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Regulatory Network of the Initiation of Chromosomal Replication in *Escherichia coli*

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ABSTRACT The bacterial chromosome is replicated once during the division cycle, a process ensured by the tight regulation of initiation at *oriC*. In prokaryotes, the initiator protein DnaA plays an essential role at the initiation step, and feedback control is critical in regulating initiation. Three systems have been identified that exert feedback control in *Escherichia coli*, all of which are necessary for tight strict regulation of the initiation step. In particular, the ATP-dependent control of DnaA activity is essential. A missing link in initiator activity regulation has been identified, facilitating analysis of the reaction mechanism. Furthermore, key components of this regulatory network have also been described. Because the eukaryotic initiator complex, ORC, is also regulated by ATP, the bacterial system provides an important model for understanding initiation in eukaryotes. This review summarizes recent studies on the regulation of initiator activity.

KEYWORDS DnaA, *oriC*, Hda, sliding clamp

INTRODUCTION

The initiation of chromosomal replication occurs only once during the cell cycle in both prokaryotes and eukaryotes. A striking difference between prokaryotic and eukaryotic cell cycles is that, in rapidly dividing prokaryotic cells, the next round of replication is initiated before the ongoing round has terminated. This feature allows fast growth, so that the cellular doubling time is shorter than the time required for one round of replication. Although the next division cycle starts before the end of the preceding cycle, chromosomal replication occurs in a timely manner and only once during each cell cycle.

It is conceivable that both positive and negative controls are responsible for establishing this "once per cell cycle" rule. For *Escherichia coli*, positive regulatory systems have not been identified for cell cycle control, and the relevance of their absence is unclear. Also, an "engine" that drives the cell cycle, comparable to eukaryotic cyclin-CDK (cyclin dependent protein kinase) complexes, has not been described in prokaryotes. In contrast, multiple negative regulatory systems have been identified.

THREE FEEDBACK CONTROL SYSTEMS

Three mechanisms mediate feedback regulation in *Escherichia coli*: (1) sequestration of the chromosomal replication origin, *oriC*; (2) titration of the

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free form of the initiator protein, DnaA; and (3) regulation of initiator activity. Because defects in each system perturb replication, all three are necessary to regulate initiation.

The minimal *oriC* region contains eleven Dam methyltransferase recognition sites (the GATC motif), at which adenine residues are methylated. When *oriC* is duplicated, GATC motifs on the newly replicated strand remain transiently unmethylated, leading to a hemi-methylated state of the DNA duplex. Although hemi-methylated *oriC* is replicated *in vitro* (Messer *et al*., 1985; Landoulsi *et al*., 1989; Boye, 1991), it is not efficiently replicated *in vivo* (Messer *et al*., 1985; Smith *et al*., 1985; Russell & Zinder, 1987). The replicated *oriC* region remains hemi-methylated for about one third of a generation, whereas other Dam recognition sites are fully methylated within about 1 minute (Ogden *et al*., 1988; Campbell & Kleckner, 1990). The discovery of SeqA, which binds to hemi-methylated *oriC*, revealed that the *oriC* region is sequestered after initiation (Lu *et al.*, 1994; von Freiesleben *et al.*, 1994). SeqA binds preferentially to two hemi-methylated sites in *oriC* and SeqA binding is then cooperatively propagated to adjacent regions, thereby excluding the DnaA initiator protein and inducing topological changes that inhibit initiation (Slater *et al.*, 1995; Brendler & Austin, 1999; Skarstad *et al.*, 2000, 2001; Taghbalout *et al.*, 2000; Torheim & Skarstad, 1999; Weitao *et al*., 2000). Prior to the next round of replication, *oriC* methylation is fully resored. Thus, from the immediate end of initiation to the end of sequestration, the potential for initiation is reduced. Another factor involved in sequestration, SeqB, a member of the membrane-bound sequestration complex, has been reported but not precisely identified and characterized (Shakibai *et al*., 1998).

The SeqA protein is a homotetramer of a 21 kDa polypeptide, and its N-terminal region is necessary for tetramer formation and for aggregation (Lee *et al*., 2001; Guarne *et al*., 2002; Han *et al*., 2003). Its C-terminal region binds to hemi-methylated GATC sequences (Guarne *et al*., 2002; Fujikawa *et al*., 2003, 2004). Each SeqA tetramer binds to two hemi-methylated GATC motifs separated by up to 31 bases, forming a stable SeqA-DNA complex (Brendler *et al*., 2000; Han *et al*., 2003). Sequential binding to hemi-methylated sites leads to higher order complex formation and induces the aggregation of free proteins onto the bound proteins (Han *et al*., 2003). Aggregated SeqA proteins can be observed to form foci by immunofluorescence microscopy, and they have also been visualized as green fluorescence protein fusions (Hiraga *et al*., 1998, 2000; Onogi *et al*., 1999; Brendler *et al*., 2000; Bach *et al*., 2003; Fossum *et al*., 2003).

