A novel regulatory mechanism couples deoxyribonucleotide synthesis and DNA replication in *Escherichia coli*

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We present evidence for a complex regulatory interplay
between the initiation of DNA replication and deoxyribo-
nucleotide synthesis. In *Escherichia coli,* the ATP-bound
DnaA protein initiates chromosomal replication. Upon
loading of the β-clamp subunit (DnaN) of the replicase,
DnaA is inactivated as its intrinsic ATPase activity is
stimulated by the protein Hda. The β-subunit acts as a
matchmaker between Hda and DnaA. Chain elongation of
DNA requires a sufficient supply of deoxyribonucleotides
(dNTPs), which are produced by ribonucleotide reductase
(RNR). We present evidence suggesting that the molecular
switch from ATP-DnaA to ADP-DnaA is a critical step
coordinating DNA replication with increased deoxyribo-
nucleotide synthesis. Characterization of *dnaA* and *dnaN*
mutations that result in a constitutively high expression of
RNR reveal this mechanism. We propose that the nucleo-
tide bound state of DnaA regulates the transcription of
the genes encoding ribonucleotide reductase (*nrdAB*).
Accordingly, the conversion of ATP-DnaA to ADP-DnaA
after initiation and loading of the β-subunit DnaN would
allow increased *nrdAB* expression, and consequently, co-
ordinated RNR synthesis and DNA replication during the
cell cycle.

Introduction

The initiation of chromosome replication is tightly regulated.
In prokaryotes, DnaA plays a key role in promoting and
regulating this process, binding directly to the origin of
replication (Kornberg and Baker, 1992; Baker and Bell,

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1998; Messer, 2002). This binding facilitates DNA strand
opening, allowing loading of helicase, primase and DNA
polymerase (pol) III holoenzyme (the chromosomal repli-
case), thus leading to chromosomal replication (Kornberg
and Baker, 1992). An early step in replicase assembly
is loading of the sliding clamp, a ring-shaped dimer of the
β-subunit (DnaN) of pol III. After encircling the DNA strand,
β-subunit stabilizes the replicase, enabling the extension of
RNA primers and processive replication (Kornberg and Baker,
1992; Baker and Bell, 1998). These steps in DNA replication
parallel those in eukaryotic cells (Bell and Dutta, 2002).

In *Escherichia coli*, DnaA protein forms a stable complex
with ATP or ADP, but only the ATP-bound form is active in
DNA strand opening during initiation (Sekimizu *et al*, 1987).
After completion of initiation, loading of the β-subunit
(DnaN) protein as a sliding clamp onto template promotes
the accelerated hydrolysis of active ATP-DnaA to inactive
ADP-DnaA, preventing premature reinitiation of chromosome
replication, a process termed 'regulatory inactivation of DnaA
(RIDA)' (Katayama *et al*, 1998). That the DNA-bound form of
β-subunit is required for DnaA-ATP hydrolysis ensures timely
coupling between DnaA inactivation and the elongation stage
of DNA replication. Also involved in this process is the
protein, Hda (Kato and Katayama, 2001). The ATPase activity
is induced by the interaction of Hda with DnaA, facilitated by
β-subunit loaded onto DNA as a sliding clamp (Su'etsugu
et al, 2005).

Another essential enzyme required for DNA replication is
ribonucleotide reductase (RNR) which catalyzes the reduc-
tion of ribonucleotides to deoxyribonucleotides (Thelander
and Reichard, 1979). *E. coli* RNR comprises two nonidentical
and coordinately synthesized subunits, R1 and R2 (Hanke
and Fuchs, 1983). The activity of the holoenzyme is con-
trolled by feedback regulation through binding of allosteric
effectors to R1 (Eliasson *et al*, 1994). However, the transcrip-
tional control of RNR synthesis is poorly understood.

The amount of RNR protein, and thus, deoxyribonucleo-
tides, is coordinated with the rate of DNA synthesis, which is
determined by the initiation events. In *E. coli* growing with
a generation time longer than the time required for chromo-
some replication, the need for RNR goes from zero to a
maximum as DNA initiation occurs. The genes encoding
the two RNR subunits, *nrdA* and *nrdB,* comprise an operon
and are cell cycle regulated. *nrd* mRNA synthesis increases at
approximately the time of DNA initiation (Sun and Fuchs,
1992), similarly to what is observed in eukaryotic organisms
(Engstrom *et al*, 1985). This regulatory mechanism generates
a controlled burst of RNR synthesis every time a new
replication fork is initiated and ensures the appropriate
amount of enzyme under any growth condition.

In addition to its role in initiation of replication, DnaA also
acts as a transcriptional regulator (Messer and Weigel, 1997).
DnaA autoregulates its own transcription and controls either

positively or negatively the expression of several other genes (Messer and Weigel, 1997). The nucleotide-bound state of DnaA is important for its activity as a transcriptional regulator. While ATP-DnaA and ADP-DnaA bind to 9-mer DnaA boxes with equal affinity, ATP-DnaA, but not ADP-DnaA, binds to a 6-mer ATP-DnaA boxes (Speck *et al*, 1999). Thus, ATP-DnaA represses *dnaA* transcription much more efficiently than ADP-DnaA (Speck *et al*, 1999). The *nrd* promoter region contains two 9-mer DnaA boxes as well as sequences conforming to ATP-DnaA boxes located immediately upstream of the *nrdAB* putative promoter (Tuggle and Fuchs, 1986; Speck *et al*, 1999). DnaA binds to these DnaA boxes and regulates transcription of the *nrdAB* operon (Augustin *et al*, 1994). Thus, DnaA would be a good candidate for coupling *nrdAB* expression in a cell cycle-dependent manner to DNA replication initiation.

