290A) were incubated with datA del5 wild type as described in Fig. 2D. The proportions of higher (C) and lower (D) complexes of ATP-DnaA or ADP-DnaA were determined as described in Fig. 2, E and F. Similar experiments were performed using DnaA R285A (E–G) and R281A (H–J).
that by datA in a linear form (Fig. 5, D and E). One molecule of supercoiled datA promoted hydrolysis of 3.3 ATP-DnaA molecules per min, whereas hydrolysis was only 1.9 ATP-DnaA molecules per min with linear datA.

DNA supercoiling slightly altered the requirement for IHF in DDAH. The maximal reaction rate was achieved with 0.15 pmol of IHF on supercoiled datA but 0.3 pmol IHF on linear datA (Fig. 5F), implying that DNA supercoiling might stimulate formation of datA-IHF complexes. The physiological ionic strength is 100–150 mM, suggesting that DNA supercoiling can stimulate DDAH activity under certain physiological conditions.

**Structural Requirements of Supercoiled datA in DDAH**—Our previous results with linear datA fragments indicated that DnaA box 2–3 and IBS are essential for DDAH, whereas DnaA box 4 is stimulatory (33). To identify the specific requirements of supercoiled datA in DDAH, the activities of various deletion derivatives of the datA WT fragment were analyzed in the presence of 150 mM NaCl (Fig. 6A). As with linear datA (33), full DDAH activity was sustained by the supercoiled form of datA del5 (pHT20), which contains DnaA boxes 6, 7, 2–4, and IBS (Fig. 6A). Furthermore, the supercoiled form of datA del6 (pHT21), which lacks DnaA box 4, also displayed full DDAH activity. As the DDAH activity of linear datA del6 DNA was moderately inhibited compared with that of the del5 DNA (33), these data suggest the possibility that DNA supercoiling stimulates DnaA oligomerization, promoting efficient DnaA-ATP hydrolysis. By contrast, the supercoiled forms of the datA del7 and del8 fragments (pHT22 and pHT23), which lack crucial DnaA box 3 and boxes 2 and 7, respectively, were largely inactive (Fig. 6A), consistent with our previous results with linear datA (33).

A similar assay was used to analyze substitution mutants of DnaA boxes and the IBS (Fig. 6B). These substitution mutants are largely inactive for DnaA and IHF binding, respectively (35, 36). Results from the DDAH assay indicated that DnaA boxes 7, 2, and 3 and IBS, but not DnaA boxes 4 or 6, were essential for DDAH even in the supercoiled form (Fig. 6B), supporting the results with the deletion derivatives (Fig. 6A). In particular, the supercoiled form of datA subDnaAbox4 DNA sustained full DDAH activity (Fig. 6B); consistently, box 4 has previously been shown to be dispensable for regulation of initiation in vivo (36).

**Modes of Inter-DnaA Interactions in DDAH with a Supercoiled Form of datA**—To further analyze the mechanism of DDAH promoted by supercoiled datA, DDAH activities were analyzed using a series of DnaA mutants and pKX40 or pACYC177 vector (Fig. 7). The DnaA L290A mutant was slightly active for DDAH with a supercoiled datA form and was substantially inactive with a linear form (Fig. 7, A and B). By contrast, the DnaA R227A mutant fully sustained DDAH activity with a supercoiled datA form and was slightly impaired with a linear form (Fig. 7, A and B). These results are consistent with...
the data shown in Fig. 3, A–D, and with the idea that assembly of ATP-DnaA molecules on datA occurs in a partly different manner to that on oriC and DARS2 (6, 33) and that supercoiling assists in functional inter-DnaA interaction on datA.

Consistent with the results of a previous study with linear datA (33), the DnaA R281A mutant induced very low DDAH activity with both the supercoiled and linear forms of datA (Fig. 7, C and D), suggesting an essential role for Box VII Arg-281-dependent inter-DnaA interactions in DDAH. By contrast, DnaA R285A showed moderately reduced activity with supercoiled datA and very low activity with the linear form (Fig. 7, C and D). These results suggest that the arginine-finger Arg-285-