The *datA* locus, a 1 kb segment located at 94.7 min on the genetic map, contains five DnaA boxes, which are specifically bound by DnaA, and this region has unusually high affinity for the DnaA protein: it can bind eight times more DnaA than can the *oriC-mioC* region, which has a similar number of DnaA boxes (Kitagawa *et al.*, 1996). Over-initiation occurs in the *datA* deletion mutant, and in a strain with increased copies of the *datA* locus, replication initiation is inhibited (Kitagawa *et al.*, 1998; Morigen *et al*., 2001). These results indicate that the *datA* locus functions as a DnaA reservoir, reducing the level of free DnaA available for binding to *oriC*. The *E. coli* chromosome contains about 300 DnaA boxes, and five regions have an unusually high affinity for DnaA *in vitro* (Roth & Messer, 1998). One is the *datA* site, but the other sites do not affect the timing of replication initiation, indicating that *datA* is a unique locus that adjusts the balance between free and bound DnaA (Ogawa *et al.*, 2002). Recently, DNA microarray analyses showed that the initiation level in the *datA* mutant cells was indistinguishable from that in wild-type cells (Camara *et al.*, 2005). Both flow cytometry and marker frequency analyses indicated that the replication pattern of a *datA* mutant is essentially the same as that of the parental strain and that deletion of *datA* site induces rifampin-resistant replication (Morigen *et al*., 2005). From these points of view, the function of *datA* should be re-examined.

Many reviews have covered the regulation of the initiation of chromosomal replication in *E. coli*, especially origin sequestration and DnaA titration (for recent reviews, Boye *et al*., 2000; Katayama, 2001; Messer, 2002; Camara & Crooke, 2005). In this review, I will focus on recent studies, particularly those concerning the systems that regulate initiator activity.

SYSTEMS REGULATING INITIATOR ACTIVITY

Regulatory Inactivation of DnaA (RIDA)

The prokaryotic initiator protein, DnaA, is widely conserved in prokaryotes and is essential for initiation

at *oriC*. In *E. coli*, DnaA proteins first bind to five specific 9-mer sequences (DnaA boxes) at *oriC*, which then promotes the cooperative binding of 20 to 40 molecules of DnaA, causing nearby 13-mer AT-rich sequences to unwind. This unwinding enables the DNA helicase DnaB to be loaded onto the template. DnaA has been extensively investigated in an *in vitro* reconstituted replication system. The observation that DnaA has a high affinity for ATP led to the important finding that only the ATP-bound form is active as an initiator protein (Sekimizu & Kornberg, 1987). The hydrolyzed, or ADP-bound, form is inactive.

Studies of a *dnaA* mutant (*dnaAcos*), in which over-initiation occurs at a non-permissive temperature, revealed that this mutant is defective in the conversion from the active ATP-bound form to the inactive ADP-bound form (Katayama & Nagata, 1991; Katayama & Kornberg, 1994; Katayama, 1994; Katayama *et al*., 1995; Katayama & Crooke, 1995; Kurokawa *et al*., 1998; for review, see Katayama, 2001). These results indicated that the hydrolysis of DnaA-bound ATP is the regulatory mechanism that enforces the once-per-cell-cycle rule.

The conversion of ATP-DnaA to ADP-DnaA was reconstituted *in vitro* and the protein factors necessary for the reaction were identified by biochemical fractionation (Katayama & Crooke, 1995; Katayama *et al*., 1998). These analyses showed that two fractions are required, one of which is the DNA polymerase III β subunit, which functions as a sliding clamp. This protein forms a stable homodimer that encircles primer RNA-DNA heteroduplex or DNA dupelx during DNA replication (Kelman & O'Donnell, 1995; O'Donnell *et al*., 2001). The clamp-loader subassembly of the polymerase opens the dimer interface of the clamp to load this protein onto the primed site. This DNA-loaded form of the clamp ensures processive replication of the DNA polymerase. After completion of the Okazaki fragment, the subassembly of the polymerase, excluding the clamp, is released from DNA, leaving the clamp loaded on the synthesized DNA. The DnaA inactivation system required the DNA-loaded form of the clamp (Katayama *et al*., 1998). This result revealed that DnaA inactivation is directly linked to the initiation of replication: active DnaA bound at *oriC* is inactivated soon after DNA polymerase III starts replication at *oriC*, thereby preventing immediate re-initiation. This mechanism was termed the regulatory inactivation of DnaA (RIDA). RIDA is a feedback regulatory system in which DNA polymerase III functions as a transducer that signals to DnaA that initiation has occurred at *oriC*.