In previous studies, we isolated suppressor mutations in the *dnaAN* locus that restored growth to a mutant depleted of the enzymes required for RNR reduction (Ortenberg *et al*, 2004). Mutations $dnaA_{A345S}$, $dnaA_{T174P}$ and $dnaN_{G157C}$ result in derepression of the operon (Ortenberg *et al*, 2004). This regulation appears to act at the transcription level as fusions of the *lacZ* gene to *nrdAB* show a similar derepression for β-galactosidase in these mutant backgrounds. In the present studies, we explore the molecular mechanism that links *nrdAB* expression and DNA replication. We investigate the *in vitro* and *in vivo* replication initiation behavior of DnaA proteins from both *dnaA* mutant strains. We show that the $DnaA_{A345S}$ and $DnaA_{T174P}$ mutant proteins are constitutively inactivated, exhibiting either high intrinsic ATPase activity

($DnaA_{T174P}$) or a defect in ATP binding combined with higher intrinsic ATPase activity ($DnaA_{A345S}$). Therefore, neither DnaA mutant protein requires Hda for its conversion to the inactive ADP form. We show that the switch of ATP-DnaA to ADP-DnaA is not only a negative feedback mechanism that prevents premature reinitiation from occurring, but is also the mechanism that allows the coupling of DNA replication initiation with synthesis of dNTPs required during the elongation step of DNA replication.

Results

Defect of ATP binding in DnaA$_{A345S}$ and high intrinsic ATPase activity in DnaA$_{T174P}$ mutant proteins

ATP binding. We speculated that the increase in RNR seen in the *dnaA* and *dnaN* mutant strains previously isolated by us (see Introduction) might be due to alterations in the switch between ATP-DnaA and ADP-DnaA, since changes in either DnaA or β-subunit might be expected to influence this process. The mutations might affect ATP binding to DnaA or hydrolysis of DnaA-bound ATP. We purified $DnaA_{A345S}$ and $DnaA_{T174P}$ mutant proteins and wild-type DnaA protein and examined them for ATP binding using a filter retention assay (Sekimizu *et al*, 1987). This assay revealed that $DnaA_{A345S}$ but not $DnaA_{T174P}$ is defective in ATP binding (Figure 1A).

ATPase activity. $DnaA_{T174P}$, which binds ATP similarly to wild-type DnaA, has a dramatically increased intrinsic ATPase activity. Bound ATP is totally hydrolyzed in less than 3 min, while, for wild-type DnaA, only half of the
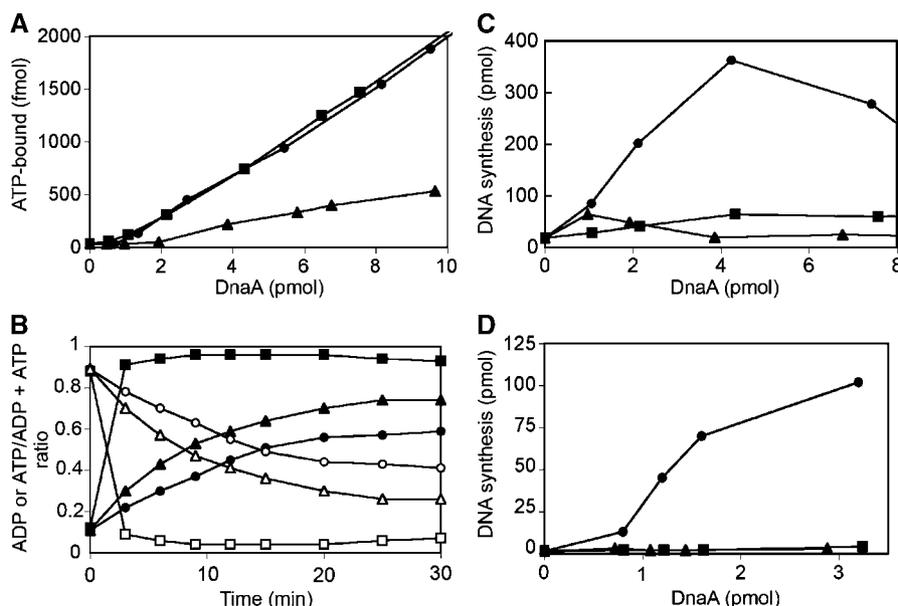

Figure 1 DnaA$_{T174P}$ and DnaA$_{A345S}$ mutant proteins with increased intrinsic ATPase activity and/or defect in ATP binding are unable to initiate replication *in vitro*. (**A**) ATP binding. DnaA$_{A345S}$ mutant is defective in its ability to bind ATP. Purified wild-type DnaA (●), mutant DnaA proteins DnaA$_{A345S}$ (▲) and DnaA$_{T174P}$ (■) were incubated with [α-^{32}P]ATP for 15 min on ice. Samples were filtered through nitrocellulose filters and the remaining protein-bound ATP was measured by liquid scintillation counting. (**B**) Intrinsic ATPase activity. Purified wild-type DnaA (●), mutant DnaA proteins DnaA$_{A345S}$ (▲) and DnaA$_{T174P}$ (■) were incubated with [α-^{32}P]ATP for 15 min on ice. The resulting ATP-bound proteins were incubated at 38°C with pBSoriC. Protein-bound ATP (open symbols) and ADP (closed symbols) were captured by filter retention, extracted from the filters, and separated by thin-layer chromatography. The ratio of extracted total bound nucleotide (ATP + ADP) for the last two time points (25 and 30 min) relative to the first two time points (0 and 3 min) for DnaA, DnaA$_{T174P}$, and DnaA$_{A435S}$ were 0.77, 0.61, and 0.53. (**C, D**) *In vitro* replication. DnaA$_{A345S}$ and DnaA$_{T174P}$ are inactive in *oriC* plasmid replication. Purified wild-type DnaA (●) and mutant DnaA proteins DnaA$_{A345S}$ (▲) and DnaA$_{T174P}$ (■) were examined for their abilities to support *in vitro* replication of an *oriC* plasmid in a system reconstituted with purified protein components (C) and in a crude extract lacking DnaA activity (D).

