Hda-mediated inactivation of the DnaA protein and *dnaA* gene autoregulation act in concert to ensure homeostatic maintenance of the *Escherichia coli* chromosome

Leise Riber,[1] Jan A. Olsson,[2] Rasmus B. Jensen,[1] Ole Skovgaard,[1] Santanu Dasgupta,[2] Martin G. Marinus,[3] and Anders Løbner-Olesen[1,4]

[1]Department of Life Sciences and Chemistry, Roskilde University, Roskilde DK-4000, Denmark; [2]Department of Cell and Molecular Biology, Uppsala University, Biomedical Centre, Uppsala SE-751 24, Sweden; [3]Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, Massachusetts 01605, USA

Initiation of DNA replication in *Eschericia coli* requires the ATP-bound form of the DnaA protein. The conversion of DnaA–ATP to DnaA–ADP is facilitated by a complex of DnaA, Hda (homologous to DnaA), and DNA-loaded β-clamp proteins in a process termed RIDA (regulatory inactivation of DnaA). Hda-deficient cells initiate replication at each origin mainly once per cell cycle, and the rare reinitiation events never coincide with the end of the origin sequestration period. Therefore, RIDA is not the predominant mechanism to prevent immediate reinitiation from *oriC*. The cellular level of Hda correlated directly with *dnaA* gene expression such that Hda deficiency led to reduced *dnaA* gene expression, and overproduction of Hda led to DnaA overproduction. Hda-deficient cells were very sensitive to variations in the cellular level of DnaA, and DnaA overproduction led to uncontrolled initiation of replication from *oriC*, causing severe growth retardation or cell death. Based on these observations, we propose that both RIDA and *dnaA* gene autoregulation are required as homeostatic mechanisms to ensure that initiation of replication occurs at the same time relative to cell mass in each cell cycle.

[*Keywords*: Chromosome replication; *dnaA* gene expression; *E. coli*; Hda protein; RIDA]

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In *Eschericia coli*, the DnaA protein is the key element in initiation of chromosome replication (Messer and Weigel 1996). Associated with either ATP or ADP, DnaA initially binds to the five high-affinity 9-mer consensus DnaA boxes located within the origin of replication, *oriC*. Further binding of DnaA to three 9-mer I-boxes, specific for DnaA–ATP, results in duplex opening (McGarry et al. 2004). Finally, the single-stranded region is stabilized by binding of DnaA–ATP to a set of 6-mer ATP–DnaA boxes (Speck and Messer 2001) and by binding of SSB (single-stranded DNA-binding) protein, allowing for recruitment of DnaB helicase, and loading of DnaG primase as well as the DNA polymerase III holoenzyme.

Chromosome replication is a process that is tightly coupled to cell growth. DnaA protein-dependent initiation of chromosome replication from *oriC* occurs once and only once each cell cycle at a constant cell mass per origin over a wide range of growth rates (Donachie 1968). In fast-growing cells, the time required to replicate the chromosome exceeds the doubling time and multiple origins are present due to overlapping rounds of replication (Cooper and Helmstetter 1968). Initiation of replication at all the origins in a cell occurs synchronously (Skarstad et al. 1986). Synchronous initiation of replication is generally explained by accumulation of the initiator, DnaA, and the sequestration of newly initiated origins that ensures that successive initiations are limited to "old origins" only (Løbner-Olesen et al. 1994).

In exponentially growing cells, the initiation occurs with remarkable precision and there is little cell-to-cell variation in the time from an initiation event in one generation to the next; i.e., the minimal interinitiation period corresponds to a constant fraction of one generation time over a wide range of growth rates (Olsson et al. 2002). Sequestration is not sufficient to explain the minimal interinitiation period, as it lasts only about one-third of a generation time (Campbell and Kleckner 1990). However, it provides a time interval during which DnaA

[4]Corresponding author.
E-MAIL lobner@ruc.dk; FAX 45-4674-3011.
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cannot access *oriC* (von Freiesleben et al. 2000). During sequestration, the cells' ability to initiate replication is reduced by the following mechanisms to prevent reinitiation at an origin in the same cell cycle (Boye et al. 2000). First, titration of DnaA protein to newly replicated DnaA boxes (Hansen et al. 1991; Kitagawa et al. 1996; Morigen et al. 2001) and sequestration of the *dnaA* gene promoter (Campbell and Kleckner 1990; Riber and Løbner-Olesen 2005) both serve to lower the intracellular concentration of free DnaA protein to a level below the threshold for initiation. Second, a process termed RIDA (regulatory inactivation of DnaA) reduces the activity of the DnaA protein by converting the active ATP-bound form to the inactive ADP-bound form by hydrolysis (Katayama et al. 1998). The latter may be analogous to assembly and disassembly of the prereplication complex in *Saccharomyces cerevisiae*, which is controlled by ATP binding and hydrolysis, respectively (Lee and Bell 2000). The Hda (homologous to DnaA) protein is essential for RIDA (Kato and Katayama 2001), and forms a complex with the DNA-loaded β-subunit sliding clamp of the polymerase III holoenzyme and the DnaA protein (Kato and Katayama 2001). In this complex, ATPase activity is promoted to convert DnaA–ATP to DnaA–ADP (Su'etsugu et al. 2004, 2005). In wild-type cells, 24% of the DnaA protein is bound to ATP, and this fraction increases to 70% in the absence of Hda (Kato and Katayama 2001). As initiation of replication generates new replication forks, and consequently more DNA-loaded β-clamps, it is thought to accelerate RIDA. It should be noted that DnaA–ADP can be rejuvenated to DnaA–ATP by a process facilitated by acidic phospholipids in the cell membrane (Sekimizu and Kornberg 1988). The role of this rejuvenation in cell cycle control is not clear.

In this study, we have evaluated the contribution of RIDA to the strict once-per-cell-cycle initiation of replication rule. This was done by altering the cellular level of Hda. Surprisingly, both Hda deficiency and overproduction were found to have only modest effects on cell cycle control, partly because they were offset by altered *dnaA* gene expression. Cells deficient in Hda were found to be very sensitive to the cellular DnaA protein level, and overproduction of DnaA in the absence of Hda led to growth retardation or cell death due to overreplication. These results lead us to propose that RIDA and *dnaA* gene autoregulation act in concert to ensure a constant minimal interinitiation time in wild-type cells.

Results

Hda-deficient cells contain suppressor mutations

The entire reading frame of the *hda* gene was replaced by a *cat* (chloramphenicol acetyl transferase) insertion using λ-Red recombineering as described in Materials and Methods. When the *hda*::*cat* deletion was transferred by P1-phage-mediated transduction into wild-type MG1655 cells with or without the *hda* plasmid pLR9 (see below), chloramphenicol-resistant colonies appeared with the same frequency in both recipients. In the absence of plasmid pLR9, the colonies were quite heterogeneous, indicating that they were initially compromised for growth and that fast-growing variants appeared over time (Fig. 1A). This observation suggested that Δ*hda* cells accumulated suppressor mutations, which we designated *hsm* (*hda* suppressor mutation). To determine whether the suppressor mutations affected DNA replication by themselves—i.e., in an Hda$^+$ background—eight independent Δ*hda* strains were transduced back to Hda$^+$ by cotransduction with the *purC80*::Tn*10* allele. Putative Hda$^+$ colonies were screened for chloramphenicol sensitivity, further verified by PCR analysis, and finally transduced to Pur$^+$.