The role of RIDA deduced *in vitro* is consistent with the expression profiles of the two forms of DnaA (Kurokawa *et al*., 1999). In synchronized cultures of the *dnaC2* mutant, the ATP-DnaA level temporarily increases around the time of initiation and decreases rapidly thereafter. The decrease in the ATP-bound form of DnaA is tightly linked to DNA replication, supporting dependence on RIDA.

Genetic Analyses

The second essential factor (provisionally termed IdaB) was identified by a genetic approach to isolate *E. coli* mutants defective in chromosomal replication and partitioning (Kato & Katayama, 2001). There have been many attempts to isolate replication-defective mutants in many organisms. A typical strategy is to isolate mutants that cannot maintain a mini-chromosome or other episome. A 9.6 kb derivative of the *E. coli* fertility (F) factor (a single-copy ~94 kb plasmid), referred to as the mini-F plasmid, has the same features as does the parental plasmid, in that it is stably maintained as a single copy. *E. coli* mutants in which the mini-F plasmid is unstable were isolated and the genes responsible for this phenotype were termed *hop* (host function of plasmid maintenance) (Niki *et al*., 1988). The *hopA*, *hopD*, and *hopE* genes were identified as *gyrB* (which encodes the B subunit of gyrase), *hupB* (encoding the HU-1 subunit of the HU protein), and *recD* (encoding the RecD subunit of RecBCD enzyme) (Ogura *et al*., 1990a,b; Niki *et al*., 1990). The same strategy was used to isolate additional mutants that could not stably maintain the mini-F plasmid at low temperature; among these, mutants that were temperature-sensitive (ts) for growth were selected (Kato & Katayama, 2001). More than 60 such mutants were isolated, and an *E. coli* genomic library was introduced into these mutants to identify plasmids that corrected the ts growth phenotype. The *hda* gene, which codes for the other essential RIDA factor, was identified on a plasmid that complements one of these mutants, the *uns36* mutant.

The *hda* gene encodes a protein related to DnaA (Kato & Katayama, 2001). The Hda protein is composed of 248 amino acids, shorter than DnaA (467 amino acids), and it shares homology with DnaA domain III, which contains motifs specific to the AAA^+ protein family. Another complementing plasmid, carried the *dnaN* gene, which codes for the β subunit

(sliding clamp) of DNA polymerase III. A third plasmid contained *oriC* and fourth carried *datA*. Because introduction of the *dnaA* plasmid into the *uns36* mutant inhibited growth, the effects of the third and fourth plasmids may be due to the titration of free DnaA molecules by plasmid-borne *oriC* and *datA* sequences. Since the *dnaN* mini-F plasmid, but not the *hda* mini-F plasmid, corrected the ts growth of the *uns36* mutant, it was suggested that the *uns36* mutant is defective in *dnaN* and that *hda* is a multi-copy suppressor.

The *hda* gene is essential, since disruption confers inviability in the absence of the *hda* plasmid (Kato & Katayama, 2001). An important clue to clarifying the function of *hda* was obtained by P1 phage-mediated transduction: the *hda* gene was found to be essential in an *rnhA* mutant but dispensable in *rnhA dnaA* and *rnhA oriC* double mutants. This result indicated that its essential function was not related to the appearance of *oriC*-independent replication (stable DNA replication in the *rnhA* mutant background) but to the disappearance of *oriC*-dependent replication. This phenotype resembles that of the *dnaAcos* mutant, which is defective in RIDA.

Hda was shown to be a factor essential for RIDA by two biochemical experiments (Kato & Katayama, 2001). In the first, disruption of the *hda* gene was found to cause accumulation of the ATP-bound active form of DnaA *in vivo*. The second experiment involved *in vitro* reconstitution. Cell lysates prepared from the *hda* disruptant were negative in a RIDA assay and activity was restored by addition of an IdaB fraction prepared from the hda^+ cells, and purified tagged Hda (MBP-Hda-Myc His) could replace the IdaB fraction.

Discovery of Hda, which is essential and specific for RIDA, allowed clarification of the *in vivo* function of RIDA (Kato & Katayama, 2001). In *hda* temperature-sensitive (ts) mutants incubated at the non-permissive temperature, the *oriC* copy number increases, indicating that RIDA represses over-initiation. However, total DNA synthesis does not increase, unlike what is seen for the *dnaAcos* mutant grown at a non-permissive temperature. This result is consistent with the interpretation that the copy number of *terC* (replicational termination site on the chromosome) per cell is reduced at non-permissive temperature, indicating that the over-initiated replication is abortive. Hda may function not only at the initiation step but also during elongation.