FIGURE 5. DNA supercoiling-dependent stabilization of DDAH. A, basic structure of supercoiled (SC) and linear (Lin) forms of datA-harboring plasmids. pKX40, a pACYC177-based plasmid harboring the datA WT fragment (991 bp), was used for analysis of DDAH activity. B and C; salt-resistant DDAH with supercoiled pKX40 ([α-32P]ATP-DnaA (1 pmol) was incubated at 30 °C for 10 min in buffer containing IHF (0.25 pmol), the supercoiled (●) or linear forms (○) of pKX40 or pACYC177, and 100, 150, or 200 mM NaCl. The proportions of ADP-DnaA to the total ATP/ADP-DnaA are indicated as percentages (%). D, DDAH activities of supercoiled and linear pKX40 in the presence of 150 mM NaCl. [α-32P]ATP-DnaA (1 pmol) was incubated at 30 °C for 10 min with the indicated amounts of supercoiled (SC; ●, ○) or linear forms (Lin; △, ▲) of pKX40 (+; ●, △) or pACYC177 (−; ○, ▲) in buffer containing 150 mM NaCl and 0.25 pmol IHF. E, time course of DDAH. Experiments similar to those shown in panel D were performed using 25 fmol of supercoiled (SC; ●, ○) or linear forms (Lin; △, ▲) of pKX40 (+; ●, △) or pACYC177 (−; ○, ▲) and 0.25 pmol IHF. F, requirement for IHF in DDAH of supercoiled pKX40. [α-32P]ATP-DnaA was incubated with the indicated amounts of IHF with 25 fmol of supercoiled (SC; ●, ○) or linear forms (Lin; △, ▲) of pKX40 (+; ●, △) or pACYC177 (−; ○, ▲).
dependent inter-DnaA interactions are less important for DDAH with supercoiled datA than with linear datA.

In DnaA-ATP hydrolysis by RIDA and DDAH with the linear datA form, DnaA AAA/H11001 sensor II Arg-334 is essential (15, 33). This residue is proposed to interact with the phosphate moiety of ATP bound to DnaA, promoting hydrolysis of ATP (15, 48, 49). DnaA R334A was substantially inactive in DDAH even with the supercoiled datA form (Fig. 7, E and F), indicating the importance of Arg-334 in DDAH.

DNA Supercoiling Stabilizes DnaA Oligomerization and IHF Binding on datA—To assess whether DNA supercoiling stabilizes DnaA oligomerization on datA, a BglII protection assay was performed (Fig. 8, A–C). The BglII recognition sequence AGATCT was introduced into the native CGATCA sequence in the region for ATP-DnaA oligomerization of pKX40 (33), yielding pKX133 (Fig. 8A). The introduced sequence had only two-base substitutions and resided in the intervening region between IBS and DnaA box 3. In the protection assay, ATP-DnaA was incubated on ice with pKX133 in 100 mM NaCl buffer in the absence of IHF followed by incubation at 30 °C with BglII. The results showed that BglII digestion of pKX133 was inhibited in a DnaA dose-dependent manner regardless of the linear or supercoiled form of datA (Fig. 8B), which indicates that extended DnaA oligomers are formed in the region and protect the DNA from BglII, consistent with the EMSA data (Fig. 3). By contrast, when similar experiments were performed in 150 mM NaCl buffer, protection of the supercoiled form was preserved, but protection of the linear form was considerably decreased (Fig. 8C). These results are consistent with the idea that DNA supercoiling stabilizes the extended DnaA oligomers and also with the changes in DDAH activities shown in Fig. 5, B–E.

A protection analysis was also performed using EcoRV to assess whether DNA supercoiling affects IHF binding to datA (Fig. 8, D–G). IHF binding to double-stranded oligonucleotides containing a datA IBS was not greatly affected by introduction of the EcoRV site within the IBS (Fig. 8E). An EcoRV protection assay with pHT25, a pKX40 derivative bearing the EcoRV site, indicated that IHF protected a supercoiled IBS region from EcoRV more effectively than a linear IBS in 100 or 150 mM NaCl buffer (Fig. 8, F and G). These results suggest that DNA supercoiling stabilizes IHF binding to datA independent of salt concentration.

Modulation of DNA Supercoiling by Novobiocin Decreases datA Function in Vivo—To investigate the in vivo role of DNA supercoiling in stimulation of DDAH, we assessed whether relaxation of negative DNA supercoiling suppresses growth inhibition caused by oversupply of datA. Consistent with previous results (44), introduction of pKX88 (pBR322-datA) severely retarded colony formation of MG1655 (WT) cells grown at 37 °C in NaCl-depleted LB medium (Fig. 9A). By contrast, KMG-5 (∆ihfA) cells bearing pKX88 formed colonies at a level similar to that of those bearing the parental vector pBR322. When similar experiments were performed in the presence of a moderate concentration (15 μg/ml) of novobiocin, an antibiotic that inhibits the function of DNA gyrase and...
relaxes DNA supercoiling (Fig. 9A) (50), MG1655 cells bearing pKX88 had a similar colony-forming ability to those bearing pBR322 or to KMG-5 (H9004 ihfA) cells. The addition of novobiocin suppressed growth inhibition caused by oversupply of datA in vivo. Flow cytometry was performed to examine the replication-initiation timing of cells harboring pKX88 (pBR322-datA) growing at 37 °C in NaCl-depleted LB medium including novobiocin (Fig. 9, B and C). The results showed that whereas MG1655 cells bearing pBR322 contained four or eight origins, those bearing pKX88 contained one, two, or four origins, and some cells experienced asynchronous initiations (Fig. 9B). In cells bearing pKX88, ori/mass was reduced to 0.73 relative to a ratio of 1.0 in cells bearing pBR322. These results are consistent with those shown in Fig. 4A and with previous studies (44) and indicate that oversupply of datA severely inhibits initiation. In KMG-5 (ΔihfA) cells bearing pBR322, regulation of initiation was disrupted, causing untimely initiations, consistent with previous reports (37, 51). Unexpectedly, introduction of pKX88 inhibited initiation in KMG-5 cells (ori/mass = 0.74), probably because of DnaA titration on datA in an IHF-independent manner. The addition of novobiocin (15 μg/ml) slightly inhibited initiation in MG1655 cells bearing pBR322. Notably, in the presence of novobiocin the oriC number per cell in MG1655 cells bearing pKX88 was increased compared with that in the absence of novobiocin, and ori/mass in those cells was only reduced to 0.91 relative to a ratio of 1.0 in MG1655 cells bearing pBR322 (Fig. 9, B and C). By contrast, the addition of novobiocin had little effect on initiation in KMG-5 cells bearing pKX88 (ori/mass = 0.78), supporting the idea that DNA supercoiling might specifically stimulate DDAH (rather than initiation per se).