Wild-type, Δ*hda*, and *hsm* (Δ*hda* transduced back to Hda$^+$) cells were analyzed by flow cytometry (Fig. 2). Wild-type cells contained mainly four origins of replication, while some contained two and some contained eight origins, indicating that origins were initiated in synchrony (Fig. 2A; Skarstad et al. 1986). All Δ*hda* mutant cells were moderately asynchronous (Fig. 2B,C) with a slightly increased average number of origins per cell (~6.0 vs. 4.9; Table 1). The size of Δ*hda* mutant cells was only slightly increased and the origin concentration was close to that of wild-type cells, indicating that the coupling between cell growth and initiation of replication was unaffected (Table 1). *hsm* cells grew with the same doubling time and were similar in size to wild-type cells (Table 1). They fell into two groups when analyzed by flow cytometry. Most clones (seven out of eight) had regained initiation synchrony and the number of origins

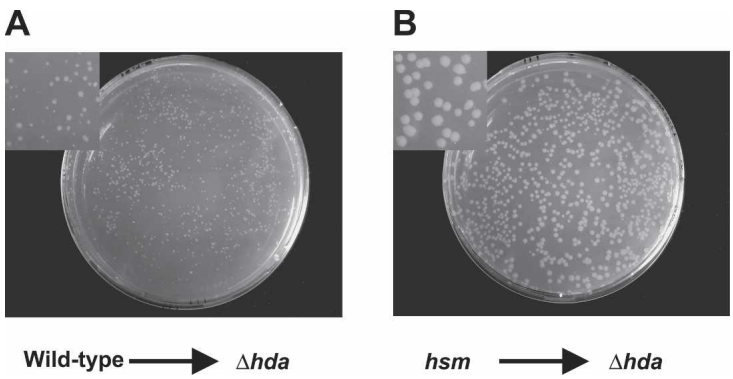

Figure 1. Hda-deficient cells contain suppressor mutations. (*A*) The *hda*::*cat* allele was transduced into MG1655 cells by P1-phage-mediated general transduction. To isolate any possible *hda* suppressor mutations (*hsm*), fast-growing MG1655 Δ*hda* mutant cells were transduced back to Hda$^+$ by cotransduction with a *purC*::Tn*10* allele. (*B*) The existence of *hsm* mutations was verified by transduction of the MG1655 *hsm* strains back to Δ*hda*. The same Δ*hda* P1 lysate was used for all strains, and the same amount of cells was plated on selective media and incubated for 24 h at 37°C. Eight individual Δ*hda* clones from *A* were tested and behaved identically.

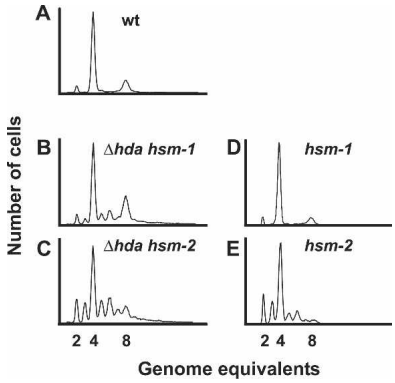

Figure 2. Two types of suppressor mutations in Hda-deficient cells. DNA histograms of the MG1655 strain (*A*), two different MG1655 Δ*hda* derivatives—ALO1917 carrying *hsm-1* (*B*) and ALO2415 carrying *hsm-2* (*C*)—and Hda$^+$ derivatives of ALO1917 (*D*) and ALO2415 (*E*). Cells were grown exponentially at 37°C in AB minimal medium supplemented with glucose and casamino acids. Cells were treated with rifampicin and cephalexin prior to flow cytometry analysis.

per cell was similar to the wild-type level. One representative of this group, *hsm-1*, is shown in Figure 2D. In the remaining clone, carrying the *hsm-2* suppressor, the number of origins per cell decreased and initiations were no longer synchronous (Fig. 2E). Because some of the putative *hsm* mutants were quite similar to wild-type cells, we decided to verify their genotype by transducing wild-type and *hsm* cells to Δ*hda* (Fig. 1). Transductants of Δ*hda* into wild-type cells were heterogeneous in colony size as expected (Fig. 1A), while transduction of Δ*hda* into cells containing *hsm* resulted in transductants that were fast growing and homogeneous (Fig. 1B). None of the *hsm* mutations cotransduced with *purC*, indicating that they were not located near *hda* on the *E. coli* chromosome (data not shown).

The *oriC* region of all eight *hsm* mutants was identical to the sequence in wild type (Blattner et al. 1997). The *dnaA* and *ygfZ* genes of four *hsm* mutants (including ALO1917 and ALO2415) were also sequenced because mutations in *ygfZ* were recently reported to suppress Hda deficiency (Ote et al. 2006). While no mutations were found in *ygfZ*, strain ALO2415 carrying the *hsm-2* suppressor had a mutation in the *dnaA* gene that resulted in substitution of phenylalanine with valine at position 349 in the protein. Because the DnaAF349V protein might be affected in nucleotide binding, it was not used any further.

For further studies, we chose the Δ*hda hsm-1* mutant (ALO1917) where the suppressor mutation did not reside in *oriC*, *dnaA*, or *ygfZ* and its isogenic *hsm-1* counterpart.

The minimal interreplication time of Δhda *mutant cells*

Hda-mediated conversion of DnaA–ATP to DnaA–ADP (RIDA) has previously been suggested as the main or the only mechanism preventing multiple initiations at *oriC* in the same cell cycle (Katayama et al. 1998; Camara et al. 2003, 2005). We decided to address this hypothesis directly by determining the minimal interreplication times in wild-type and Hda-deficient cells. In *E. coli*, the minimal interreplication period corresponds to the minimum time interval between two successive initiations from the same origin; multiple reinitiations in the same cell cycle should lower the value of this period.

A Meselson-Stahl density-shift experiment was used to determine the length of the minimal interreplication time during exponential growth of wild-type and Δ*hda* cells (Fig. 3; Olsson et al. 2002). Briefly, cells were grown exponentially in "heavy" medium, were shifted to "light" medium, and the distribution of a specific chromosome segment among DNA fractions separated in a CsCl density gradient followed after the density shift. Previous experiments have demonstrated that the minimal interreplication time for origin-proximal and distal sequences are the same in *E. coli* (Olsson et al. 2002) and we chose the *tus* locus for our experiments. Before the density shift, cells contained only two heavy DNA strands (HH) and consequently *tus* was found in this fraction. When shifted to light medium, *tus* appeared in fractions of hybrid density DNA (HL) with a corresponding decline of *tus* in HH-DNA. Further replication of HL-DNA resulted in *tus* appearance in Light (LL)-DNA as well. Figure 3 shows the distribution of *tus*-specific DNA in HH-DNA, HL-DNA, and LL-DNA fractions exactly one generation after the density shift. For both the wild-type and Hda-deficient cells, almost all of *tus*-specific DNA was found in the HL fraction (Fig. 3), as expected when cells initiate each origin once and only once per cell cycle. The minimal interreplication time was estimated either from the maximum value of the HL-DNA (as described in Olsson et al. 2002) or from the time after the shift corresponding to the first appearance of the LL-DNA, and this was determined to be 0.55 generation for wild-type cells (Fig. 3) in agreement with previous experiments (Olsson et al. 2002). In Δ*hda* mutant cells, the minimal interreplication time was reduced slightly compared with wild-type cells (0.45 vs. 0.55) (Fig. 3), indicating that Δ*hda* mutant cells only rarely initiate the same origins more than once within the same cell cycle. This explains why the cellular number of origins and the initiation asynchrony for Hda-deficient cells are only slightly increased (Fig. 2; Camara et al. 2003). In a separate set of experiments, we used an origin-proximal probe (*dnaA*) with essentially the same result (data not shown). For comparison, *seqA* cells deficient in origin sequestration have a minimal interreplication time of 0.1 generation, and *dnaA46* cells displaying complete initiation asynchrony have an interreplication time of 0.3 generation (Olsson et al. 2003), corresponding to the sequestration period (Campbell and Kleckner 1990).

We conclude that Hda-mediated conversion of DnaA–ATP to DnaA–ADP is unlikely to be the sole or the main mechanism to prevent multiple initiations in the same cell cycle. When reinitiation of replication occurs in Hda-deficient cells, it does not coincide with the end of origin sequestration.

Table 1. *Cell cycle parameters for* hda::cat *mutant cells and their Hda⁺ derivatives*

Strain	Relevant genotype	Doubling time (min)	No. of origins/cell	Relative cell mass	Relative number of origins/cell mass
MG1655	wild type	34	4.9	1.0	1.0
ALO1917	*hda::cat*	35	6.1	1.1	1.1
ALO2415	*hda::cat*	34	5.6	1.1	1.0
ALO2852	*hsm-1*	35	4.7	1.0	1.0
ALO2854	*hsm-2*	36	3.8	1.2	0.7

Cells were grown at 37°C in AB minimal medium supplemented with glucose and casamino acids. The cell cycle parameters for these strains are representative of all strains included in the experiment. ALO2852 and ALO2854 are strains ALO1917 and ALO2415, respectively, transduced back to Hda⁺.

Overproduction of the Hda protein delays initiation of replication

To study the effect of different levels of Hda protein on initiation of replication and initiation synchrony, we constructed plasmid pLR9, which carries the *hda* gene under the control of the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible $lacP_{A1\text{-}04/03}$ promoter (Lanzer and Bujard 1988). Wild-type cells containing either the vector plasmid (pFH2102) (von Freiesleben et al. 2000) or pLR9 were grown exponentially with different steady-state concentrations of IPTG, treated with rifampicin and cephalexin, and analyzed by flow cytometry (Fig. 4).