The *hda* gene was also identified as a suppressor of the toxicity of a peptide derived from an initiation protein, TrfA, of the broad-host-range plasmid RK2 (Kim *et al*., 2003). The product of the *hda* gene (here termed Dp) physically interacts with TrfA and the two proteins co-localize in the inner membrane fraction. The *hda* gene has also been reported to be dispensable for cell growth and viability (Camara *et al*., 2003). This discrepancy with respect to other studies has not yet been clarified, but it may be due to a short region of the gene remaining in the mutant constructed by Camara and colleagues.

Biochemical Analyses

Identification of Hda made it possible to investigate the functions of each factor and of the reaction mechanism of RIDA reconstituted with purified factors. The β subunit of DNA polymerase III is necessary for RIDA, and its ability to promote DnaA inactivation depends on its assembly as a sliding clamp on DNA (Katayama *et al*., 1998). When a single clamp is loaded, at least 40 bp of duplex DNA flanking the clamp is necessary for DnaA inactivation, suggesting a direct interaction between ATP-bound DnaA and duplex DNA flanking the clamp (Su'etsugu *et al*., 2004). DnaA-ATP hydrolysis is inhibited *in vitro* when DnaA is tightly bound to an oligonucleotide bearing a DnaA box (T. Katayama, personal communication).

Hda was recently purified and characterized (Su'etsugu *et al*., 2005). In solution Hda forms a homodimer. A single cell contains about 50 Hda dimers per cell, as measured with the anti-Hda antibody and purified Hda. This level is significantly lower than that of DnaA (500 to 2000 molecules per cell) (Katayama & Kornberg, 1994; Sekimizu *et al*., 1988; Chiaramello and Zyskind, 1989; Hansen *et al*., 1991) or that of the β-clamp (400–5000 molecules per cell) (Kawakami *et al*., 2001; Leu *et al*., 2000). Probably, the Hda-clamp complex is recycled for the DnaA-ATP hydrolysis. Reaction modes *in vitro* are consistent with this idea (Katayama *et al*., 1998; Su'etsugu *et al*., 2005).

One or two Hda dimers associate with a single β-clamp molecule and this complex is necessary for DnaA-ATP hydrolysis (Su'etsugu *et al*., 2005). The interaction between the β-clamp and Hda is mediated by an Hda N-terminal motif, the clamp-binding consensus (Kurz *et al*., 2004; Su'etsugu *et al*., 2005). Hda is composed of this N-terminal region and the C-terminal domain that contains the of AAA^+ superfamily-specific motifs (Neuwald *et al*., 1999). The "Arg finger," which is found in the Box VII motif of AAA^+ proteins, is proposed to directly participate in ATP hydrolysis (Ogura

et al., 2004). The Hda Arg finger is R168, a residue involved in DnaA-ATP hydrolysis and stable homodimer formation (Su'etsugu *et al*., 2005). The DnaA N-terminal domain is also essential for the functional interaction between DnaA and the Hda-clamp complex. Like Hda, DnaA is a member of AAA^+ superfamily (Neuwald *et al*., 1999). The AAA^+ Box VIII motif or Sensor II, DnaA R334 is required for the DnaA-ATP hydrolysis (Nishida *et al*., 2002). ATP bound to DnaA may be catalytically hydrolyzed at a binding interface between the AAA^+ domains of DnaA and Hda.

The RIDA system exploits the unique role of the ATP-bound form of DnaA as an active initiator. Only DnaA-ATP can promote strand opening and subsequent replication steps, although both DnaA-ATP and DnaA-ADP participate in similar nucleoprotein complexes (Sekimizu *et al*., 1987). There are two possibilities to explain the requirement for the ATP-bound form in *oriC* unwinding. One is that only DnaA-ATP has a conformation appropriate for opening the DNA duplex, although both DnaA-ATP and DnaA-ADP bind the same sites at *oriC*. The other possibility is that only DnaA-ATP binds to a specific site at *oriC*. Although each of the five DnaA boxes at *oriC* is bound by inactive DnaA-ADP and active DnaA-ATP with equal affinity, (Sekimizu *et al*., 1987; Schaper & Messer, 1995), several other 9-mer DnaA-binding motifs, termed I sites, were identified as sites that are preferentially recognized by DnaA-ATP but not DnaA-ADP (McGarry *et al*., 2004). The I sites differ from the DnaA-box consensus at 3 to 4 positions, and single-base changes in these sites produce two phenotypes: blockage of *oriC* unwinding and reduction of ATP specificity, indicating that the I sites are functionally important.