bound ATP is hydrolyzed in 15 min (Figure 1B). Detectable increases in the extent and the rate of the low amount of bound ATP hydrolysis are also seen with the DnaA$_{A345S}$ protein (Figure 1B).

Mutations in dnaA and dnaN reduce initiation efficiency

In vitro oriC replication. Since the ATP-bound form of DnaA is thought to initiate chromosomal replication, the two mutant DnaA proteins should be defective for replication initiation, as they exhibit either reduced ATP binding or high intrinsic ATPase activity. In agreement with this presumption, both DnaA$_{A345S}$ and DnaA$_{T174P}$, in contrast to wild-type DnaA, are inert for *in vitro* DNA replication from *oriC* in a system reconstituted with purified proteins (Figure 1C) or when added to crude cell extracts lacking DnaA activity (Figure 1D), behaving similarly to ADP-bound wild-type DnaA protein (data not shown).

Flow cytometry analysis. Despite the *in vitro* replication initiation deficiency of the DnaA$_{A345S}$ and DnaA$_{T174P}$ proteins, the cells carrying either of the *dnaA* mutations present only a slight reduction in growth rate (Figure 2A) and form normal-sized colonies on solid media (data not shown). This finding suggests that the mutant DnaA protein can support the required functions of DnaA *in vivo*. Thus, the lack of initiation activity *in vitro* is likely not due to the mutations adversely affecting other essential DnaA functions. In order to further assess the *in vivo* effects of *dnaA$_{A345S}$*, *dnaA$_{T174P}$* and *dnaN$_{G157C}$* mutations on replication initiation, we performed flow cytometry on these strains to determine whether the mutants 'under-replicate', that is, replicate fewer chromosomes per mass.

These measurements show a decrease in DNA content per cell and also per mass in the *dnaA$_{A345S}$*, *dnaA$_{T174P}$* and *dnaN$_{G157C}$* mutant cells compared to wild-type cells (Figure 2A). The increased mass/DNA ratio suggests under-replication in the *dnaA$_{A345S}$*, *dnaA$_{T174P}$* and *dnaN$_{G157C}$* mutants, and indicates that these cells have problems initiating replication. To further investigate the replication pattern, exponentially growing cells were treated with rifampicin and cephalexin to inhibit initiation of replication and cell division. Growth was continued for four to five generations to allow ongoing rounds of replication to finish. The number of
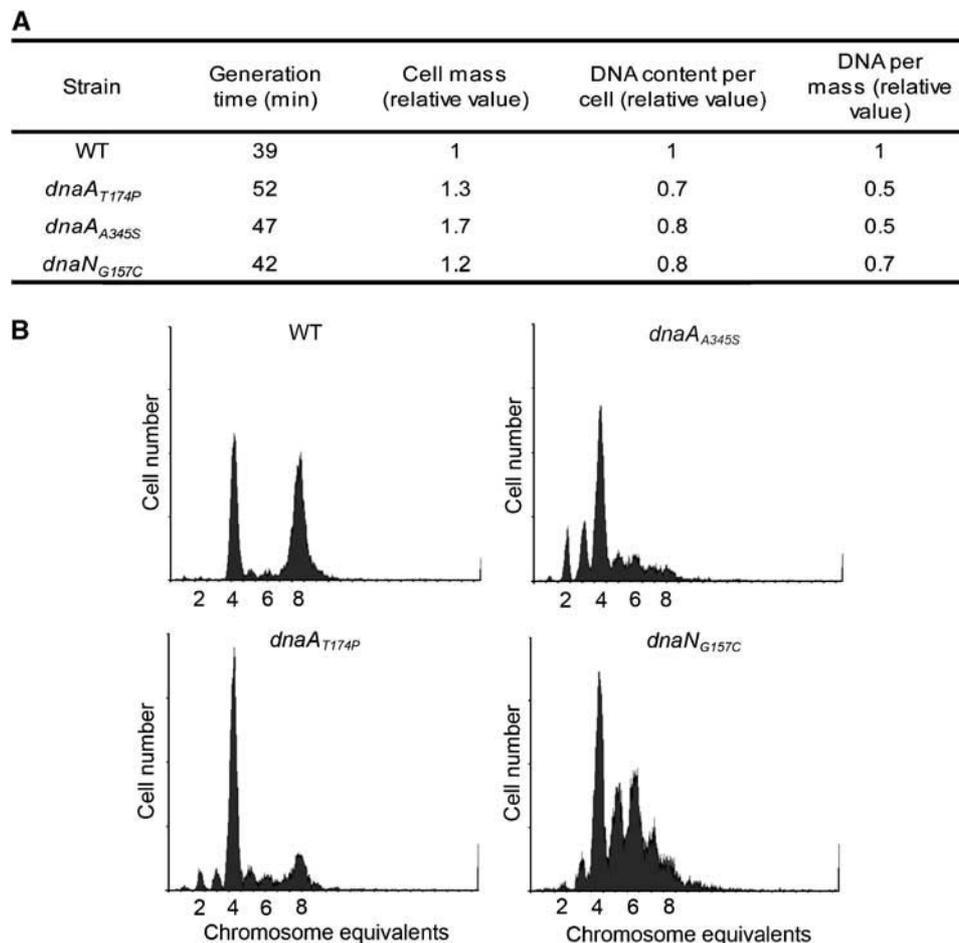

