Novel mechanisms of specific DnaA-binding sequences in the Escherichia coli chromosome for cell cycle-coordinated regulation of the replication initiation

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Novel mechanisms of specific DnaA-binding sequences in the *Escherichia coli* chromosome for cell cycle-coordinated regulation of the replication initiation

（大腸菌染色体における複製開始因子 DnaA の特異的結合部位による、細胞周期に応じた新規複製開始制御メカニズム）

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【Introduction】

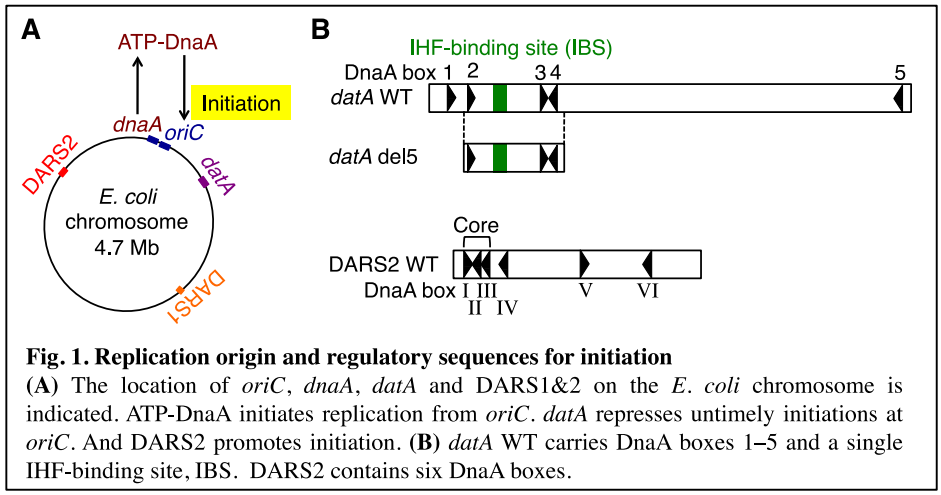

Fig. 1. Replication origin and regulatory sequences for initiation
(A) The location of *oriC*, *dnaA*, *datA* and DARS1&2 on the *E. coli* chromosome is indicated. ATP-DnaA initiates replication from *oriC*. *datA* represses untimely initiations at *oriC*. And DARS2 promotes initiation. **(B)** *datA* WT carries DnaA boxes 1–5 and a single IHF-binding site, IBS. DARS2 contains six DnaA boxes.

In *E. coli*, the initiation of chromosomal DNA replication is regulated to occur only once per cell cycle, in a timely manner. The timely and cell cycle-coordinated initiation is sustained by multiple regulatory systems for DnaA and *oriC*, the replication origin (Fig. 1*A*). DnaA is a member of AAA+ protein family which contains specific ATP/ADP-binding and ATPase motifs. DnaA activity for initiation is regulated by its bound nucleotide. ATP-bound DnaA (ATP-DnaA) forms specific multimers on *oriC*, resulting in duplex DNA unwinding and replication initiation. In contrast, ADP-DnaA doesn't initiate replication, although it can form multimers on *oriC*. Key residues for formation of the ATP-DnaA specific multimer include the AAA+ Arg-finger Arg285, which plays a key role in the ATP-dependent interaction between DnaA protomers that activates DnaA complexes during initiation, and AAA+ Box VII Arg281, which stabilizes inter-DnaA interactions. Along with ATP-DnaA, IHF, a nucleoid-associated protein (NAP), binds to *oriC* at a specific IHF-binding site (IBS) and stimulates initiation.

The cellular ATP-DnaA level is oscillated through cell cycle and temporarily increases only before initiation. Immediately after ATP-DnaA induces initiation, DnaA-bound ATP (DnaA-ATP) is hydrolyzed by the complexes of Hda and the DNA-loaded clamp subunit of DNA polymerase III holoenzyme, yielding ADP-DnaA. This feedback system is termed RIDA (regulatory inactivation of DnaA) and is required for repressing extra initiations. In addition, specific chromosomal locus, termed *datA,* which carries five DnaA-binding sites (DnaA boxes) and a single IBS, is crucial for repressing untimely initiation events (Fig. 1*B*). *datA*-deleted cells perform untimely initiations at a level that does not inhibit cell growth. Originally, *datA* was speculated to bind ~370 DnaA molecules and was thought to reduce the number of DnaA molecules accessible to *oriC*, thereby inhibiting untimely initiations. However, the speculation was deduced from indirect measurements not yet supported by direct evidence.