To assess whether relaxation of DNA supercoiling occurred in cells cultivated in NaCl-depleted LB medium containing
novobiocin, the structures of plasmid topoisomers and the copy number of plasmids were examined. To analyze topoisomers, plasmids were subjected to electrophoresis in agarose gels containing chloroquine, which induces positive DNA supercoiling into plasmids. Consistent with a previous report (52), the addition of 15 μg/ml novobiocin relaxed negative DNA supercoiling of pBR322 (Fig. 9D). Plasmid copy numbers were analyzed by quantitative PCR. As a control for chromosome number, we quantified the signal intensity of the ter locus, a termination site of DNA replication (53). Quantification of relative plasmid signal by amplifying the tet gene of pBR322 revealed that the addition of novobiocin had little effect on plasmid copy numbers (Fig. 9E). Previous transcriptome analysis revealed that relaxation of DNA supercoiling by drug addition had little effect on the expression of IHF (54), implying that suppression of growth inhibition by oversupply of datA was caused by reduction of DNA supercoiling, resulting in inhibition of plasmid datA-derived DDAH.

**Discussion**

This study revealed the functional structure of ATP-DnaA oligomers associated with datA and IHF and demonstrated the fundamental role of negative DNA supercoiling in the regulation of DDAH. Our results identified that datA DnaA box 7 as well as DnaA AID-2 Leu-290 were required for DDAH (Fig. 2B, 3A, 4, 6B, 7, A and B, and summarized in Fig. 10A). Binding of ATP-DnaA on datA DnaA box 7 is probably assisted by head-to-tail inter-DnaA interaction with DnaA bound to the neighboring DnaA box 2, which has the same orientation as box 7 (Fig. 2A) (7). Consistently, the DnaA arginine-finger Arg-285 and Box VII Arg-281, which are required for formation and stabilization, respectively, of ATP-DnaA-specific oligomers on...
oriC (13, 16, 33) were also essential for DDAH promoted by linear datA (Figs. 3A and 10A). These results are supported also by the 3-bp interval between boxes 2 and 7 in datA, which is preferable in the interaction between DnaA molecules bound to adjacent boxes (Fig. 2A) (8). Furthermore, formation of ATP-DnaA oligomers on datA depends on the arginine-finger motif but not on AID-2 (Fig. 3, B–G). Similar mechanisms might be also involved in inter-DnaA interaction for DnaA bound to the right-half oriC subregion (Fig. 10A) (6). In the left-half oriC subregion, a functional ATP-DnaA oligomer is sustained by the arginine-finger- and AID-1 and -2-dependent tight cooperative binding between two DnaA boxes with a 2-bp interval, which is required for DUE unwinding (Fig. 10A) (6). These observations suggest the possibility that ATP-DnaA oligomers formed on DnaA boxes 2 and 7 with a 3-bp interval might engage a relatively loose inter-DnaA contact, triggering interaction between DnaA sensor II Arg-334 and ATP and activating DnaA-ATP hydrolysis specifically on datA. Our observation that oligomers of ADP-bound DnaA L290A mutant were somehow stabilized on datA suggests a possibility that DnaA AID-2 Leu-290 participates in a process of dissociation of ADP-DnaA from datA in DDAH reactions (Fig. 3, B–D). Different requirements for specific