A

Strain	Generation time (min)	Cell mass (relative value)	DNA content per cell (relative value)	DNA per mass (relative value)
WT	39	1	1	1
dnaA$_{T174P}$	52	1.3	0.7	0.5
dnaA$_{A345S}$	47	1.7	0.8	0.5
dnaN$_{G157C}$	42	1.2	0.8	0.7

Figure 2 Under-replication in *dnaA$_{T174P}$*, *dnaA$_{A345S}$* and *dnaN$_{G157C}$* mutants. (**A**) Mutations *dnaA$_{T174P}$*, *dnaA$_{A345S}$* and *dnaN$_{G157C}$* lead to reduced chromosome content. Cells (DHB4, SMG275, SMG217 and SMG219) grown exponentially were stained and analyzed by flow cytometry. Average cell mass and DNA contents were derived from the flow cytometry histograms (see Materials and methods). The average numbers from three experiments are given. (**B**) Cells growing exponentially in AB glucose-CAA medium with uridine (100 μg/ml) were treated with rifampin and cephalexin to block initiation but allow elongation. Distinct peaks represent the accumulation of cells with integral numbers of chromosomes that reflect the numbers of origins at the time of drug action. Top left, wild-type (MG1655); top right, *dnaA$_{A345S}$* (SMG379); bottom left, *dnaA$_{T174P}$* (SMG378); bottom right, *dnaN$_{G157C}$* (SMG380).

completed chromosomes after rifampicin–cephalexin treatment reflects the number of origins present at the time the inhibitors were added. In a culture of cells with synchronous initiation, the number of chromosomes is 2, 4, 8, etc. depending on the growth rate, whereas in a culture with asynchronous initiation some of the cells also contain 'irregular' numbers of origins (Skarstad *et al*, 1986). Wild-type cells were found to initiate synchronously and at four origins early in the cell cycle such that the older part of the population contained eight origins (Figure 2B). The $dnaA_{A345S}$, $dnaA_{T174P}$ mutant cells mostly contained four origins (Figure 2B), indicating that initiation of replication still occurs fairly synchronously in these cells, but later in the cell cycle so that few cells contain eight origins. The average number of origins per cell was about six in the wild-type, compared to about four in the $dnaA_{A345S}$ and $dnaA_{T174P}$ mutant strains and about five in the $dnaN_{G157C}$ mutant, indicating that all three mutants initiate less efficiently. The $dnaN_{G157C}$ culture contained a substantial number of five or six chromosome cells in addition to four chromosome cells, indicating that initiation was asynchronous. The reason for an increased asynchrony phenotype in the $dnaN_{G157C}$ cells is not clear.

For all three strains, the decrease, compared to wild type, in both average number of origins per cell and in DNA concentration (Figure 2A), indicates that initiation occurred less efficiently. The result suggests that mutant cells tend to underinitiate, consistent with the results for *in vitro* replication.

Deletion of hda leads to a strong growth defect in a wild-type strain

Stimulated hydrolysis of DnaA-bound ATP by Hda requires DNA-bound β-subunit, which serves as a matchmaker between DnaA and Hda. The $DnaA_{A345S}$ and $DnaA_{T174P}$ mutant proteins, being constitutively inactivated, are already mainly either in the ADP-bound or nucleotide-free form. Therefore, we predicted that the $dnaA_{A345S}$ and $dnaA_{T174P}$ mutant strains should bypass the requirement for Hda to regulate initiation. In contrast, the wild-type strain should still be affected by a loss of *hda*.

To test this hypothesis, we transduced the $\Delta hda::$Tet^R allele into the wild-type strain, DHB4. Only very small transductants appear on rich media plates and only after 2 days of incubation at 37°C, although the frequency with which these colonies appear is normal (data not shown and Camara *et al*, 2003). Importantly, transductants picked from these plates do not form isolated colonies upon restreaking on fresh medium (Figure 3A). Transductants of the *hda* mutation into a wild-type DHB4 strain carrying a plasmid with a complementing copy of *hda* (pSMG19) grew normally in contrast to the strain with an empty vector (data not shown). Thus, in the wild-type strain, the *hda* deletion confers a severe growth defect.

However, when we transduced the *hda* deletion into the $dnaA_{A345S}$ or $dnaA_{T174P}$ mutants (in a DHB4 background), the transductants grew to normal colony size after 1 day (Figure 3B); both *dnaA* mutants eliminate the requirement for Hda. It appears that, since these *dnaA* mutations result in a shift in DnaA from the ATP-bound to the ADP-bound state, Hda is no longer required for the switch to occur. In contrast, transduction of the *hda* deletion into the $dnaN_{G157C}$ mutant
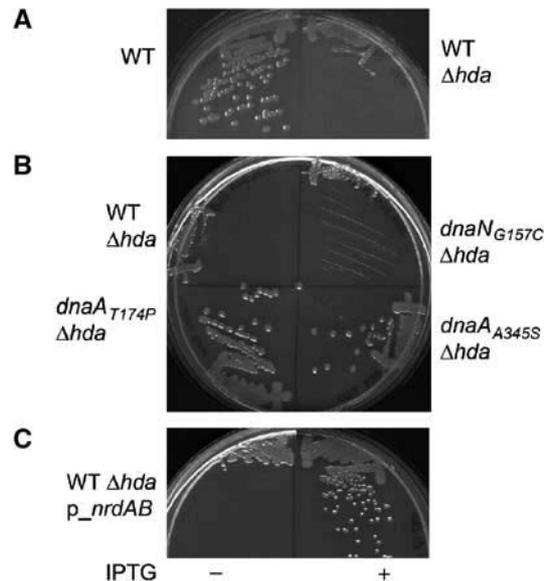