In addition, our recent study revealed that other specific chromosomal sequences termed DARS1 (DnaA reactivating sequence 1) and DARS2 convert ADP-DnaA to ATP-DnaA by nucleotide exchange (Fig. 1*A*). DARS1 and DARS2 are crucial for increase of cellular ATP-DnaA level, and DARS2 affects the level more largely than DARS1. DARS1 and DARS2 share a highly conserved core region which consists of oppositely-orientated three DnaA boxes and is essential for DnaA-ADP release (Fig. 1*B*). We previously identified a certain protein as DARS2 stimulator (Kasho, master's thesis). However, the cell cycle-coordinated regulation of DARS2 remains unclear.

In this study, I report that *datA* efficiently stimulated DnaA-ATP hydrolysis in a RIDA-independent manner. I termed this novel mechanism DDAH (*datA*-dependent DnaA-ATP hydrolysis). IHF was specifically required for DDAH. I reconstituted DDAH with purified proteins and analyzed molecular mechanism using DnaA mutants and mutant *datA* fragments. Also, I analyzed *in vivo* DnaA nucleotide forms and found that defect of DDAH increased ATP-DnaA level. Cell cycle analysis revealed that IHF binds to *datA* just after initiation. In addition, I revealed the cell

cycle-coordinated regulation of DARS2 stimulation by a certain protein. Based on these findings, I propose a model for cell cycle-coordinated regulation of initiation by *datA* and DARS2.

【Experimental procedures】

In vitro *reconstitution of DnaA-ATP hydrolysis by datA* [α-^{32}P]ATP-DnaA (1 pmol) was incubated at 30°C with *datA* DNA and/or IHF. DnaA-bound nucleotides were recovered on nitrocellulose filters and analyzed by TLC (thin layer chromatography).

In vivo *DnaA-bound nucleotide analysis* Cells were grown at 37°C in medium containing [^{32}P]orthophosphate. DnaA was immunoprecipitated from cell lysates by anti-DnaA antiserum, and recovered DnaA-bound nucleotides were analyzed by TLC.

Electrophoretic mobility shift assay (EMSA) ATP- or ADP-DnaA was incubated at 15°C for 5 min with the *datA* del5 DNA fragment (0.15 pmol), a minimal *datA* region for DDAH (see below), in the presence of 6 pmol of IHF. DNA and DNA-protein complexes were analyzed by 2% agarose gel electrophoresis, followed by staining with GelStar.

Chromatin immunoprecipitation (ChIP) with synchronized cells *dnaC2* (Ts) cells cultivated at 30°C, a permissive temperature, in supplemented M9 medium were transferred to 38°C, a restrictive temperature, and incubated for 90 min in order to arrest cell cycle at initiation period. The cells were then transferred to 30°C, incubated for 5 min to initiate replication, and further incubated at 38°C to inhibit the next round of initiation. IHF-bound DNA was immunoprecipitated from each cell lysates using antiserum and the recovered levels for *oriC*, *datA*, and *ylcC* (as a negative control for IHF-ChIP) were determined using real-time qPCR.

【Results】

1-1. Cell cycle-coordinated function of *datA*-IHF complexes for DnaA-ATP hydrolysis

***datA*-IHF complexes promote DnaA-ATP hydrolysis**

To assess whether *datA*-IHF complexes stimulate DnaA-ATP hydrolysis, I incubated ATP-DnaA with a *datA* WT (wild-type) DNA and purified IHF. Interestingly, most of the input ATP-DnaA molecules (1 pmol) were converted into ADP-DnaA by 0.05 pmol of *datA* after only 10 min (Fig. 2). This activity was specific to *datA*-IHF complexes, because an *oriC* fragment or HU, another NAP, was inactive in this reaction. 1.6 molecules of ATP-DnaA were hydrolyzed per min per *datA* molecule, suggesting that *datA* catalytically promotes DnaA-ATP hydrolysis.