FIGURE 9. Modulation of DNA supercoiling decreased datA function in vivo. A, suppression of datA-dependent growth inhibition by novobiocin (Novo). Strains MG1655 (WT) and KMG-5 (ΔihfA) were transformed with pBR322 (Vector) or pKX88 (datA) and incubated at 37 °C for 12 h on NaCl-depleted LB agar plates containing 50 μg/ml ampicillin and 0 or 15 μg/ml novobiocin. Colony formation with each set of cells is shown. B and C, suppression of datA-dependent repression of replication initiation by novobiocin. B, MG1655 or KMG-5 cells harboring pBR322 or pKX88 were cultivated at 37 °C in NaCl-depleted LB medium containing 50 μg/ml ampicillin and 0 or 15 μg/ml novobiocin and analyzed by flow cytometry. C, mean cell mass and the ori/mass ratio (relative to MG1655 cells with pBR322) are shown in the histograms. D, relaxation of plasmid by addition of novobiocin. 50 ng of pBR322 topoisomers collected from cells cultivated in NaCl-depleted LB containing 50 μg/ml ampicillin and 0 or 15 μg/ml novobiocin were separated by 0.65% (w/v) agarose-gel electrophoresis and stained by ethidium bromide. The gel image is shown in black and white inverted mode. E, relative copy number of plasmid DNA. The cultures, prepared in the experiments shown in Fig. 9D, were boiled, and supernatants were collected. The ratio of pBR322 tet gene per chromosomal terC locus in the supernatants was determined using quantitative PCR. Duplicated samples were used in this experiment.
**Mechanism of DnaA-ATP Hydrolysis by Supercoiled datA**

**A**

| DNA    | Arg-finger (Arg285) | Box VII (Arg281) | Sensor II (Arg334) | AID-1 (Arg227) | AID-2 (Leu290) |
|--------|---------------------|------------------|--------------------|----------------|----------------|
| oriC   | +                   | +                | −                  | +              | +              |
| datA   | +                   | +                | +                  | −              | +              |
| DARS1  | −                   | −                | −                  | n.d.           | n.d.           |
| DARS2  | ±                   | −                | ±                  | ±              | +              |

**B**

1. **ATP-DnaA oligomer formation**
   - Inter-ATP-DnaA interaction by Arg285
   - Stabilization by Arg281, Leu290, and DNA supercoiling

2. **ATPase activation**
   - Conformational change of DnaA by Arg281
   - ATP hydrolysis by Arg334

3. **ADP-DnaA dissociation**
   - Inhibition of inter-DnaA interaction by Leu290

**FIGURE 10. Molecular mechanism for activating DDAH.** A, summary of the functional requirements of DnaA motifs for oriC, datA, DARS1, and DARS2. For functional DnaA oligomerization in oriC, roles for AID motifs are different between the left-half (left) and right-half (right) subregions. +, essential; ±, stimulatory; −, nonessential; n.d., not determined. See the Introduction and “Results” for references. B, mechanistic models of the datA-IHF-DnaA complex for activating DDAH. ATP-DnaA oligomers are formed on the datA minimal region containing DnaA boxes 7, 2, and 3. For simplicity, only DnaA domain III (blue or red polygons), the arginine-finger (pink triangle), and domain IV (orange square) are shown. DnaA molecules forming extended complexes are omitted. Negative DNA supercoiling assisted DnaA oligomerization and IHF binding on datA. Sharp DNA bending by IHF might stimulate the intermolecular contact of oligomers formed around DnaA boxes 2 and 3. Cooperative ATP-DnaA binding on datA-DnaA box 7 might induce conformational change of the DnaA nucleotide pocket to activate DnaA-ATP hydrolysis (Model 1). Alternatively, in the oligomer formed around DnaA box 2, DnaA-ATP hydrolysis might be activated by interaction with the oligomer formed around DnaA box 3 (Model 2). In both models DnaA AID-2 Leu-290 residue is important for ATP-DnaA-specific interactions, especially for destabilizing the resultant ADP-DnaA protomers to enable further binding and hydrolysis of ATP-DnaA. In the schematic, DnaA domain III colored with blue, but not red, promotes hydrolysis of bound ATP.

DnaA motifs in functions at oriC (DUE unwinding), datA, and DARSs are consistent with differentiation in structure–function relationship of DnaA complexes on those sites (Fig. 10A).

Our results showed that negative DNA supercoiling increases the efficiency of the DDAH reaction and stabilizes it against conditions of 150–200 mM NaCl (Fig. 5, B and C). Mechanistic analysis revealed that DNA supercoiling assisted DnaA oligomerization as well as IHF binding on datA in 150–200 mM NaCl, which is consistent with the results of previous studies of IHF binding to ilvPG promoters and IS1 (55, 56). The necessary elements within datA and DnaA for DDAH with supercoiled datA were almost the same as for DDAH with linear datA, except that with supercoiled datA the DnaA arginine-finger was stimulatory but not essential (Figs. 6 and 7). DNA supercoiling stabilized oligomerization of ATP-DnaA (Fig. 8, A–C). These results suggest that DnaA AID-2 Leu-290 and Box VII Arg-281 are more important for ATP-DnaA-specific oligomerization and inter-DnaA interactions formed in supercoiled datA than the interaction between the arginine-finger and ATP.