Figure 3 Growth defect due to *hda* deletion in a wild-type DHB4 strain is suppressed by increased amounts of dNTP. (**A**) Deletion of *hda* leads to growth defect in wild-type DHB4. The *hda*::Tet^R allele was transduced into the wild-type DHB4 strain and transductants were selected at 37°C on NZ plates containing tetracycline. Small transductants that appeared after 2 days were restreaked on new NZ plates containing tetracycline (right). (**B**) Deletion of *hda* did not lead to growth defect in $dnaA_{T174P}$ and $dnaA_{A345S}$ mutant strains. The *hda*::Tet^R allele was transduced into the wild-type DHB4, $dnaA_{T174P}$, $dnaA_{A345S}$ and $dnaN_{G157C}$ strains. Transductants were selected at 37°C on NZ plates containing tetracycline and restreaked on identical fresh plates. (**C**) Overexpression of RNR suppresses the growth defect in the wild-type DHB4. A wild-type DHB4 strain containing a plasmid carrying the *nrdAB* operon under IPTG control (pSMG7) was transduced with the *hda*::Tet^R allele in presence of IPTG. Transductants were restreaked on NZ plates containing (right) or not (left) 100 μM IPTG.

strain led to the same growth defect as observed with the wild-type strain (Figure 3B), indicating that the $dnaN_{G157C}$ mutant strain is still dependent on Hda. In contrast to the *dnaA* mutations, the $dnaN_{G157C}$ mutation presumably affects DnaA indirectly by altering the Hda–DnaA interaction, so that Hda is still required for the switch to occur.

Overexpression of ribonucleotide reductase (nrdAB) suppresses the growth defect in the hda deletion strain

Two quite different properties shared by the $dnaA_{A345S}$ and $dnaA_{T174P}$ mutants—elimination of the Hda requirement and the high levels of RNR (Ortenberg *et al*, 2004)—may provide an explanation for the growth defect of the *hda* mutant. Deletion of *hda*, which prevents the conversion of ATP-DnaA to ADP-DnaA, may interfere with the normal necessary derepression of RNR. A lack of dNTP's to complete a round of DNA replication could lead to DNA fork stalling during the elongation step of chromosomal replication and failure to grow. Overexpression of RNR in such a strain would then be expected to restore growth. To test this hypothesis, plasmid pSMG7, which contains the *nrdAB* operon under IPTG control (Ortenberg *et al*, 2004), was introduced into the wild-type DHB4 strain. The *hda*::Tet^R allele was then transduced into this strain in the presence of IPTG. Transductants were restreaked on media with or without IPTG. As shown in Figure 3C, transductants overexpressing RNR grow normally,

in contrast to transductants that do not overexpress RNR (no IPTG). These results show that high levels of RNR suppress the growth defect in the wild-type strain whose *hda* has been deleted, supporting the proposal that the growth defect of *hda*-deleted cells is due to a lack of dNTPs to complete the elongation step of DNA replication.

The correlation between RNR levels and *hda* was further analyzed by determining the RNR levels in wild-type, *dnaA*$_{A345S}$, *dnaA*$_{T174P}$ and *dnaN*$_{G157C}$ strains deleted for *hda*. As deletion of *hda* leads to a strong growth defect in wild-type and *dnaN*$_{G157C}$ strains (Figure 3B), we performed the Western blot analysis on cells from the beginning of the streak (cells do not form isolated colonies but PCR analysis of the bacteria from the top of the streak confirm the presence of the *hda*::Tet^{R} allele). The levels of RNR are high in *dnaA*$_{A345S}$, *dnaA*$_{T174P}$ and *dnaN*$_{G157C}$ strains carrying wild-type *hda* in comparison to the wild-type DHB4 strain (Figure 4A), with the cytoplasmic protein thioredoxin serving as a loading control. In the absence of *hda*, RNR levels are still high in both *dnaA* mutant strains and no growth defect is observed. In contrast, deletion of *hda* in the *dnaN*$_{G157C}$ strain leads to low levels of RNR and a strong growth defect. These results are consistent with our proposal that the growth defect in the *hda* null mutant is due to a lack of RNR.

One attractive explanation for our findings is that Hda not only has a role in preventing the premature reinitiation of DNA replication but has also a role in coordinating the expression of RNR with replication initiation. Interestingly, in the *dnaA*$_{T174P}$ mutant strain the derepression of *nrdAB* expression is totally independent of *hda*, whereas derepression in the *dnaA*$_{A345S}$ mutant is still partially dependent on *hda*, as shown by a decrease of RNR levels in the *hda* deletion strain (Figure 4A). This result is confirmed by assays of β-galactosidase activity of an *nrdA'–'lacZ* fusion in
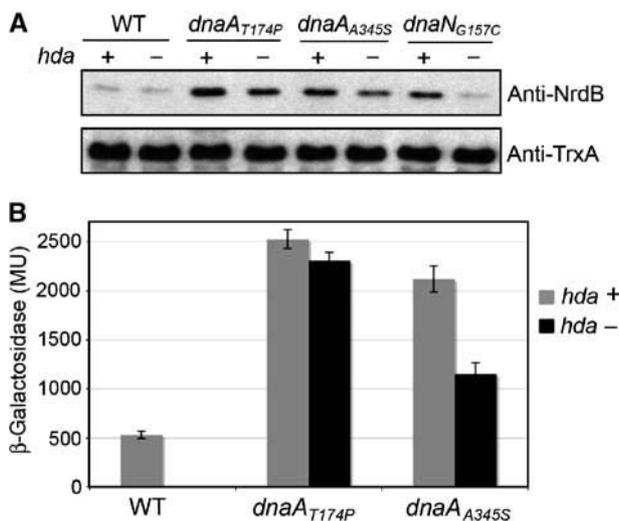