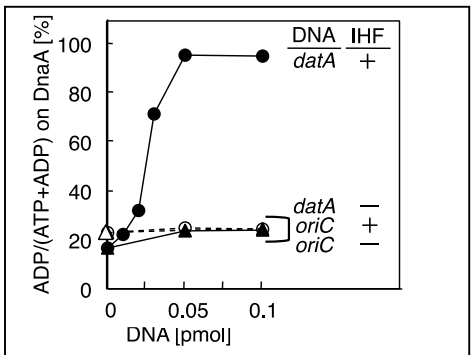

Fig. 2. *datA*-dependent DnaA-ATP hydrolysis
[α-^{32}P]ATP-DnaA (1 pmol) was incubated with indicated amount of *datA* WT DNA and purified IHF (0.2 pmol) for 10 min at 30°C . Most of the input ATP-DnaA molecules were converted into ADP-DnaA in the presence of *datA* and IHF.

***datA* and IHF function for reducing ATP-DnaA levels in cells**

I investigated whether the *datA*-IHF complexes repress ATP-DnaA levels *in vivo*. Previous analyses indicated that RIDA inactivation via the deletion of the *hda* gene or inactivation of the clamp increases the cellular ATP-DnaA level to 70–80%, but never to 90–100%. Here, I examined whether DDAH affects cellular ATP-DnaA levels by introducing Δ*datA* or Δ*ihf* into RIDA-defective cells. For this analysis, Δ*oriC* Δ*rnhA* cells were used as a standard. Deletion of *oriC* represses lethal overinitiation caused by RIDA inactivation, whereas deletion of the *rnhA* gene encoding RNaseH I activates alternative origins, allowing the growth of cells lacking *oriC*. In the standard cells, ATP-DnaA levels were low (27%). Deletion of *hda* increased the ATP-DnaA level to 72%. Importantly, deletion of *datA* or *ihf* further increased the ATP-DnaA levels to 88–97%, indicating that DDAH is also functions in cells.

Coordination between initiation from *oriC* and IHF-binding to *datA*

To assess whether IHF-binding to *datA* is regulated through cell cycle, I performed ChIP assay using synchronized cells. Consistent with previous study, IHF bound to *oriC* at an initiation period and dissociated immediately after initiation. Different from *oriC*, IHF dissociated from *datA* in the pre-initiation stage, bound to this site immediately after initiation, and dissociated again 20–30 min after initiation, suggesting that DDAH timely inactivates

DnaA after initiation.

1-2. Mechanistic analyses for DDAH

Oligomerization of ATP-DnaA on the minimum *datA* for DDAH

Mutant analysis of *datA* using a reconstituted DDAH assay revealed that full DDAH activity was sustained even by *datA* del5 DNA containing DnaA boxes 2–4 and IBS, described in Fig. 1*B*. To determine whether DnaA oligomers are formed in this fragment (*datA* del5), I performed EMSA. ATP-DnaA formed complexes carrying at least six DnaA molecules per *datA* del5. ADP-DnaA formed complexes on *datA* less efficiently than ATP-DnaA; it predominantly formed complexes on *datA* carrying only 1–3 DnaA molecules.

DDAH requires DnaA AAA+ motifs for ATPase and for inter-DnaA interactions

To show how the *datA*-IHF complexes stimulate DnaA-ATP hydrolysis, I analyzed the DDAH activity of DnaA mutants *in vitro*. In RIDA, DnaA AAA+ Sensor II Arg334 is crucial for DnaA-ATP hydrolysis. This residue is proposed to participate directly in catalysis of ATP hydrolysis. Significantly, DnaA R334A was completely resistent to DDAH, suggesting that this residue plays a crucial role in DDAH, as it does in RIDA. Arg-finger Arg285 and Box VII Arg281 play important roles in inter-DnaA interactions within the *oriC* initiation complex. Interestingly, DDAH also required Arg281 and Arg285. In contrast, DnaA Asn44 and Lys54, which are required for DnaA-Hda interaction in RIDA, were dispensable for DDAH. These results support the idea that specific inter-DnaA interactions are crucial for DDAH.

2. Cell cycle-coordinated stimulation of DARS2

To assess whether stimulation of DARS2 by a certain protein is regulated through cell cycle, I performed ChIP assay using synchronized cells. As a result, a certain protein dissociated from DARS2 in the pre-initiation stage, bound just after initiation, and dissociated again after initiation, indicating that stimulation of DARS2 is also regulated in the replication cycle-coordinated manner.