On the basis of our results, we propose a mechanistic model of DDAH (Fig. 10B). In the first step of this model, ATP-DnaA cooperatively binds on the left-hand subregion of datA that includes DnaA boxes 2 and 7, and on the right-hand subregion that includes DnaA box 3. The two subregion oligomers might interact with each other in a head-to-tail manner, with the aid of DNA bending by IHF. The DnaA arginine-finger is also essential for the cooperative ATP-DnaA oligomerization on linear datA (Fig. 3, E–G), and DnaA Box VII Arg-281 supports tight inter-DnaA interactions in complexes involving datA and IHF. The arginine-finger side of DnaA orients toward the 5′ end of the DnaA-box consensus sequence, and the ATP-bound side of DnaA has the opposite orientation (7). Negative DNA supercoiling stabilizes ATP-DnaA oligomers against dissociation caused by high salt concentrations and promotes IHF binding (Fig. 8), probably via stabilization of the DNA loop. DnaA would also bind to DnaA boxes 4 and 6, although their roles in DDAH are minimal. Then, in this complex, DnaA-ATP hydrolysis by DnaA Sensor II Arg-334 is activated by the two possible mechanisms (Fig. 10B); in Model 1, loose interaction of ATP-DnaA protomers bound on DnaA boxes 7 and 2 might induce a conformational change of the nucleotide pocket after interaction with Box 3-bound ATP-DnaA to activate DnaA-ATP hydrolysis of box 7-bound DnaA. Sharp DNA bending by IHF might stimulate the interaction between ATP-DnaA protomers bound to DnaA boxes 2 and 3. In another mechanism (Model 2), DNA bending by IHF induces functional interaction between the protomers bound to DnaA boxes 2 and 3, thereby activating DnaA-ATP hydrolysis of ATP-DnaA oligomer on the
left-hand subregion (Fig. 10B). In either of the cases, DnaA Box VII Arg-281 might modulate the structure of ATP-DnaA oligomers on the complexes. Finally, the resultant ADP-DnaA might be destabilized and dissociated from datA via the action of DnaA AID-2 Leu-290. The present data that we have cannot allow for distinction of these possible mechanisms. Those could be further elucidated using a chimeric DnaA molecule that can bind to a different DnaA box sequence (7); we consider that this might be an important future study.

Our observations of DNA supercoiling-dependent stimulation of IHF binding (Fig. 8, D-G) are consistent with the possibility that cell cycle-coordinated IHF binding to datA and DARS2 is regulated by changes in DNA supercoiling. Our previous finding that the addition of rifampicin inhibits timely dissociation of IHF from datA (33) is consistent with the current observations; rifampicin is an antibiotic drug that dissociates running RNA polymerases from the chromosome and reduces DNA supercoiling (57). Studies have also demonstrated that transcription of yjeV (ORF2.1) and queG (ORF43) genes can pass through the datA essential region (43), and thus alteration of datA DNA supercoiling by transcription is a potential mechanism for regulation of timely IHF binding.

Unlike DnaA from E. coli and Bacillus subtilis, DnaA from H. pylori efficiently assembles on the supercoiled form of the cognate oriC rather than the relaxed form (27, 58, 59). Similarly, in Drosophila melanogaster, supercoiled DNA has 30-fold higher affinity than linear DNA for the Origin recognition complex (60). As such, supercoiling of DNA might be of general importance for regulation of initiator-DNA binding. In many bacterial species including B. subtilis and Streptomyces coelicolor, DnaA box cluster-dependent repression systems for replication initiation are suggested to have roles similar to those of DDah (61, 62), and supercoiling-mediated regulation could be important for DnaA assembly on the DnaA-box clusters underlying these systems.

Like IHF, HMG1 protein, which belongs to the high mobility group family, a eukaryotic counterpart of NAPs, as well as MC1 protein, which is an archaeabacterial histone-like protein, have higher affinity for supercoiled DNA than for relaxed DNA (63, 64). DNA supercoiling-dependent regulation of the binding of chromosome-structuring proteins could be conserved widely in prokaryotic and eukaryotic species.

**Experimental Procedures**

Proteins, DNA, and E. coli Strains—DnaA proteins and IHF were overexpressed and purified from E. coli cells, as previously described (9, 33).