Figure 4 Deletion of *hda* leads to decreased RNR levels in *dnaN*$_{G157C}$ but not in *dnaA*$_{A345S}$ and *dnaA*$_{T174P}$ mutant strains. (**A**) Equal amounts of total cellular protein from wild-type DHB4, *dnaA*$_{A345S}$, *dnaA*$_{T174P}$ and *dnaN*$_{G157C}$ mutant strains harboring (+) or not (−) the *hda* gene were separated by SDS–15% PAGE. Subunit R2 (*nrdB*) of the RNR was detected by Western blotting. As loading control, cytoplasmic thioredoxin was also detected by Western blotting. (**B**) β-Galactosidase activity of the *nrdA'–'lacZ* fusion in wild-type DHB4, *dnaA*$_{A345S}$ and *dnaA*$_{T174P}$ strains harboring (gray) or not (black) the *hda* gene.

dnaA$_{A345S}$ and *dnaA*$_{T174P}$ strains (Figure 4B). One main difference between the two DnaA mutants is that DnaA$_{T174P}$ has a very high intrinsic ATPase activity which leads to almost instant hydrolysis of ATP, whereas DnaA$_{A345S}$ presents an intermediate phenotype, since it exhibits a diminished ATP binding combined with a slight but significant increase of its ATPase activity. Thus, the DnaA$_{T174P}$ mutant protein is likely to be mainly in the ADP-bound form, whereas in the DnaA$_{A345S}$ mutant the ratio of [ATP−DnaA]/[DnaA total] is lower than in a wild-type strain. The *in vivo* stability of DnaA$_{A345S}$ and DnaA$_{T174P}$ are comparable to that of wild-type DnaA (data not shown), ruling out the possibility that *ndrAB* derepression is caused by rapid degradation of DnaA$_{A345S}$ or DnaA$_{T174P}$. These observations strengthen the notion that the nucleotide-bound state of DnaA plays a central role in the regulation of RNR, with DnaA serving as a transcriptional regulator. However, these results do not allow us to determine whether ATP-DnaA acts as a repressor or if ADP-DnaA acts as an activator.

ATP-DnaA may act as a repressor of nrdAB transcription

If ATP-DnaA is a repressor, overexpression of the wild-type DnaA in a *dnaA*$_{T174P}$ mutant strain (where levels of RNR are abnormally high) might be expected to lead to decreased amounts of RNR, while there may be no effect in a wild-type strain. In contrast, if ADP-DnaA is an activator, overexpression of the DnaA$_{T174P}$ mutant protein (mainly ADP-DnaA) may increase RNR expression in a wild-type strain. An empty plasmid or a plasmid expressing either wild-type DnaA, DnaA$_{A345S}$ or DnaA$_{T174P}$ were introduced into a wild-type and a *dnaA*$_{T174P}$ mutant strain. Equivalent amounts of cell extracts from these strains were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) and Western blot analysis was performed using antibodies raised against NrdB (cytoplasmic protein thioredoxin serving as a loading control). As shown in Figure 5A, expression of DnaA$_{T174P}$ in a wild-type strain does not affect the RNR levels when compared to a strain carrying the empty vector. However, overexpression of the wild-type DnaA in the *dnaA*$_{T174P}$ mutant strain leads to lower amounts of RNR, while the empty vector or the vector overexpressing DnaA$_{T174P}$ has no effect (Figure 5A). Overexpression of DnaA$_{A345S}$ in the *dnaA*$_{T174P}$ mutant strain leads to partial reduction of RNR levels. With this mutant protein, which exhibits an ATP binding defect, overexpression leads to accumulation of some ATP-bound form and can explain the partial decrease of RNR levels. These results are consistent with the proposal that the ATP-bound form of DnaA acts as a repressor, which regulates RNR expression.