Cells carrying the vector pFH2102 contained mainly four and eight origins of replication, irrespective of the IPTG concentration (Fig. 4A). This indicates that four origins were initiated in synchrony in these cells (Skarstad et al. 1986). The growth rate of cells carrying plasmid pLR9 decreased with increasing *hda* expression, indicating that this is somewhat detrimental to the cells as previously described (Table 2; Su'etsugu et al. 2004). Cells carrying plasmid pLR9 also contained mainly four origins of replication at all IPTG concentrations, but in addition some cells had five, six, and seven origins (Fig. 4B). Because few cells contained less than four or more than eight replication origins, initiations in these cells took place on four replication origins and each origin was initiated only once per cell cycle. The presence of a significant amount of cells containing five, six, and seven replication origins indicates that the initiation interval—i.e., the time from initiation of the first origin to initiation of the last origin—took up a larger fraction of the cell cycle relative to wild-type cells. This replication pattern was observed even without addition of IPTG, indicating that the $lacP_{A1\text{-}04/03}$ promoter is somewhat leaky. As the IPTG concentration increased, the synchrony pattern remained the same (Fig. 4B), whereas the average number of origins per cell increased gradually up to 14% at 1 mM IPTG (from 5.0 to 5.7) (Table 2). However, the relative cell mass also increased gradually up to

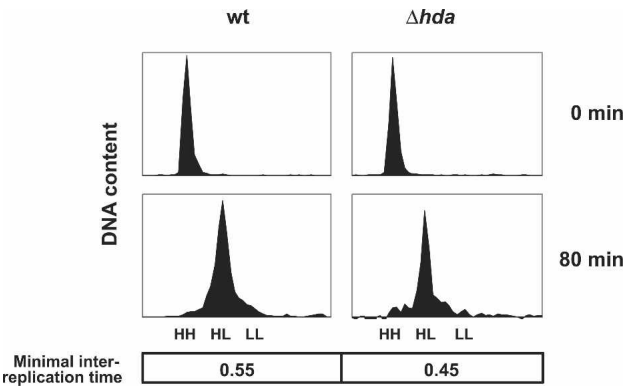

Figure 3. Separation of HH-DNA, HL-DNA, and LL-DNA by equilibrium centrifugation in CsCl density gradients. MG1655 and MG1655 Δ*hda* mutant (ALO1917) cells were grown exponentially at 37°C in heavy glucose-minimal medium for at least 10 generations and then shifted to light minimal medium. Samples were taken at intervals and the chromosomal DNA was isolated, sheared, and subjected to density-gradient centrifugation. About 40 fractions were collected from each gradient. The amount of DNA in each fraction was determined by hybridization with a *tus*-specific radiolabeled probe. The *top* and *bottom* rows show the results at zero and one generation (80 min) after the density shift. The minimal interreplication time was estimated from a complete time-course experiment (Olsson et al. 2002), as the peak value reached by the HL-DNA curve as the asymptotic nature of the appearance of LL-DNA makes a direct estimation of the minimal interreplication time too difficult.

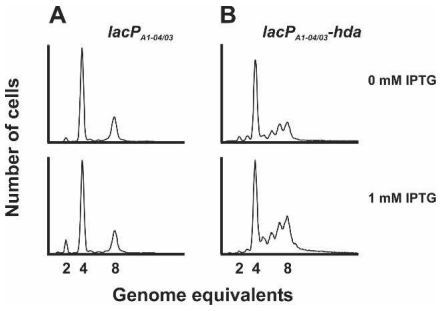

Figure 4. Initiation of replication is delayed by increased expression of the *hda* gene. DNA histograms of MG1655 cells carrying the control plasmid pFH2102 (*A*), and MG1655 cells carrying the *hda* expression plasmid pLR9 (*B*). Cells were grown with or without 1 mM IPTG at 37°C in AB minimal medium supplemented with glucose and casamino acids. Cells were treated with rifampicin and cephalexin prior to flow-cytometric analysis.

Table 2. *Cell cycle parameters for Hda-overproducing strains*

Host strain	Plasmid	IPTG (μM)	Doubling time (min)	No. of origins/cell	Relative cell mass	Relative cell mass/origin
MG1655	pFH2102	0	35	4.9	1.0	1.0
		1000	36	4.8	0.9	0.9
MG1655	pLR9[a]	0	36	5.0	1.3	1.3
		30	39	5.1	1.3	1.2
		100	42	5.3	1.5	1.4
		300	47	5.4	1.6	1.5
		1000	54	5.7	1.7	1.5
MG1655 *hsm-1*	pFH2102	0	35	4.6	1.0	1.1
		1000	36	4.4	1.0	1.1
MG1655 *hsm-1*	pLR9[a]	0	35	5.0	1.4	1.3
		30	39	5.0	1.5	1.4
		100	46	5.2	1.6	1.5
		300	52	5.6	1.7	1.5
		1000	56	5.4	1.8	1.6

Cells were grown at 37°C in AB minimal medium supplemented with glucose, casamino acids, and the indicated concentrations of IPTG.

[a]Plasmid pLR9 is a pFH2102 derivative (von Freiesleben et al. 2000) carrying a copy of the *hda* gene downstream from the $lacP_{A1\text{-}04/03}$ promoter (Lanzer and Bujard 1988).

30% (from 1.3 to 1.7) (Table 2). Consequently, the cell mass per origin at the time of initiation (initiation mass) was increased with increasing *hda* gene induction level (Table 2). Cells remained homogeneous without extensive filamentation, indicating that the SOS response was not turned on to any great extent. Hda overproduction in the *hsm-1* cells had similar effects on growth rate, number of origins per cell, and cell mass as in wild-type cells (Table 2).

Increased *hda* gene expression therefore leads to a delay in initiation of replication. The asynchrony pattern observed during Hda overproduction is consistent with a situation in which the initiation interval is increased, and each origin is initiated once per cell cycle only.

The Hda protein affects dnaA *gene expression*

To determine whether the effects of the Hda protein on DNA replication were mediated through the initiator protein DnaA, we quantified the cellular content of DnaA in strains that expressed different levels of Hda protein. For this quantification, we constructed and used an isogenic wild-type and Δ*hda* strain pair of MG1655 Δ*lac* lysogenized with λRB1. This λ phage carries a *dnaA–lacZ* translational fusion (Braun et al. 1985). In such cells, we can quantify the DnaA protein directly by Western blot analysis or indirectly by the *lacZ* reporter gene activity. In Hda-deficient cells, we found that the DnaA protein concentration was reduced to ~50%–60% of wild-type level (Fig. 5A), and there was a good correlation between data derived from immunoblotting (Fig. 5A, Relative DnaA protein) and from reporter gene activity (Fig. 5A, *dnaA* gene expression). The presence of *hsm-1* alone lowered the DnaA concentration relative to wild-type cells, but not to the same level as in Hda-deficient cells (data not shown).

Next, we quantified the DnaA protein level in wild-type cells that overproduced the Hda protein. For this we used plasmid pLR9 and grew cells to steady state in the presence of various concentrations of IPTG. We observed an increase in DnaA protein concentration that correlated with the Hda induction level (Fig. 5B). Again, there was a good correlation between levels of DnaA protein determined by immunoblotting and increased levels of *dnaA* gene expression measured as β-galactosidase activity (Fig. 5B). Overproduction of Hda in *hsm-1* cells had similar effect on *dnaA* gene expression as was observed for wild-type cells (data not shown).

We conclude that there is a direct correlation between *dnaA* gene expression and Hda protein level in both wild-type and *hsm-1* cells, such that *dnaA* gene expression is reduced by Hda deficiency and increased by Hda overproduction. This was true for cells grown in both minimal medium (Fig. 5) and in rich medium (LB) (data not shown).