The following plasmids were described previously (33): pKX62 (datAΔIBS), pKX48 (subDnaAbox1), pKX41 (subDnaAbox2), pKX43 (subDnaAbox3), pKX47 (subDnaAbox4), pKX49 (subDnaAbox5), and pKX42 (subIBS). A WT data fragment (991 bp) was cloned into pACYC177 plasmid to yield pKX40 as described (33). pKX40 derivatives containing datA sequence substitutions were constructed by inside-out PCR from pKX40 with the following primers: subL1-U and subL1-L for pKX120 (subDnaAbox8), tkn126datA-W and tkn128datA-R for pHT28 (subDnaAbox7), 40BglII-U and 40BglII-L for pKX133 (IBS-DnaA box 3 linker), and tkn124IBS EcoRV-F and tkn125IBS EcoRV-R for pHT25 (IBS_{ECORV}). The data fragment (991 bp) and its derivatives, which were used in Fig. 2B, were amplified from pKX40, pKX48 (subDnaAbox1), pKX120 (subDnaAbox6), pHT28 (subDnaAbox7), and pKX41 (subDnaAbox2) with primers datA-1 and datA-2 (Table 1). The data minimal region for DDah (del5) and its derivatives, which were used in EMSA, were amplified from pKX40, pKX62 (ΔIBS), and pHT25 (IBS_{ECORV}) with primers datA-3 and datA-6 and from pKX40 with primers datA-3 and datA6subDnaAbox7 for subDnaAbox7 (Table 1). Derivatives of datA 672 fragments that were used for EMSA (Fig. 2F) are shown in Table 1, and these oligonucleotides were annealed at room temperature overnight (672WT-U and 672WT-L for WT sequence, sub672-U and sub672-L for subDnaAbox6, 6sub72-U and 6sub72-L for subDnaAbox7, and 6sub72-U and 6sub72-L for subDnaAbox2). For construction of chromosomal datA mutants, pTH5 plasmid was digested with EcoRI, and the resultant frt-kan gene (the kanamycin resistance gene flanked by FRT site-specific recombination sequences) was cloned into the EcoRI sites of an inside-out PCR fragment amplified from pKX40 with primers datAEcoRI-U and datAEcoRI-L, resulting in pKX40kan. Truncated datA derivatives were amplified by PCR from pKX40 or pKX62 (ΔIBS) with the following primers: tkn121datA-1 and tkn116datA-3 for del2, tkn122datA-2 and tkn117datA-6 for del3, tkn116datA-3 and tkn117datA-6 for del5, del5IBS, tkn117datA-6, and tkn120datA-10 for del6, tkn117datA-6 and tkn119datA-9 for del7, and tkn116datA-3 and tkn118datA-7 for del8 (see Table 1 for each sequence). These fragments were inserted into the BamHI and HindIII sites of pACYC177 to yield pHT17 (del2), pHT18 (del3), pHT20 (del5), pHT24 (del5IBS), pH71 (del6), pH72 (del7), and pHT23 (del8). A datA WT fragment (991 bp) was also cloned into the NruI site of pBR322 plasmid to yield pKX88.

All E. coli strains used in this study are listed in Table 2. ΔdatA::kan mutation, derived from RSD448 cells, was introduced into MG1655 cells using P1 transduction, yielding strain MIT128. Chromosomal datA mutants with DnaA-box substitutions were introduced into MG1655 cells harboring pKD46 (65); DNA fragments carrying the datA mutation and frt-kan gene were amplified from pKX40kan with the following primers: ChdatAKn and datA-2 for data WT-frt-kan, ChdatAKn and ChdatAsub6 for subDnaAbox6-frt-kan, ChdatAKn and ChdatAsub7 for subDnaAbox7-frt-kan, and ChdatAKn and ChdatAsub2 for subDnaAbox2-frt-kan. The resultant mutants (data WT-frt-kan, subDnaAbox6-frt-kan, subDnaAbox7-frt-kan, and subDnaAbox2-frt-kan) were introduced into MG1655 cells by P1 transduction. kan gene sequences were removed by introduction of pCP20 plasmid (65), yielding strains SR01 (WT-frt), SR10 (subDnaAbox6-frt), SR30 (subDnaAbox7-frt), and SR06 (subDnaAbox2-frt), respectively. Medium was supplemented with 0.2% glucose, 0.2% casamino acids, and 5 μg/ml thiamine; 100 μg/ml ampicillin was included, if required.

**In Vitro Reconstitution of DnaA-ATP Hydrolysis—**This was performed basically as we previously described (33). For DDah reconstitution using PCR fragments (Figs. 2B and 3A), [α-32P]ATP-DnaA was prepared by incubation of nucleotide-free DnaA (apo-DnaA) on ice for 15 min in buffer containing 3 μM [α-32P]ATP. [α-32P]ATP-DnaA (1 pmol) was then incu-
Mechanism of DnaA-ATP Hydrolysis by Supercoiled datA