It was reported that the sites with consensus AGATCT in the AT-rich region of *oriC* were preferentially bound by DnaA-ATP resulting in stabilization of single-stranded regions (Speck & Messer, 2001). This 6-mer consensus sequence is called as "ATP-DnaA box," which was originally identified in the study of the *dnaA* promoter region (Speck *et al.*, 1999). However, this interaction between DnaA-ATP and those sites withib the AT-rich region of *oriC* was not observed in the experiment, in which four-fold lower concentrations of DnaA-ATP were used (McGarry *et al*., 2004).

Recently, the analyses of the DnaA R285A mutant proteins showed that this does not interact with ATP-DnaA-specific binding sites such as the I sites (Kawakami *et al.*, 2005). The DnaA Arg-285 residue is proposed to play a unique role in the ATP-dependent conformational activation of an initial complex. This residue may be involved in recognizing ATP bound to DnaA and in modulating the structure of the DnaA multimer to allow interaction with ATP-DnaA specific binding sites in the complex.

Rejuvenation of Inactive DnaA

In determining the level of the active form of initiator, not only the inactivation of the DnaA-ATP but also the reactivation of DnaA-ADP is important. The reactivation reaction has been clearly demonstrated *in vitro*. Studies of the conversion of DnaA-ADP into DnaA-ATP started with the interesting finding that acidic phopholipids in a fluid bilayer promote the exchange of tightly bound ADP for ATP (Sekimizu *et al*., 1988). Reactivation is facilitated in the presence of *oriC* DNA (Crooke *et al*., 1992). The DnaA domain essential for the membrane reactivation was indicated to be within the region defined by amino acids 300 to 380 (Garner & Crooke, 1996), and this domain was found to be inserted into the hydrophobic portion of the lipid bilayer during nucleotide release (Garner *et al*., 1998).

The presence of the reactivation system was suggested from two *in vivo* studies. First, mutations of the *pgsA* gene, which encodes phosphatidylglycerophosphate synthase, are suppressed by mutation of the *rnhA* gene, which encodes RNaseH (Xia & Dowhan, 1995). In *pgsA* mutants, synthesis of major anionic phospholipids is limited, resulting in cell growth arrest, and *rnhA* mutations induce constitutive stable DNA replication that is independent of *oriC* and DnaA. Therefore, this suppression is explained by a close link between DnaA protein function and cellular membrane status. The second *in vivo* study involved analyses of the cellular levels of DnaA-ATP and DnaA-ADP (Kurokawa *et al*., 1999). Regeneration activity was detected in the *dnaN* ^{ts} mutant incubated at non-permissive temperature, at which RIDA is inhibited, and in the presence of chloramphenicol, which prevents *de novo* DnaA synthesis. Regeneration was dependent on a plasmid carrying a *dnaA* cistron, which over-expresses DnaA protein.

Definitive proof of the existence of a biologically functional reactivation system required isolation of a *dnaA* mutant defective in re-activation, much as identification of the *dnaAcos* mutant firmly established RIDA. Mutations in *dnaA* that bypass the growth arrest conferred by the *pgsA* mutation under conditions

of over-production of the mutant DnaA were isolated (Zheng *et al*., 2001). However, one of the mutant proteins, DnaA (L366K), was found to be independent of the capacity for nucleotide binding and exchange (Li *et al*., 2005).

Recently, it was found that pBluescript (a plasmid) DNA promotes the dissociation of ADP from DnaA-ADP (Fujimitsu & Katayama, 2004). This interesting effect is due to a plasmid-borne sequence (termed DARS, for DnaA-reactivating sequence). The DARS sequence contains three motifs, a typical DnaA-binding 9-mer motif (DnaA box) and two DnaA box-like sequences, and these motifs and their orientations are crucial for reactivation. A potential sequence with similar activity was identified in the *E. coli* genome. If this site is involved in the reactivation of DnaA-ADP *in vivo*, characterization of mutations affecting its sequence may confirm the biological significance of the reactivation system.

OTHER REGULATORY NETWORKS

The RIDA reaction can be reconstituted with two previously identified factors, the β-clamp and Hda. The *in vitro* system is a simplified system derived from the complex *in vivo* network. Several lines of investigation using these identified factors have gradually unveiled the complex network that regulates RIDA.

Suppressor Analyses of *hda* Mutations

Suppressor mutations conferring temperature resistance were isolated from the *hda*^{ts} mutants, and the *ygfZ* gene was identified as a suppressor (Ote *et al*., submitted). This suppression is not allele-specific. Interestingly, the *ygfZ* null mutation suppresses a null mutation of the *hda* gene, which is otherwise essential. The *ygfZ* gene is not essential but the *ygfZ* disruptant grows slowly, especially at low temperature, suggesting that this gene is important for cell growth. YgfZ is a member of a large protein family conserved from bacteria to eukaryotes, in contrast to Hda, which is conserved only in most alpha, beta, and gamma proteobacteria. The biological function of YgfZ has not been clarified but its crystal structure has been determined (Teplyakov *et al*., 2004). The protein has a three-domain architecture with a central hydrophobic channel, which has been identified as a folate-binding site. The ability of YgfZ to bind folate derivatives was confirmed experimentally. Although its structure is very similar to that of bacterial dimethylglycine oxidase, an enzyme of the glycine betaine pathway, YgfZ is not thought to be an aminomethyltransferase because it lacks amino acid conservation at the folate-binding site. Therefore, YgfZ is speculated to be a folate-dependent regulatory protein.