【Discussion】

Significance of DDAH for repressing untimely initiations

In this study, I identified a novel DnaA inactivation mechanism by the *datA*-IHF complexes, termed DDAH (Fig. 3). Deletion of *datA* or *ihf* further increased ATP-DnaA levels in RIDA-defective cells, supporting the idea that DDAH assists RIDA in regulating ATP-DnaA levels and restricts initiation of replication in a manner independent of RIDA. The chromosomal location of *datA* is relatively close to those of *oriC* and *dnaA* (Fig. 1*A*), which might enhance interaction of *datA* with ATP-DnaA molecules expressed from the *dnaA* locus and localized in the *oriC*-proximal space. DDAH might be a mechanism common to many bacterial species including *B. subtilis* and *S. coelicolor* whose genomes contain DnaA box clusters for repressing untimely initiations.

Cell cycle-coordinated regulation of *oriC*, *datA*, and DARS2

Moreover, I found that IHF binds to *datA* immediately after initiation and that it dissociates from *datA* 20–30 min after initiation. This is consistent with observations that ATP-DnaA levels increase during the pre-initiation stage and decrease to their basal level about 20 min after initiation, and that IHF binds to *oriC* to promote initiation. From these results, I conclude that IHF is crucial for the timely activation of *datA* after initiation.

I also revealed the cell cycle-coordinated stimulation of DARS2. A certain protein dissociated from DARS2 at an initiation period, and temporarily bound after initiation. This

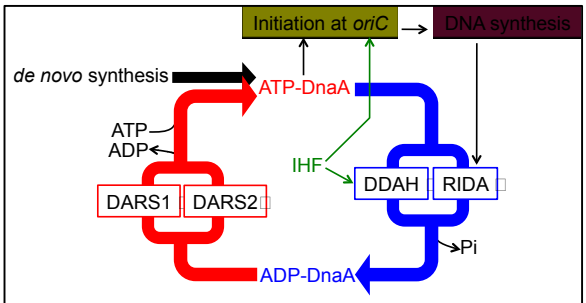

Fig. 3. Cell cycle-coordinated regulation of DnaA by DDAH and DARS2
After initiation, ATP-DnaA is hydrolyzed by two independent pathways, RIDA and DDAH, yielding inactive ADP-DnaA. The resultant ADP-DnaA is reactivated by DARS1&2. IHF temporarily stimulates initiation at *oriC* and DDAH. A certain protein also stimulates DARS2 in a cell cycle-coordinated manner.

dissociation timing was consistent with previous results that for the second round of initiation, ATP-DnaA level is increased again 20 min after the first initiation. Based on these finding, I suggest an idea that stimulation of DARS2 by a certain protein might be related with ATP-DnaA level in a feedback manner. This feature would be crucial for sustaining coordinated fluctuations in the activities of DnaA and *oriC*.

Molecular mechanism of DDAH

Previous study showed that DnaA bound less stably to *datA* than to *oriC*, which could increase the requirement for IHF in formation of DnaA oligomers on *datA* (Fig. 4). DNA looping by IHF might also promote inter-DnaA interaction between DnaA oligomers on DnaA boxes 2 and 3. From mutational analyses of DnaA, AAA+ Arg-finger Arg285 and Box VII Arg281 that support specific inter-DnaA interaction are required for DDAH, suggesting that, like the ATP-DnaA-Hda interaction in RIDA, the inter-DnaA interaction in the ATP-DnaA-*datA* complex is not as tight as that in the ATP-DnaA-*oriC* complex. This would result in a conformational change of DnaA, permit the interaction of Sensor II Arg334 with ATP and stimulate DnaA-ATP hydrolysis. The resultant ADP-DnaA molecules would engage more weakly in cooperative binding, and therefore dissociate from *datA*. This mechanism would support catalytic function of *datA* in DDAH.

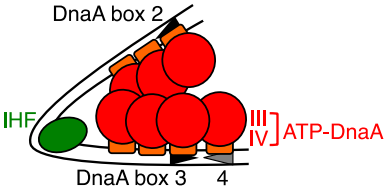

Fig. 4. Model for moleculer mechanism of DDAH
Predicted ATP-DnaA multimers formed at minimal *datA* region for DDAH (*datA* del5) are indicated. For simplicity, nucleotide-binding domain (domain III; III), and DNA-binding domain (domain IV; IV) are indicated.

【References】

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