In order to obtain further evidence for the role of ATP-DnaA as a repressor of RNR levels, we attempted to measure the effects of ATP-DnaA and ADP-DnaA protein on *nrdAB* operon expression in an *in vitro* transcription assay. We used a 608 bp DNA fragment containing the *nrdA* promoter and we also used, as a positive control, a 381 bp DNA fragment containing both *dnaA* promoters. The regulation of both *dnaA* promoters is known to be under repression control of the ATP-bound form of DnaA (Speck *et al*, 1999). *In vitro* run-off transcription by RNA polymerase from the fragment containing both *dnaA* promoters generates two main fragments for the *dnaA* promoter: two transcripts 290 and 200 bp in length transcribed from *dnaAp1* and *dnaAp2* promoters, respectively (Figure 5B). The ATP-DnaA protein represses
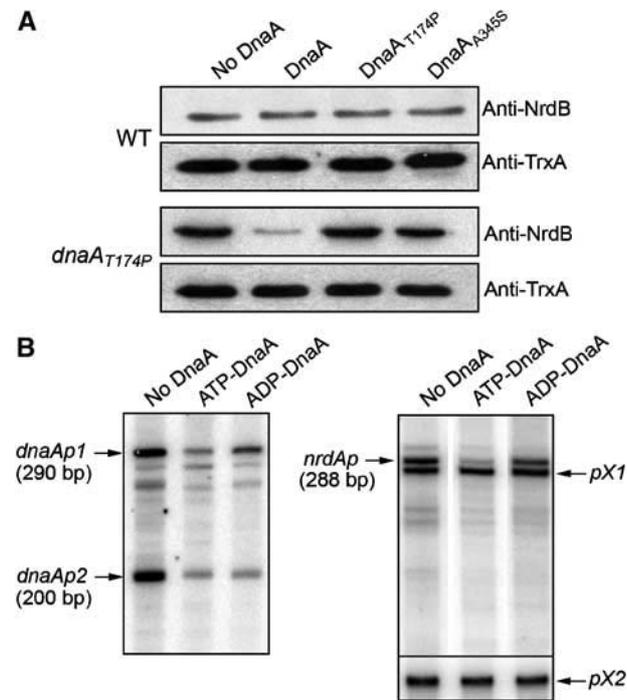
Figure 5 ATP-DnaA represses the *nrdAB* promoter more effectively than ADP-DnaA. (**A**) Western blot analysis. Wild-type DHB4 strain (top) or *dnaA*$_{T174P}$ mutant strain (bottom) containing an empty vector or a plasmid carrying either *dnaA*, *dnaA*$_{A345S}$ or *dnaA*$_{T174P}$ gene under IPTG control were grown in the presence of 100 μM of IPTG to mid exponential growth. Equal amounts of total cellular proteins were separated by SDS–15% PAGE. Subunit R2 (*nrdB*) of the RNR was detected by Western blotting. As loading control, the cytoplasmic thioredoxin was also detected by Western blotting. (**B**) *In vitro* transcription. Run-off *in vitro* transcripts from a 381 bp *Fok*I–*Cla*I restriction fragment that contains promoters *dnaAp1* and *dnaAp2*, and from a 608 bp PCR fragment that contains the nrdAB promoter and two additional uncharacterized promoters *pX1* and *pX2*. Purified ATP- or ADP-DnaA wild-type protein (200 nM) was preincubated with DNA template for 5 min, then the RNA synthesis was started by addition of RNA polymerase (66 nM) and NTPs. Samples were separated by 6%-urea polyacrylamide gel electrophoresis. The transcripts *dnaAp1*, *dnaAp2* and *nrdAp* (290, 200 and 288 bp, respectively) are indicated. Transcription from *pX1* and *pX2* are used as internal control for the experiments.

transcription from both *dnaA* promoters, *dnaAp1* and *dnaAp2*, very efficiently. In contrast, ADP-DnaA protein represses both promoters rather weakly, *dnaAp2* better than *dnaAp1* (Figure 5B). These results are similar to those previously published by Speck *et al* (1999) and serve as a positive control to confirm that our preparation of purified DnaA protein behaves as expected. In parallel, *in vitro* run-off transcription by RNA polymerase from the fragment containing the *nrdAB* promoter generates an expected transcript of 288 bp in length (Figure 5B). Two additional transcripts of shorter length are also detected on the gel. The promoter of the *nrdAB* operon has been well characterized by primer extension analysis but no additional transcript has been identified (Tuggle and Fuchs, 1986). However, it is not surprising to find an additional site of transcription initiation *in vitro*. We used these two additional promoters, named *pX1* and *pX2* as internal controls for the experiment. As shown on Figure 5B, the transcription from these two promoters are not modified by the addition of the DnaA protein. In comparison, ATP-DnaA represses transcription from the 288 pb fragment

of *nrdAB* promoter, whereas the ADP-DnaA represses the *nrdAB* promoter rather weakly (Figure 5B), suggesting that the effect of the ATP-DnaA on the *nrdAB* promoter is specific. Taken together, the *in vivo* studies, indicating the dominant effects of the wild-type DnaA over the two mutant alleles, and the *in vitro* transcription results are consistent with DnaA acting as a repressor of NrdAB expression. We did not attempt to assess the activity of the DnaA mutant proteins in the *in vitro* studies, since the ATP binding defects and/or the increased hydrolysis of ATP of these proteins make it difficult to conceive of how to obtain preparations of the protein that would reflect their *in vivo* state.

We also have attempted to obtain further *in vitro* evidence for our proposal that DnaA-ATP acts as a repressor of the *nrdAB* operon. However, footprinting experiments did not allow us to define a DnaA-binding site. These results may indicate that other protein factors are involved in the transcription regulation of *nrdAB*.

High levels of RNR lead to increased mutation rate

In *E. coli*, the major need for deoxyribonucleotide triphosphates is to allow DNA replication. Thus, the required amount of RNR, the sole enzyme that is capable of generating deoxyribonucleotides in *E. coli* grown aerobically, is directly proportional to the rate of DNA synthesis, which is determined by the rate of DNA initiation. If a strain overexpresses RNR, it may accumulate high levels of dNTPs. Indeed, in yeast and mammalian cells, high levels of dNTP pools are responsible for misincorporation of dNTPs which results in DNA mutagenesis. No studies examining this phenomenon have been carried out in *E. coli*. To address this issue, we measured the spontaneous mutagenesis frequency rate using the frequency of rifampicin resistance in a wild-type DHB4 strain overexpressing or not RNR (SMG300 and SMG301) under IPTG control. The frequency of rifampicin-resistant cells is increased by 10-fold in the strain overexpressing RNR, in comparison to the strain carrying the empty vector (data not shown). The frequency of rifampicin-resistant cells is also increased by three-fold over the wild type in the *dnaA*$_{T174P}$ and *dnaA*$_{A345S}$ mutant strains that exhibit constitutively high RNR levels (data not shown). These results show that high levels of dNTP pools increase the mutagenesis rate in *E. coli*, suggesting that dNTP pools may have to be regulated to avoid deleterious mutagenic effects on the cells.