There are two general possibilities to explain *ygfZ* suppression: true suppression and phenotypic suppression. In the former case, defects due a lack of Hda may be corrected and in the latter case cell growth may be phenotypically suppressed despite the Had deficiency. To address the nature of the suppression, the copy numbers of *oriC* and *terC* were examined, and both over-initiation and abortive elongation due to the *hda* mutation were found to be partially suppressed. This finding indicated either that the accumulation of DnaA-ATP due to the *hda* mutation is reduced or that over-initiation and abortive elongation are phenotypically suppressed despite an accumulation of DnaA-ATP. To discriminate between these two possibilities, the cellular levels of DnaA-ATP and DnaA-ADP were examined, and the level of DnaA-ATP was found to be decreased even in the absence of Hda. Hda is essential for the *in vitro* RIDA reaction, but DnaA-ATP may be converted to DnaA-ADP *in vivo* in the absence of Hda. Alternatively, YgfZ may be necessary for the rejuvenation of DnaA-ADP, explaining why the cellular level of DnaA-ATP is reduced in its absence.

A clue to YgfZ function was obtained by further genetic analysis. The *mnmE* gene, which is involved in tRNA modification (Elseviers *et al*., 1984; Cabedo *et al*., 1999), was identified as a suppressor of the *hda*^{ts} *ygfZ* double mutation, suggesting that YgfZ is also involved in tRNA modification. Analysis of modified nucleosides revealed that the levels of 2-methylthio N^6-isopentenyladenosine (ms^2i^6A), 2-methyladenosine (m^2A), and queosine are decreased in the *ygfZ* disruptant. YgfZ may be primarily involved in stable RNA modification and a deficiency in the modified tRNA may lead to a decrease in the DnaA-ATP level (Figure 1).

Suppressor Analyses of the *dnaAcos* Mutation

A series of suppressor analyses of the *dnaAcos* mutation allowed identification of two novel genes. One of these, *cedA*, is a multicopy suppressor of the low