**TABLE 1**

*List of oligonucleotides*

| Primers | Sequences |
|---------|-----------|
| subL1-U | GCAACAAACACCCAGCATATACCC |
| subL1-L | ACCATGCTTCCCTTCTGGAAACAGATTCCAGCTACCG |
| tknl26datAow-F | CTCAAGGCTTTGTAACTTCAAGATAATACTTCAAGCAGAA |
| tknl28datAow-B | GCTTGGTCAATTTCAGTAAAAACTTTGTTAGGAGG |
| 408bhL-U | GAGAATCTTCCAGAGTTTTTTTTTTTGTTCAGAAGT |
| 408bhL-L | GAGAATCTTCCAGAGTTTTTTTTTTTGTTCAGAAGT |
| tknl21BSecoRV-F | GTGAATAGTATTTTTGCCTATTGATATCTAAGTTAAAAATTAAGATTC |
| tknl21BSecoRV-R | CTCAGGCTTTGTAACTTCAAGATAATACTTCAAGCAGAA |
| datA-1 | CCCGTCTCCAATTTCTTCTCA |
| datA-2 | TTTTCTGAGCAGGTTTATCAG |
| datA-3 | GATATCTTCCAGAGTTTTTTTTTTTGTTCAGAAGT |
| datA-6 | GATATCTTCCAGAGTTTTTTTTTTTGTTCAGAAGT |
| datA6subDnaAbox7 | GTTGGTCTTCAACACTCAGAACAGT |
| datAEcoRI-U | CGGAATCTGAAATTTCTGGAGGAGCTTAGG |
| datAEcoRI-L | GCAATTTGTCCTCCATAAAATATCAGAGGACG |
| 672WT-U | GGACGGTGTGATTTTTTTTTTTTGTTCAGAAGT |
| 672WT-L | GAGGCTGTTGATTTTTTTTTTTTGTTCAGAAGT |
| sub672-U | TTTTCATGTTTTCTTAAAGAATTTCTTTCAGAAGT |
| sub672-L | GAGGCTGTTGATTTTTTTTTTTTGTTCAGAAGT |
| 6sub72-U | GAGGCTGTTGATTTTTTTTTTTTGTTCAGAAGT |
| 6sub72-L | GAGGCTGTTGATTTTTTTTTTTTGTTCAGAAGT |
| 67ub2-U | TTTTCTGAGCAGGTTTATCAG |
| 67ub2L | GAGGAGGTTTATCAGTTTCAATAGGAGTTATCCACAGCCTC |
| tknl6edatA-3 | CGGAATCTGAAATTTCTGGAGGAGCTTAGG |
| tknl7datA-6 | CCCGAAGTTCCTTCTGCTGAAAACCTTCTTAAAGACAG |
| tknl1datA-9 | CCCGAATCTGAAATTTCTGGAGGAGCTTAGG |
| tknl20datA-10 | GGACGGTGTGATTTTTTTTTTTTGTTCAGAAGT |
| tknl21datA-1 | CCCGAATCTGAAATTTCTGGAGGAGCTTAGG |
| tknl22datA-2 | CGGAATCTGAAATTTCTGGAGGAGCTTAGG |
| ChdatAkn | GCACTATGATCCAGGCTTTGTACGTCAGTCAAGTGAGG |
| ChdatAsub6 | GCACACCAGCAGGCTTTGTACGTCAGTCAAGTGAGG |
| ChdatAsub7 | GAACTATGATCCAGGCTTTGTACGTCAGTCAAGTGAGG |
| ChdatAsub2 | GAACTATGATCCAGGCTTTGTACGTCAGTCAAGTGAGG |
| ER_2 | TACCTGGTCCAATGAGCC |
| SMT-7 | GACATCCGCGATATG |
| RTPR322 | CAAGGCTTCACAACCTTACTG |

**TABLE 2**

*List of E. coli strains*

| Strain | Relevant genotype | Source |
|--------|------------------|--------|
| MG1655 | Wild type | Laboratory stock |
| SR01 | Wild-type datA-frt | This work |
| MIT128 | datA-kan | This work |
| SR10 | datA subDnaAbox6-frt | This work |
| SR30 | datA subDnaAbox7-frt | This work |
| SR06 | datA subDnaAbox2-frt | This work |
| KMG-5 | ∆ihfA-frt-kan (32) | |

Bated with various amounts of DNA and/or proteins as indicated in 15 μl of buffer H (20 mM Tris–HCl (pH 7.5), 10 mM magnesium acetate, 2 mM ATP, 8 mM diethiothreitol, and 100 μg/ml bovine serum albumin) including 100 mM NaCl instead of 100 mM potassium glutamate (33). DnaA-bound nucleotides were recovered on nitrocellulose filters and extracted by 1 M HCOOH and analyzed by thin-layer chromatography as described previously (33).

For DDAH reconstitution with plasmid DNA (Figs. 5–7), [α-32P]ATP-DnaA (1 pmol) was incubated with various amounts of DNA and/or proteins in 15 μl of buffer H containing 150 mM NaCl. To construct linear forms of pKX40 derivatives and pACYC177, as a negative control, these plasmids were digested at a unique site with a restriction enzyme PstI, which specifically cleaves DNA at CTGCA sequences.