Increased levels of DnaA protein are detrimental to Δhda *mutant cells.*

Hda-deficient cells were able to initiate replication at a cell mass comparable to wild-type cells and some origins were even reinitiated within the same cell cycle, despite having their DnaA protein concentration reduced to half of the wild-type level. This suggests that Δ*hda* cells are capable of a more efficient utilization of DnaA protein for initiation than their wild-type counterparts, as if the initiator activity of DnaA was repressed by Hda. We therefore decided to examine the consequences of overexpressing DnaA in Δ*hda* cells. We constructed plasmid pLR40, which carries the *dnaA* gene under control of the wild-type *lac* promoter, as well as a copy of *lacI* for efficient repression in the absence of inducer. When introduced into a *dnaA46* host strain, the presence of pLR40 complemented the temperature sensitivity only in the presence of IPTG, demonstrating that the *dnaA* gene is efficiently repressed by *lacI* (data not shown). As a con-

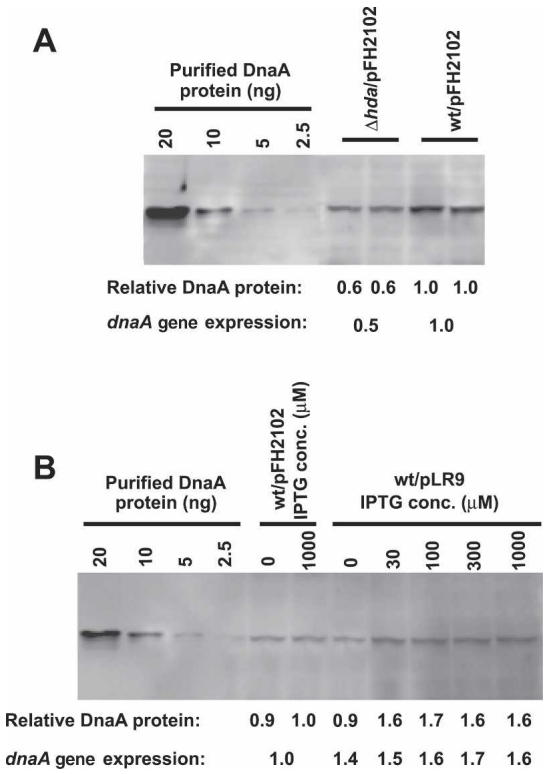

Figure 5. *dnaA* gene expression is affected by the level of Hda protein. MG1655 (λRB1) cells carrying plasmid pFH2102, MG1655 (λRB1) Δ*hda* mutant cells carrying the control plasmid pFH2102, and MG1655 (λRB1) cells carrying the *hda* expression plasmid pLR9 were grown exponentially at 37°C in AB minimal medium supplemented with glucose and casamino acids. Cells in *A* were grown without IPTG. In *B*, the indicated concentrations of IPTG were added to the growth medium. Samples for immunoblotting and for measurement of β-galactosidase activity were taken. For immunoblotting, 12 μg of total protein per lane was loaded on the gel. The control lanes contain the indicated amounts of purified DnaA protein. The relative amount of DnaA protein was quantified for each sample and normalized to wild-type cells in the same experiment (Relative DnaA protein). The β-galactosidase activity was used as an estimate of the level of *dnaA* gene expression as described in Materials and Methods. A β-galactosidase activity level of 1 corresponds to 33 Miller units.

trol plasmid, we used pLR41 that carried a 121-base-pair (bp) deletion in the *dnaA* gene but was otherwise identical to pLR40.

Wild-type, Δ*hda hsm-1*, and *hsm-1* mutant cells carrying the DnaA-overproducing plasmid pLR40 or pLR41 were streaked on minimal medium plates without or with 1 mM IPTG (Fig. 6A). While the viability of wild-type and *hsm-1* cells was unaffected by the addition of IPTG, the viability or growth rate of Δ*hda* cells was clearly reduced; in this case, only slow-growing heterogeneous colonies appeared on the plate (Fig. 6A).

To verify that the DnaA protein was overexpressed to the same level in wild-type and Δ*hda* strains, we grew cells exponentially prior to addition of IPTG to a final concentration of 1 mM. All cells grew with the same doubling time (~40 min) before and after IPTG addition, except the Δ*hda* cells carrying pLR40 (which had a reduced growth rate ~120 min after IPTG addition). Samples for immunoblotting were collected at different time points before and for 2 h following induction of the plasmid-borne *dnaA* gene (Fig. 6B). The immunoblot confirmed that the cellular DnaA protein content of Δ*hda* cells is about half of the wild-type level, in agreement with previous data (Fig. 5). For both wild-type and Δ*hda* cells carrying pLR40, the DnaA protein concentration started to increase 40 min following IPTG induction (Fig. 6B). After 120 min induction, the level of DnaA protein was increased two- to threefold relative to wild-type cells carrying the control plasmid, pLR41, where the DnaA concentration remained unchanged.

Therefore, the DnaA protein could be overproduced two- to threefold in Hda$^+$ cells without any apparent effect on the cellular growth rate, whereas a similar overexpression in Hda-deficient cells was either lethal or resulted in markedly reduced cell growth.

Initiation of replication of Δhda *cells is highly sensitive to changes in DnaA protein concentration*

Because Δ*hda* cells have most of their DnaA protein in the ATP-bound form (Kato and Katayama 2001), which is also the active repressor for a number of genes—including *dnaA* itself (Braun et al. 1985), *nrdAB* (Gon et al. 2006), and possibly *rpoH*, *uvrC*, and *polA* (Messer and Weigel 1997)—the inhibitory effect of DnaA protein overproduction on growth of Δ*hda* cells could result either from efficient transcriptional repression of these genes or from chromosome replication defects.

To evaluate the effect of overproducing DnaA on chromosome replication of wild-type, Δ*hda hsm-1*, and *hsm-1* cells, we induced DnaA protein synthesis in cells containing plasmid pLR40 by addition of 1 mM IPTG. Samples were taken prior to and at various times after induction, treated with rifampicin and cephalexin, and analyzed by flow cytometry (Fig. 7). In agreement with earlier results, we found that overproduction of DnaA protein in wild-type cells resulted in additional initiations from *oriC* and a reduction in initiation synchrony (Fig. 7A; Løbner-Olesen et al. 1989; Morigen et al. 2003). Note that the number of cellular origins started increasing at the same time the level of DnaA protein was raised above wild-type level—i.e., ~40 min (cf. Figs. 6B and 7A)—and continued to increase to an average of 6.9 per cell or 1.7-fold relative to noninduced cells. Overproduction of DnaA in *hsm-1* cells was similar to DnaA overproduction in wild-type cells and resulted in a 1.5-fold increase in number of origins per cell, from 4.3 to 6.3 (data not shown).

For Hda-deficient cells, the situation was different (Fig. 7B). Initiation of replication was stimulated more than in its wild-type counterpart, and at the time when Δ*hda* cells contained an amount of DnaA protein comparable to wild type (30–40 min after IPTG induction) (Fig. 6B) the number of origins had almost doubled (Fig. 7B). Sixty minutes after induction of DnaA synthesis,

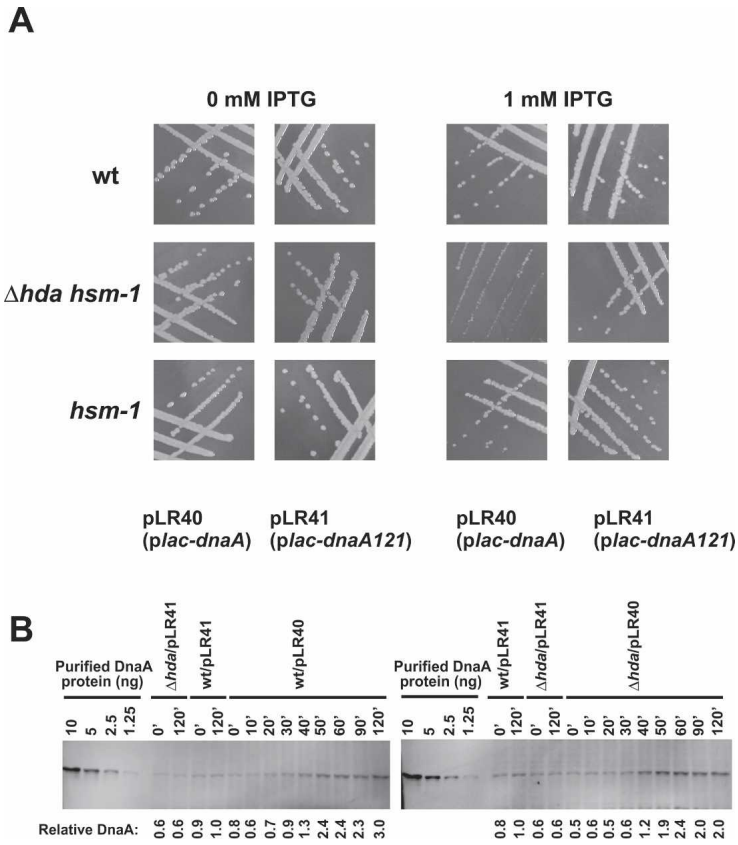

Figure 6. Growth impairment of *hda* mutant cells at an increased DnaA protein level. (*A*) MG1655, MG1655 Δ*hda* (ALO1917), and MG1655 *hsm-1* (ALO2852) cells carrying either the DnaA-overproducing plasmid pLR40 or the control plasmid pLR41 were streaked on AB minimal medium plates supplemented with glycerol and casamino acids with or without 1 mM IPTG. (*B*) Wild-type and Δ*hda* mutant cells carrying either plasmid pLR40 or plasmid pLR41 were grown exponentially at 37°C in AB minimal medium supplemented with glycerol and casamino acids. At $t = 0$, IPTG was added to a final concentration of 1 mM and samples for immunoblotting were collected at different time points following induction. The total protein content was determined, and 5 µg were loaded on the gel. The control lanes contain the indicated amounts of purified DnaA protein. The relative amount of DnaA protein was quantified for each sample and normalized to wild-type cells carrying the plasmid pLR41 at 120 min.