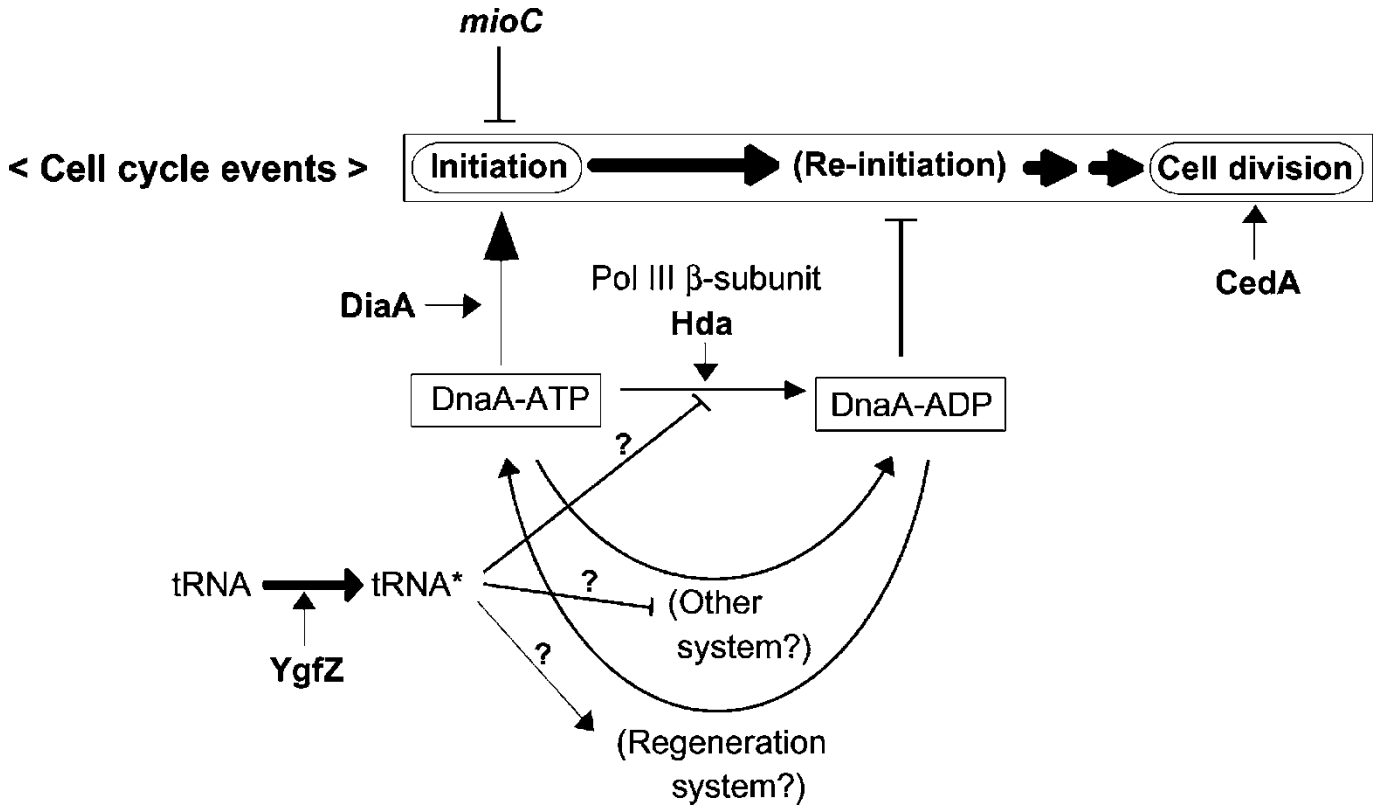

FIGURE 1 **Possible regulatory network. The YgfZ protein primarily participates in tRNA modification. This modified tRNA functions positively and negatively during cell division and during the inactivation of DnaA, respectively. When the *ygfZ* gene is disrupted, the absence of modified tRNA results in a reduction of the active form of DnaA and in a delay of cell division.**

temperature growth defect of the *dnaAcos* mutant (Katayama *et al.*, 1997). Over-initiation due to the *dnaAcos* mutation is not corrected in the presence of a multicopy plasmid carrying the *cedA* gene, indicative of phenotypic suppression. An analysis of cell number and morphology showed that an excessive level of this gene stimulates cell division, resulting in cell growth with an abnormally elevated chromosomal copy number. These results suggested that the inhibition of cell division caused by chromosomal over-replication in the *dnaAcos* mutant is bypassed when cell division is stimulated due to the overproduction of CedA.

The other novel gene, *diaA*, was identified following Tn5 random-insertion mutagenesis (Ishida *et al.*, 2004). Although the *diaA*::Tn*5* mutation suppresses the growth defect of the *dnaAcos* mutant at low temperature, it inhibits the growth of other *dnaA*^{ts} mutants at semi-permissive temperature and reduces the stability of minichromosomes, suggesting that the *diaA*::Tn*5* mutation inhibits initiation at *oriC* and thus that DiaA is involved in initiation at *oriC*. The results are consistent with results showing that purified DiaA, which forms a stable homodimer, stimulates *in vitro oriC* replication, especially in the presence of limited levels of DnaA. Purified DiaA directly binds to the N-terminal domain of DnaA. Flow cytometry analyses revealed that the timing of replication initiation is disrupted in both the *diaA* mutant and the DiaA overproducer, indicating that *diaA* is involved in the regulation of chromosome replication.

Another suppressor mutant obtained by Tn*5* random-insertion mutagenesis has an insertion mutation of the *mioC* promoter (*mioC*p) (Su'etsugu *et al.*, 2003). Like the *diaA*::Tn*5* mutation described above, the *mioC*p insertion suppresses the growth defect of the *dnaAcos* mutant at low temperature but inhibits growth of the *dnaA*^{ts} mutants at semi-permissive temperature, suggesting that the *mioC*p::Tn*5* mutation inhibits initiation at *oriC*. The transcription level of *mioC* and *oriC* is not significantly changed by the *mioC*p::Tn*5*. The transcription of *mioC* is coordinated with the replication cycle: expression increases after initiation and rapidly decreases prior to initiation (Theisen *et al.*, 1993; Ogawa & Okazaki, 1994; Bogan and Helmstetter, 1996, 1997). In the *mioC*p::Tn*5* mutant, the transcription of *mioC* is

constitutive and does not fluctuate during the cell cycle. These results indicate that replication initiation is impaired by the constitutive transcription of *mioC*.