*EMSA—*This was performed basically as we previously described (33). For the experiments shown in Fig. 2, C–F, and Fig. 3, B–F, various amounts of ATP-DnaA or ADP-DnaA (WT or mutated) were incubated at 15 °C for 5 min with the datA del5 DNA fragment (0.15 pmol) in the presence of 150 mM NaCl, 2 mM ATP or ADP, and 150 ng of phage λDNA (as a nonspecific competitor) with or without 6 pmol of IHF. DNA and DNA-protein complexes were analyzed by electrophoresis on 2% (w/v) agarose gels for 120 min in Tris borate buffer, with staining with ethidium bromide, as described previously (33).

For the experiments shown in Fig. 2G, the indicated amounts of ATP-DnaA were incubated with datA 672 WT fragment (containing DnaA boxes 6, 7, and 2) or its derivatives at 30 °C for 5 min in 10 μl of buffer H including 150 mM NaCl and the indicated amounts of DNA followed by analysis with 7% polyacrylamide-gel electrophoresis (PAGE) at 90 V for 60 min in Tris borate buffer, with staining by Gel-Star.

For the experiments shown in Fig. 8E, the indicated amounts of IHF were incubated with IBS DNA at 30 °C for 5 min in 10 μl of buffer H containing 150 mM NaCl and the indicated amounts of DNA followed by analysis with 5% PAGE at 90 V for 60 min in Tris borate buffer.

*Flow Cytometry Analysis—*This was performed basically as we previously described (33). Cells were cultivated at 37 °C in LB medium, supplemented M9 medium, or NaCl-depleted LB medium including 100 μg/ml ampicillin and 0 or 15 μg/ml novobiocin until the A600 (absorbance at 600 nm) reached 0.1–0.2 followed by further incubation at 37 °C for 4 h in the presence of 300 μg/ml rifampicin and 10 μg/ml cephalaxin for run-out replication. The resultant cells were fixed, stained with...
SYTOX Green (Life Technologies), and analyzed with FACS Calibur (BD Biosciences), as described previously (37, 38).

BgIII Analysis of DnaA Binding—The linear or supercoiled forms of plasmid pKX133 (10 fmol), which contains a dataA sequence engineered with a BgIII-recognition site between the IBS and DnaA box 3 (dataA<sub>rgbI</sub>), were incubated on ice for 10 min with various amounts of DnaA in 10 μl of buffer H including 100 or 150 mM NaCl and 1 mM ATP or ADP followed by further incubation at 30 °C for 45 min with BgIII (New England BioLabs, 2.5 units). The reaction was terminated in SDS loading buffer, and DNA fragments were analyzed by 1% (w/v) agarose-gel electrophoresis.

EcoRV Analysis of IHF Binding—The linear or supercoiled forms of pHT25 (10 fmol), which contain a dataA sequence engineered with an EcoRV recognition site flanking the IBS (dataA<sub>EcoRV</sub>), were incubated at 30 °C for 5 min with various amounts of IHF in 10 μl of buffer H including 100 mM or 150 mM NaCl followed by incubation at 30 °C for 10 min with EcoRV (TOYOBO, 0.5 units). The resultant DNA fragments were analyzed as described above.

Chloroquine Gel Electrophoresis and Quantitative PCR—MG1655 cells containing pBR322 were cultivated at 37 °C in 100 ml of NaCl-depleted LB medium including 100 μg/ml ampicillin and 0 or 15 μg/ml novobiocin until A<sub>660</sub> reached 0.2; then pBR322 was isolated. To assess the effects of novobiocin on DNA supercoiling, plasmid topoisomers were separated by 2.5 μg/ml chloroquine, 0.65% (w/v) agarose-gel electrophoresis with Tris borate-EDTA buffer. Approximately 50 ng of plasmid DNA was loaded onto the gel using loading buffer (5% glycerol and 4 μg/ml bromphenol blue). Topoisomers were separated by electrophoresis at 20 V (1.5 V/cm) for 15 h and stained by ethidium bromide.

For analysis of the plasmid copy number, cell suspensions were boiled, and the supernatants were collected by centrifugation. The levels of chromosomal ter loci and pBR322 tet gene copies in the supernatants were quantified by real-time qPCR using SYBR Premix Ex TaqII (Perfect Real Time) (Takara Bio) and the following primers: TER_2 and SUUterRev1 for ter and SMT-7 and RTpBR322 for tet (see Table 1 for each sequence).

Author Contributions—K. K. and T. K. designed the study. K. K. and H. T. performed most of the experiments. R. S. performed part of the in vitro and in vivo experiments. All authors analyzed and discussed the data. K. K. and T. K. wrote the manuscript. All authors reviewed the manuscript and approved the final version.

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