chromosome replication could no longer proceed to completion in the presence of rifampicin and cephalexin and the number of origins per cell could not be determined. Surprisingly, we consistently observed a decrease in DNA content of rifampicin- and cephalexin-treated cells after longer periods of DnaA protein synthesis (Fig. 7B, 120 min). This decrease may result from either DNA degradation or a semisynchronous cell division induced by DNA replication. For both wild-type and Δ*hda* cells containing the control plasmid pLR41, there was no effect of IPTG addition on DNA replication (Fig. 7C,D).

These data clearly show that initiation of replication in Δ*hda* cells increased rapidly in response to additional DnaA protein. Significant overinitiation in these cells was observed at near-wild-type DnaA protein levels. This result is in agreement with a reduced DnaA protein requirement for initiation of replication in Δ*hda* cells.

Increased level of DnaA protein leads to uncontrolled replication initiation in Δhda *cells*

The inability of DnaA-overproducing Δ*hda* cells to complete chromosome replication during the rifampicin and cephalexin incubation period and the eventual decrease in DNA content could be explained if elongation or initiation of replication was compromised when DnaA protein concentration increased above a critical level.

To assess whether initiation of replication was affected, we determined the origin-to-terminus ratio for wild-type cells, Δ*hda hsm-1*, and *hsm-1* cells carrying the DnaA-overproducing plasmid pLR40 by Southern blot hybridization and quantitative PCR (Q-PCR) before and after induction of DnaA protein synthesis (Fig. 8A).

All cells analyzed had similar *ori*/*ter* ratios of ~2 before *dnaA* gene expression was increased. Following IPTG induction, the *ori*/*ter* ratio for wild-type and *hsm-1* cells overproducing the DnaA protein increased gradually to ~5–6 (Fig. 8A), slightly higher than the increase observed by flow cytometry (Fig. 7A). For the Δ*hda* cells overproducing DnaA, the *ori*/*ter* ratio continued to increase throughout the experiment, ending at a value of 15–20 after 120 min induction. There was a good correlation between the *ori*/*ter* ratios determined by Q-PCR and by Southern blotting (Fig. 8A).

Finally, whole genome microarrays were used to characterize the massive increase in *ori*/*ter* ratio for Δ*hda* cells overproducing DnaA (Fig. 8B,C). Genomic DNA was prepared, fragmented by limited DNaseI digestion, labeled, and hybridized to Affymetrix microarrays (Affymetrix, Inc.) containing all known *E. coli* ORFs as well as probes specific to intergenic sequences. All samples were normalized to DNA from wild-type cells treated with rifampicin and cephalexin, and the signal from each probe set was plotted against chromosomal position in such a way that *oriC* was centered (Løbner-Olesen et al. 2003). As expected, the relative abundance of chromosomal loci diminished with increasing distance from *oriC* for all samples, confirming that initiations occurred

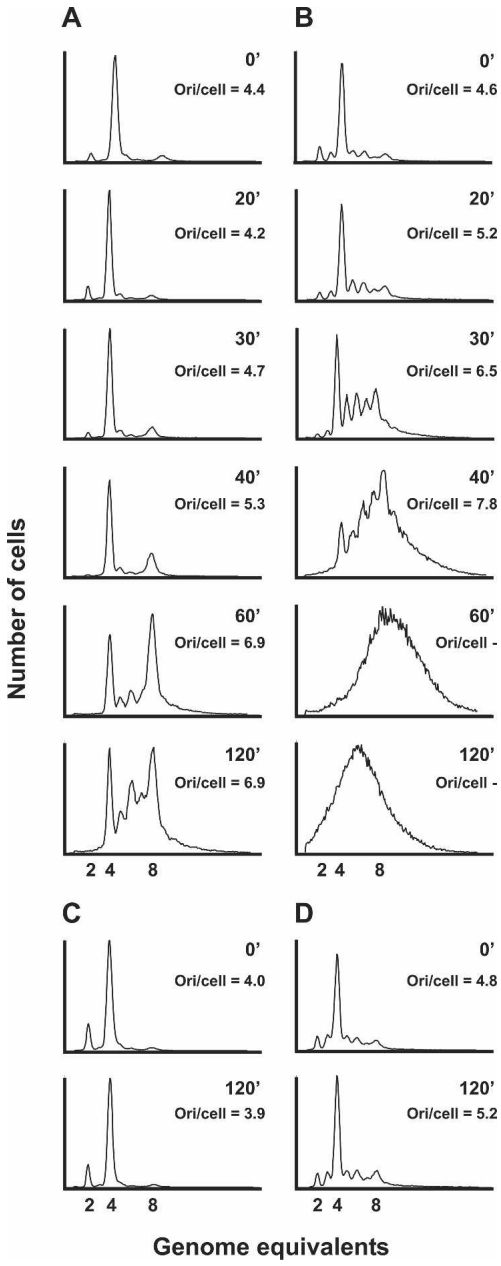

Figure 7. Overproduction of DnaA protein in wild-type and Hda-deficient cells. DNA histograms of MG1655 cells carrying pLR40 (*A*), MG1655 Δ*hda* (ALO1917) cells carrying pLR40 (*B*), MG1655 cells carrying pLR41 (*C*), and MG1655 Δ*hda* cells (ALO1917) carrying pLR41 (*D*). Cells were grown at 37°C in minimal medium supplemented with glycerol and casamino acids. At $t = 0$, IPTG was added to a final concentration of 1 mM. Samples were taken at different times following induction and treated with rifampicin and cephalexin prior to flow-cytometric analysis.

at *oriC* and that replication proceeded bidirectionally. DNA from exponentially growing *seqA* mutant cells was included as a control (Fig. 8B). In agreement with previous reports (von Freiesleben et al. 1994), the *seqA* cells had an increased *ori/ter* ratio. Furthermore, the shape of the curve indicated either that some replication forks collapse before reaching the terminus or that origin-proximal sequences are replicated slower than those closer to the terminus. The fact that *seqA* cells are somewhat SOS-induced (Lu et al. 1994) lends some support to the first explanation.

Wild-type and Δ*hda* mutant cells had a similar chromosome replication pattern prior to induction of *dnaA* gene expression, with an *ori/ter* ratio of ~2 (Fig. 8C). Following induction of *dnaA* gene expression in wild-type cells, the gene dosage of origin-proximal genes was increased about twofold relative to the uninduced level and in agreement with earlier data (Figs. 7, 8; Løbner-Olesen et al. 1989). Hda-deficient cells were different. A huge increase in copy number of chromosomal loci near *oriC* was observed 120 min after *dnaA* gene induction (Fig. 8C), although the *ori/ter* ratio derived by this array analysis was somewhat lower than observed by Southern blot hybridization or Q-PCR (Fig. 8A). The copy number increase was localized to the origin and ~1 megabase (mb) on either side, whereas the *terC*-containing half of the chromosome was little affected. This indicates either that some replication forks collapse before reaching the terminus or that origin-proximal sequences are replicated slower than those closer to the terminus. Because we observed a decrease in DNA content of rifampicin- and cephalexin-treated Δ*hda* cells upon prolonged DnaA overproduction (Fig. 7B, 120 min), we believe that replication forks frequently collapse in these cells, generating double-stranded DNA breaks that in turn lead to excessive DNA degradation during the rifampicin and cephalexin incubation period.