PERSPECTIVES

The tight regulation of replication initiation is achieved by multiple mechanisms, which include *oriC* sequestration, titration of free DnaA, and inactivation and rejuvenation of initiator activity. The timing of replication initiation is perturbed by defects in any of these systems, which does not necessarily mean that they are interdependent. For example, RIDA and *datA* regulation are independent (Katayama *et al*., 2001). Tight regulation can be achieved by coordination of multiple systems, which play complementary roles in preventing untimely initiation (Katayama, 2001; Katayama *et al*., 2001; Camara & Crooke, 2005). *oriC* is rapidly sequestered while the level of DnaA-ATP decreases slowly (Kurokawa *et al*., 1999). Sequestration lasts for only about 10 minutes, and requires both a reduction in free DnaA levels and inactivation of DnaA. There may be regulatory systems other than these three mechanisms involved in initiation regulation; for example, the novel *diaA* gene may represent an additional regulatory component.

The mechanisms regulating replication initiation are diverse in prokaryotes. For *oriC* sequestration, Dam methylation is essential for discriminating between old and new replication origins. Sequestration mediated by Dam methylation is restricted to Enterobacteriaceae; there is no Dam-like methylation system nor SeqA homologe in *Bacillus subtilis* (Seror *et al*., 1994). In Bacilli, co-localization of *oriC* and the *dnaA* gene makes this region function as a large sink with a high capacity for DnaA binding, effectively reducing the level of free DnaA (Ogasawara *et al*., 1985). The interaction between *oriC* and the cell membrane may also be important for regulation (Firshein, 1989). In *Caulobacter crescentus*, untimely replication is inhibited by binding of the CtrA protein to *oriC* (Quon *et al*., 1998). The level of this protein is controlled in a cell cycle-dependent manner by transcription regulation and by proteolysis.

In eukaryotes, the initiator origin recognition complex (ORC) is a conserved multiprotein complex (Bell, 2002). ORC recruits other replication proteins, Cdc6, Cdt1, and Mcm2-7, to the origin to form a prereplicative complex (pre-RC), an essential intermediate in initiation (Mendez & Stillman, 2003). Here, ATP regulates pre-RC formation. Ten of the 14 proteins (Orc 1,4, and 5, Mcm 2-7, and Cdc6) that are required for pre-RC formation belong to the AAA^+ family, which include proteins with nucleotide binding motifs (Neuwald *et al*., 1999). In particular, ATP binding by Mcm2-7, Orc1, and Cdc6 is essential (Bell & Dutta, 2002). Although it has been shown that there are two ATP-requiring steps during pre-RC formation (Seki & Diffley, 2000) and that ATP hydrolysis is required for at least one of these steps (Gillespie *et al*., 2001; Harvey & Newport, 2003), it is unclear how nucleotide binding and hydrolysis control pre-RC formation. In *Saccharomyces cerevisiae*, the Orc1 and 5 subunits bind ATP (Klemm *et al*., 1997). ATP binding by Orc1 is required for origin-specific DNA binding and cell viability, and ATP hydrolysis by ORC requires the coordinate function of the Orc1 and Orc4 subunits (Bowers *et al*., 2004). Prevention of ORC ATP hydrolysis inhibits reiterative loading of the putative helicase complex, Mcm2-7, at origins (Bowers *et al*., 2004). Studies of *Drosophila* and human ORCs suggest that the regulation of ORC by ATP is conserved throughout eukaryotic organisms (Chesnokov *et al*., 1999; Remus *et al*., 2004; Vashee *et al*., 2001). Since it has not been determined when ATP hydrolysis by Orc1 is required *in vivo*, the *E. coli* RIDA system may serve as a model system for understanding the eukaryotic regulatory system.

Two general models have been proposed for DNA replication. One is that the replicase moves along DNA like a train on a track, and the other is that it is stationary like a factory. Analyses of the sub-cellular localizations of replicase subunits in *B. subtilis* first indicated that the catalytic subunit is localized at a discrete intracellular position, predominantly midcell or nearby, rather than being distributed randomly, indicating that the replicase is anchored (Lemon & Grossman, 1998, 2000). Similar results were also obtained for *E. coli* (Espeli*et al*., 2003). The factors involved in anchoring have not been identified, and the molecular nature of the replication factory has not yet been clarified. The process of construction of this factory may be important for the regulation of initiation.

To identify factors involved in the regulatory network of the replication initiation, a forward genetics approach is necessary, for example, entailing the isolation and characterization of suppressor mutants. However, it is difficult to know whether the systems composed of the identified factors are sufficient for the tight regulation observed *in vivo*. Recently, *E. coli* strains with a reduced

genome were engineered, consisting of a series of large-scale chromosomal deletion mutants lacking from 2.4% to 29.7% of the parental chromosome (Hashimoto *et al.*, 2005). Flow cytometry analyses showed that at least in a strain deleted for about 24% of chromosomal regions, the timing of replication initiation is not markedly perturbed, indicating that the set of genes remaining in this strain is sufficient for initiation regulation. Both forward genetics and genome science will be necessary to understand the global network relevant to initiation regulation.

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