We conclude that the loss of Hda function by itself does not significantly alter the chromosome replication pattern. However, absence of Hda function renders *oriC* much more sensitive to the initiation activity of DnaA; less DnaA is required to initiate replication, and DnaA overproduction leads to massive overinitiation from *oriC* and compromised cell growth.

Discussion

We have examined the importance of Hda (RIDA) in regulating initiation of replication from the chromosomal origin and found no gross cell cycle defects in either Hda-deficient or Hda-overproducing cells. The cellular level of Hda was directly correlated with *dnaA* gene expression, such that Hda deficiency led to reduced *dnaA* gene expression and overproduction of Hda led to DnaA overproduction. Hda-deficient cells were highly sensitive to variations in DnaA protein content, and DnaA overproduction led to uncontrolled initiation from *oriC*. This suggests that RIDA and *dnaA* gene autoregulation act in concert to secure once-per-cell-cycle initiation.

Viability of Hda-deficient cells

The *hda* gene was previously reported to be essential (Kato and Katayama 2001) or dispensable (Camara et al.

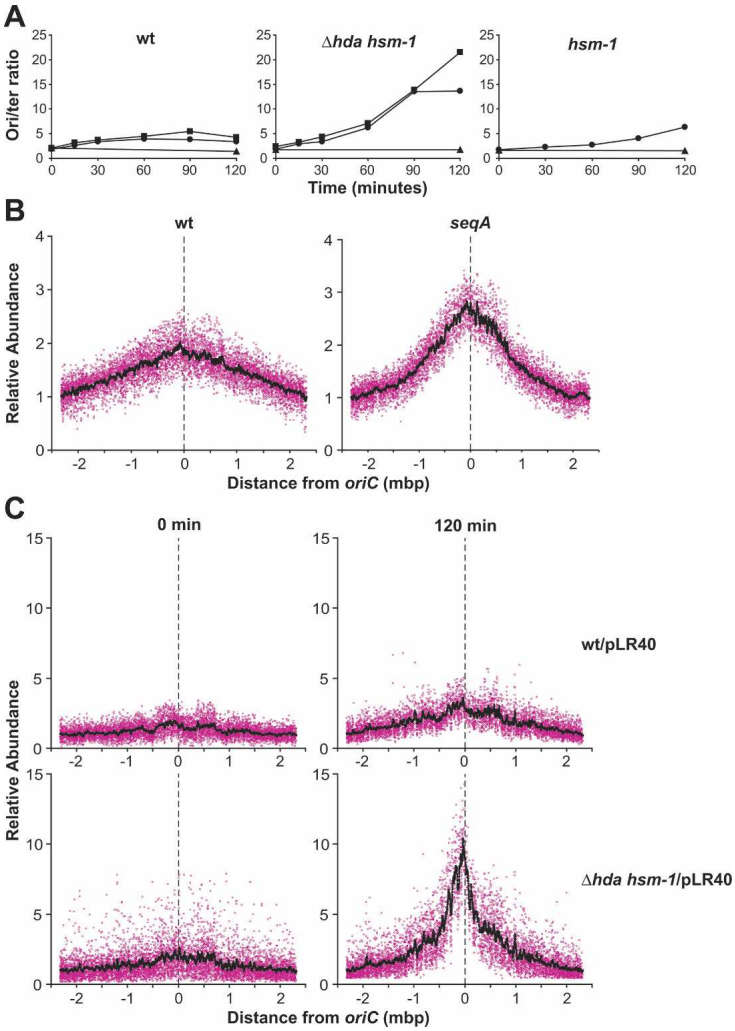

Figure 8. Uncontrolled initiation from *oriC* in Δ*hda* mutant cells overproducing the DnaA protein. (*A*,*C*) MG1655, MG1655 Δ*hda hsm-1* (ALO1917), and MG1655 *hsm-1* (ALO2852) cells carrying either the DnaA-overproducing plasmid pLR40 or the control plasmid pLR41 were grown exponentially at 37°C in AB minimal medium supplemented with glycerol and casamino acids. At $t = 0$, IPTG was added to a final concentration of 1 mM. (*B*) MG1655 and MG1655 *seqA* mutant cells were grown at 37°C in minimal medium supplemented with glucose and casamino acids. (*A*) Quantification of the origin-to-terminus ratio by Southern blot hybridization and Q-PCR (see Materials and Methods) for samples taken at the indicated times after induction. (■) *ori*/*ter* ratio obtained from Southern blot hybridization for cells carrying pLR40. (●) *ori*/*ter* ratio obtained from Q-PCR for cells carrying pLR40. (▲) *ori*/*ter* ratio obtained from Q-PCR for cells carrying pLR41. Microarray analysis of genomic DNA prepared from MG1655 and MG1655 *seqA* mutant cells (*B*) or from MG1655 and MG1655Δ*hda hsm-1* (ALO1917) cells carrying the DnaA-overproducing plasmid, pLR40 (*C*). DNA samples were treated as described in Materials and Methods and hybridized to Affymetrix *E. coli* Antisense Genome Arrays. The *X*-axis represents the chromosomal location of each probe set in megabase pairs (mbp), with *oriC* located in the center of each plot at 0 mbp, as represented by the vertical dashed black lines. The *Y*-axis represents relative gene dosage (abundance). The abundance of terminus DNA was set to 1 in all panels. The black lines represent the average of a moving window of 50 probe sets. Arrays for cells carrying the control plasmid pLR41 were similar to those obtained for cells carrying pLR40 at $t = 0$.

2003) for cell growth. We found that Δ*hda* mutant strains carried suppressor mutations that improved growth. This strongly suggests that the *hda* gene is either essential or that Hda deficiency leads to severe growth impairment so suppressors are readily generated. Recently, a null mutation in the *ygfZ* gene, encoding a folate-binding protein, has been identified as an extragenic suppressor of *hda* mutations (Ote et al. 2006). Accumulation of DnaA–ATP in an Hda-deficient cell was counteracted by disruption of the *ygfZ* gene by a yet unknown process, indicating that YgfZ is somehow involved in regulating the DnaA–ATP level in the cell. However, none of the *hsm* strains showed the phenotypes of *ygfZ* mutants or DNA sequence alterations in the *ygfZ* gene. We also sequenced the *oriC* region and the *dnaA* gene in the Δ*hda hsm* strains, and no mutations were found except in the *hsm-2* strain, which had a mutation in the *dnaA* gene (DnaAF349V). Position 349 is in the amphipathic α-helix thought to interact with phospholipids (Garner et al. 1998). Another mutation that alters the protein in the same region, DnaAA345S isolated by its ability to increase expression of the *nrdAB* genes (Ortenberg et al. 2004; see below), was reported to be deficient in both ATP binding and hydrolysis (Gon et al. 2006). We therefore consider it likely that nucleotide binding by the DnaAF349V protein is altered, which would also explain the initiation asynchrony of cells carrying this mutation (Skarstad et al. 1986) and the fact that dnaAF349V gene expression was not reduced in Hda-deficient cells (data not shown). The properties of the *dnaAF349V* mutant will be described in detail elsewhere.

The *hsm-1* mutation used in this study represented the most frequent class of Δ*hda* suppressor mutations. The only difference observed between *hsm-1* and wild-type cells was a slightly reduced total DnaA protein concentration in the former. *hsm-1* cells were, however, similar to wild-type cells with respect to all cell cycle parameters analyzed, including doubling time, cell size, DNA content, initiation synchrony, sensitivity to additional Hda protein, Hda-dependent *dnaA* gene expression, and sensitivity to variations in DnaA protein. We therefore consider it unlikely that the suppressor mutation contributes to the cell cycle effects we ascribe to *hda*, although we cannot completely rule out that *hsm* plays a role that is only observed in Hda-deficient cells.

Furthermore, this indicates that the lethality of Hda deficiency may not result from overinitiation from *oriC* as previously suggested (Kato and Katayama 2001), but could be associated with the gene regulation function of DnaA–ATP within the cell. It was reported recently that a multicopy plasmid carrying the *nrdAB* genes, encoding ribonucleotide reductase, suppresses an *hda* mutation (Gon et al. 2006). The upstream region of the *nrdAB* operon contains ATP–DnaA boxes, and transcription of the *nrdAB* operon is regulated by the level of DnaA–ATP (Gon et al. 2006). In Hda-deficient cells, where most of the DnaA protein is bound to ATP (Kato and Katayama 2001), the *nrdAB* genes are efficiently repressed and cells do not synthesize sufficient ribonucleotide reductase to supply precursors for DNA synthesis. Therefore, some or all Hda suppressor mutations may compensate for the limited dNTP synthesis.

Numerous other genes including *rpoH*, *uvrC*, and *polA* also contain DnaA boxes in their promoter regions (Messer and Weigel 1997) and, provided that DnaA–ATP is also an active repressor of these, their expression could also be dramatically reduced in Δ*hda* cells.

The Hda protein affects dnaA *gene expression*

The *E. coli dnaA* gene is transcribed from two promoters, P1 and P2, with a consensus DnaA box between them (Hansen et al. 1982). There is also a second DnaA box with a mismatch and four DnaA–ATP boxes located between the promoters (Speck et al. 1999). DnaA–ATP binds to all boxes, and this form of the DnaA protein is a much better repressor for *dnaA* gene transcription than ADP-bound DnaA protein, which only binds to the consensus DnaA boxes (Speck et al. 1999; Gon et al. 2006).

Therefore, a likely explanation for the decreased level of total DnaA protein in Δ*hda* mutant cells is that they contain an increased level of DnaA–ATP relative to wild-type cells (Kato and Katayama 2001) that in turn efficiently represses *dnaA* gene transcription. Increased *dnaA* gene expression upon Hda overproduction can be explained by the same rationale. The extra Hda protein accelerates RIDA and results in an increased level of DnaA–ADP and a reduced level of DnaA–ATP. Because DnaA–ADP is inefficient as a repressor of the *dnaA* gene, its transcription is increased. This suggestion implies that the level of Hda protein is limiting for RIDA activity in wild-type cells.

RIDA and immediate reinitiation in E. coli

RIDA is often suggested as a mechanism for preventing reinitiation of replication in *E. coli* (Katayama et al. 1998; Kato and Katayama 2001; Camara et al. 2003, 2005). However, sequestration-deficient *seqA* cells frequently initiate replication in the same cell cycle (Olsson et al. 2002), resulting in asynchrony (Lu et al. 1994), and have an increased origin-to-terminus ratio, such as observed here (Fig. 8B). SeqA-deficient cells are wild type with respect to RIDA, and conversion of DnaA–ATP to DnaA–ADP is therefore insufficient for limiting initiation at each origin to once per cell cycle. Cells where *dnaA* and *oriC* are not sequestered simultaneously also show a high degree of asynchrony and an elevated origin-to-terminus ratio (Riber and Løbner-Olesen 2005). In such cells DnaA protein accumulates during origin sequestration. Because the cellular ATP/ADP ratio is high (Petersen and Møller 2000), and DnaA protein has the same affinity for ATP and ADP, this newly synthesized DnaA protein is believed to be ATP bound, which in turn stimulates initiation at the end of the sequestration period. Therefore, RIDA is insufficient to prevent origins from being reinitiated in these cells as well.

In Δ*hda* mutant cells, inactivation of DnaA–ATP does not take place as a consequence of initiation, yet asynchrony and overinitiation are modest (Camara et al. 2003; this study). On the occasions where reinitiation takes place, it does not immediately follow sequestration; i.e., the minimal interreplication time is 0.45 generation whereas sequestration lasts 0.3 generation (Campbell and Kleckner 1990). It is conceivable that the DnaA-boxes generated by replication during origin sequestration can titrate sufficient DnaA–ATP away from *oriC* to prevent immediate initiation at the end of sequestration. Therefore, a period of de novo DnaA protein synthesis is necessary to reach the initiation level even in Hda-deficient cells. The limited effect on initiation from *oriC* by deleting *hda* suggests that the primary function of RIDA is not in preventing immediate reinitiation, but rather to increase the fidelity of the initiation process by "fine-tuning" *dnaA* gene expression (see below). It should be noted that our results and conclusions disagree with a recent publication where extensive reinitiation was found in Δ*hda* but not in *seqA* cells (Camara et al. 2005).

The reason for this is not differences in growth conditions, because we also observed limited overinitiation in Hda-deficient cells grown in LB medium (data not shown). The twofold increase in *ori/ter* ratio for *hda* mutants (Camara et al. 2005) is not consistent with earlier Rif run-out results from the same laboratory (Camara et al. 2003) or those presented here. Additionally, the important parameter for measuring overinitiation is origin concentration, and this was not measured in either paper. The different results could be due to the presence of different suppressors in the cells analyzed by the different laboratories. It should be noted that none of our eight isolated Δ*hda* mutants behave like the one previously described (Camara et al. 2005). The microarray experiments done by Camara et al. (2005) with *seqA* cells do not show any increase in origin/terminus ratio relative to the wild-type strain. This disagrees with the data obtained in this study, as well as with flow cytometry data from a number of laboratories that have clearly shown a higher DNA content and an elevated number of *oriC* per cell mass of *seqA* cells (Lu et al. 1994; von Freiesleben et al. 1994). Furthermore, direct measurements of interinitiation time in *seqA* cells by density-shift analysis indicate multiple initiations per cell cycle, consistent with overinitiation (Olsson et al. 2002).

Multiple regulatory mechanisms are necessary for controlled once-per-cell-cycle initiation

The influence of the Hda protein on cell cycle control is, however, still crucial; the concentration of total DnaA protein in Δ*hda* mutant cells was only half the wild-type level, and yet Δ*hda* mutant cells seem to initiate replication at the same average initiation mass (cell mass per origin) as wild-type cells. Therefore, Hda-deficient cells initiate replication at a lower amount of DnaA protein per chromosomal origin. However, this DnaA protein is mainly in the ATP-bound form (Kato and Katayama 2001). An increased activity of DnaA–ATP in vivo correlates well with earlier in vitro data indicating that the ATP-bound form of the DnaA protein is absolutely required, and more efficient than DnaA–ADP for the initiation process (Sekimizu et al. 1987).

Our data from cells overproducing the Hda protein also suggest that DnaA–ATP is most active in the initiation process. In these cells the level of total DnaA protein is increased by 60%, yet initiations take place at an increased mass per origin. The total amount of DnaA protein required for initiation at an origin is therefore increased, presumably because the majority is in the ADP-bound less-active form. Because the rate of DnaA–ATP protein accumulation in Hda-overproducing cells is slow, the time required to initiate all origins within each single cell—i.e., the initiation interval—is also increased. This is in good agreement with the observation that both ATP- and ADP-bound DnaA protein can bind the origin R-boxes (Sekimizu et al. 1987). DnaA–ADP can therefore augment a limited amount of DnaA–ATP in the initiation process despite being inert for initiation by itself (Yung et al. 1990).

In Hda-deficient cells that cannot convert DnaA–ATP to DnaA–ADP, the DnaA protein is always active and the effects of DnaA protein overproduction are dramatic: uncontrolled initiation from *oriC*, replication fork collapse, and impaired growth. Even at wild-type levels of DnaA protein, the stimulation of initiation of replication in Δ*hda* mutant cells was notable (Figs. 6, 7, 30–40 min). This is in agreement with an earlier report where temperature-dependent inactivation of *hda* led to increased initiations from *oriC* (Kato and Katayama 2001); these cells also contained near-wild-type levels of DnaA. DnaA-dependent overinitiation in Δ*hda* cells resembles the overinitiation observed when the DnaAcos (Simmons et al. 2004) or DnaAR334A (Nishida et al. 2002) proteins are overexpressed in wild-type cells. The DnaAcos protein is hyperactive because it binds neither ATP nor ADP (Katayama 1994), whereas the DnaAR334A is unable to hydrolyze bound ATP (Nishida et al. 2002), and consequently both fail to be inactivated by RIDA. Thus, it is the controlled supply of the active initiator protein (DnaA–ATP) that is the predominant mechanism for copy-number control of the *E. coli* chromosome, and Hda-mediated conversion of DnaA–ATP to DnaA–ADP fine-tunes the feedback regulation, limiting the supply of the initiation factor to the *oriC* replicon. The sensitivity of Hda-deficient cells to variations in the DnaA protein level demonstrates that cells containing only the active form of the initiator protein are likely to display increased cell-to-cell variations in the timing of the initiation process, simply due to stochastic variation in the initiator concentration within single cells. This explains why the interinitiation period is slightly reduced in Δ*hda* mutant cells.

In Hda$^+$ cells, overproduction of DnaA protein also increases initiation at *oriC*, but the cellular origin content is only raised two- to threefold. An explanation for this dampened copy-number increase is that, although the newly synthesized ATP-bound DnaA protein stimulates initiation of replication, the initiation process itself generates new replication forks that accelerate RIDA to convert DnaA to the less-active ADP-bound form. RIDA thus limits overinitiation to a level that can be tolerated by the cells. Based on these observations, we propose that RIDA may serve as a homeostatic mechanism that serves to maintain the copy number of the chromosome even in wild-type cells. This would be a situation analogous to the monomer dimer competition in plasmid P1 replication (Das et al. 2005).

At least three homeostatic mechanisms serve to respond to an increase in initiator and *oriC* copy number in *E. coli* by a decrease in initiation rate. All of these affect the DnaA initiator protein. First, the initiation process generates new replication forks that promote RIDA to decrease the overall activity of the DnaA protein. Second, autoregulation of *dnaA* gene expression ensures that de novo DnaA synthesis is slowed when the level of DnaA–ATP increases within the cell. Third, titration of DnaA protein to binding sites outside the origin serves to reduce the free initiator concentration (Hansen et al. 1991; Kitagawa et al. 1996; Morigen et al. 2001). Each of the three homeostatic mechanisms would also respond to a decrease in initiator and *oriC* copy number by increasing the initiation rate. It is therefore likely that these homeostatic mechanisms act in concert to ensure timely initiation; i.e., maintain a constant interinitiation time in wild-type cells.

Materials and methods

Strains

All strains used were *E. coli* K-12 and derived from MG1655 (λ$^-$F$^-$) (Guyer et al. 1981). The *hda*::*cat* mutants were constructed by deletion of the chromosomal *hda* gene by homologous recombination with a linear DNA fragment. The *cat* gene was amplified from plasmid pACYC184 (Chang and Cohen 1978) using the oligonucleotides 5′-AAGGCGTTCGCGCCG CATCCGACAATAAACACCTTATCTAGCACCAGGCGTTT AAGGGCACC-3′ and 5′-GTACCGACCGGGCAGTGTTGC TGCCCGGTTCAAACATCATGTAAGTTGGCAGCATCACC CG-3′ as primers. The 5′ ends of the primers (40 bp) were homologous with DNA sequences flanking the wild-type *hda* gene. Homologous recombination using the λ *red* system (Yu et al. 2000) resulted in replacement of *hda* with *cat*. The *hda*::*cat* mutation was later transferred to either the MG1655 strain or the MG1655 λRB1 strain by P1-phage-mediated transduction (Miller 1972). The

hda::*cat* mutants were converted into Hda$^+$ cells by cotransduction with the *purC80*::Tn*10* allele derived from strain CAG18470 (Singer et al. 1989), followed by transduction to Pur$^+$.

Plasmids

The pBR322-based vector pFH2102 (von Freiesleben et al. 2000) carries the *lacI* gene and the $lacP_{A1\text{-}04/03}$ promoter (Lanzer and Bujard 1988) together with the *bla* gene. Plasmid pLR9 is a pFH2102 derivative carrying the *hda* gene downstream the $lacP_{A1\text{-}04/03}$ promoter. The MG1655 chromosomal *hda* gene was amplified by PCR with oligonucleotides 5′-ATGCTCTAGAC GGTTCAAACATCATGGGATTC-3′ and 5′-TTCGCGCCG GATCCGACAATAAAC-3′ as primers. This fragment was digested with XbaI and BamHI, and ligated into pFH2102 digested with the same restriction enzymes. Sequence analysis later revealed that the *hda* gene was 100% identical to the wild-type sequence (Blattner et al. 1997).

Plasmid pALO12 is a pBR322 derivative carrying the *dnaA* gene exclusively under *lac* promoter control. Plasmid pALO13 is pALO12 containing a 121-bp deletion internally in the *dnaA* gene (Løbner-Olesen et al. 1989). Plasmids pLR40 and pLR41 are derivatives of pALO12 and pALO13, respectively, carrying a copy of the *lacI* gene encoding the Lac repressor. The MG1655 chromosomal *lacI* gene including promoter was amplified by PCR with the primers 5′-CGTATCCCGAGCCGTAATCATG GTCATAGCTG-3′ and 5′-GATAGTCCTTGGGACACCATC GAATGGCGCAAAAC-3′. The PCR fragment was digested with AvaI and StyI, and ligated into either pALO12 or pALO13 digested with the same restriction enzymes.

Growth conditions

Cells were grown in LB medium or AB minimal medium (Clark and Maaløe 1967) supplemented with 0.2% glucose or 0.2% glycerol, and 10 µg/mL thiamine and, when indicated, Casamino Acids were added to a final concentration of 0.5%. Adenosine was added to 30 µg/mL where indicated. All cells were cultured at 37°C.

Flow cytometry

Exponentially growing cells (OD_{450} = 0.15–0.25) were treated with rifampicin (300 µg/mL; Norvatis Pharma, Inc.) and cephalexin (36 µg/mL; Sigma Chemical Co.) to inhibit initiation of DNA replication and cell division, respectively (Skarstad et al. 1986; Boye and Løbner-Olesen 1991). Flow cytometry was performed as described previously (Løbner-Olesen et al. 1989) using an Apogee A10 instrument (Apogee, Inc.). For all samples a minimum of 60,000 cells were analyzed.

Density-shift experiments

Meselson-Stahl density-shift experiments were performed as previously described (Olsson et al. 2002).

β-*Galactosidase assay*

Exponentially growing cells (OD_{450} = 0.3–0.4) were permeabilized by toluene, and β-galactosidase activity was determined as described (Miller 1972).

Immunoblotting

Immunoblotting was performed as described previously (Riber and Løbner-Olesen 2005), with a polyclonal anti-DnaA antibody. The membrane was scanned on a Storm 840 imaging system (Molecular Dynamics, Inc.), and quantification was carried out using ImageQuant version 5.2 software (Molecular Dynamics, Inc.).

Southern blot analysis

Southern blot analysis was done as described previously (Riber and Løbner-Olesen 2005).

Q-PCR

Sodium azide (300 µg/mL; Fluka BioChemika) was added to exponentially growing cells (OD_{450} = 0.1–0.4) to prevent further growth. Cells were spun down and adjusted to the same density (OD_{450}) in 0.9% sodium chloride for direct use as DNA template in the PCR reaction. For quantification of the origin, we used primers that amplified part of the *gidA* gene: 5′-TTCGAT CACCCCTGCGTACA-3′ and 5′-CGCAACAGCATGGCGA TAAC-3′. For the terminus, we used primers that amplified part of the *dcp* gene: 5′-TTGAGCTGCGCCTCATCAAG-3′ and 5′-TCAACGTGCGAGCGATGAAT-3′. For the PCR reaction, we used LightCycler FastStart DNA Master^{PLUS} SYBR Green I ready-to-use-reaction mix (Roche, Inc.). Q-PCR was performed in a LightCycler 2.0 Instrument (Roche, Inc.). By use of crossing points and PCR efficiency only, a calibrator-normalized relative quantification analysis was performed using LightCycler Software version 4.0 (Roche, Inc.) for determining the *ori*/*ter* ratio of each sample. These results were normalized to the *ori*/*ter* ratio of a sample treated with rifampicin and cephalexin in which the *ori*/*ter* ratio was expected to be close to 1.

Microarray analysis

Total cellular DNA was isolated from exponentially growing cells as described previously (Løbner-Olesen and von Freiesleben 1996). The DNA was denatured by treatment with 0.25 M NaOH for 30 min at 65°C, followed by neutralization with HCl. Denatured DNA was fragmented by incubating for 10 min at 37°C with 0.15 U of DNase I (Amersham Biosciences, Inc.) per microgram of DNA. The average fragment sizes were estimated by gel electrophoresis to ~50–200 bases. For each array, 2–4 µg of fragmented single-stranded DNA was labeled and hybridized to Affymetrix *E. coli* Antisense Genome Array, as recommended by the supplier (Affymetrix, Inc.). Data were processed using the Microarray Suite version 5.0 software (Affymetrix, Inc.) and raw data were exported to Microsoft Excel 2002. All data points were normalized to data points obtained with DNA from MG1655 cells treated with rifampicin and cephalexin, thereby containing fully replicated chromosomes only. Outlying data points with values more than three standard deviations from the average values of the 20 surrounding probes were removed. Finally, the data were normalized to the average value of the probes opposite the origin to give a relative abundance of the terminus region of 1.

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