Expression of RNR is negatively regulated by the dNTP pools

We asked whether there might be other modes of regulation of RNR. To determine whether the RNR NrdAB (or its products) might regulate its own synthesis, we introduced into the wild-type DHB4 strain carrying the *nrdA'–'lacZ* fusion, the plasmid expressing *nrdAB* under the control of an IPTG-inducible promoter (pSMG12) or an empty plasmid (pSMG11). We then examined the effect of overproduction of RNR on its own promoter by following the expression of the *nrdA'–'lacZ* fusion. Overexpression of *nrdAB* led to a five-fold decrease of *nrdA'–'lacZ* fusion expression when compared to the uninduced strain (Figure 6A).

To determine whether the feedback regulation of RNR on its own synthesis might be operating via the DnaA regulation of RNR, we examined the effects of overproduction of RNR in *dnaA*$_{A345S}$ and *dnaA*$_{T174P}$ cells. We found that the *dnaA*
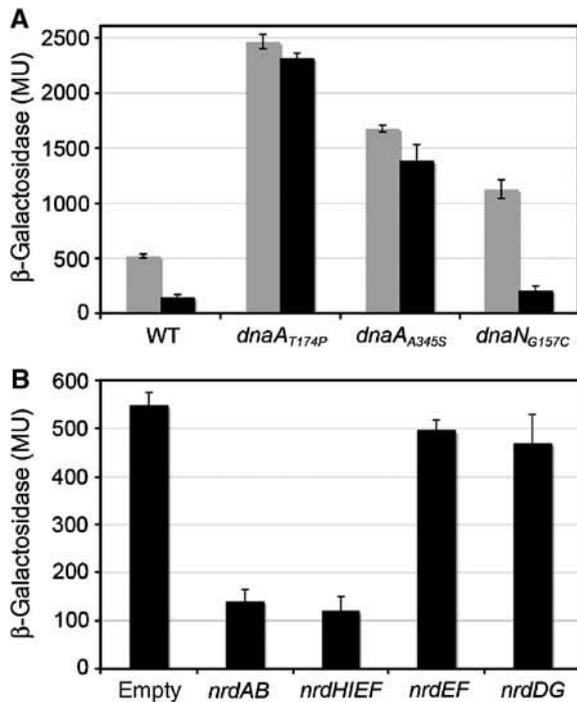
Figure 6 Increased dNTP levels lead to repression of the *nrdAB* promoter in the wild-type DHB4 and *dnaN$_{G157C}$* strains but not in the *dnaA$_{A345S}$* and *dnaA$_{T174P}$* mutant strains. (**A**) β-Galactosidase activity, expressed in Miller units, of the *nrdA'–'lacZ* fusion of a wild-type strain (DHB4), *dnaA$_{A345S}$*, *dnaA$_{T174P}$* and *dnaN$_{G157C}$* strains containing an empty vector (gray) or a plasmid carrying the *nrdAB* operon under IPTG control, grown in presence of 100 μM IPTG to mid exponential growth. (**B**) β-Galactosidase activity, expressed in Miller units, of the *nrdA'–'lacZ* fusion of a wild-type strain (DHB4) containing an empty vector (Ctrl) or a plasmid carrying either *nrdAB*, *nrdHIEF*, *nrdEF* or *nrdDG* under IPTG control grown in the presence of 100 μM IPTG to mid exponential growth.

mutations eliminate the feedback regulation by over-expressed RNR, whereas the *dnaN$_{G157C}$* mutation does not (Figure 6A). These results are consistent with a model in which negative regulation of *nrdAB* triggered by overproduction of RNR operates through DnaA. The finding that the feedback mechanism is not eliminated in *dnaN$_{G157C}$* cells could be due to the fact that this strain is expressing wild-type DnaA, in contrast to the two dnaA mutant strains. In the *dnaA* mutant strains, newly formed DnaA rapidly adopts the inactive ADP-bound form or remains nucleotide-free. In contrast, in *dnaN$_{G157C}$* cells, newly synthesized DnaA is likely in its active ATP-DnaA form, the conformation of DnaA that is responsible for *nrdAB* repression.

Overexpression of RNR leads to higher amounts of RNR as well as higher dNTP pools. Therefore, either the dNTPs or the RNR enzyme itself could be responsible for the negative feedback mechanism. To address this question, we artificially overexpressed two other *E. coli* RNRs, NrdDG, which is active in anaerobiosis, and NrdEF, whose structure is similar to NrdAB, but whose expression is cryptic. In the case of NrdEF, we can distinguish between the regulatory effects of the synthesis of dNTPs and of the enzyme itself, since NrdEF only synthesizes dNTPs if NrdH, a specific reductant of NrdEF, is expressed at the same time. Overexpression of NrdEF allows synthesis of the enzyme whereas overexpression of the entire operon *nrdHIEF* leads to dNTP synthesis

as well (unpublished data). Plasmids expressing *nrdEF* (pSMG13), *nrdHIEF* (pSMG14) or *nrdDG* (pSMG15) under the control of an IPTG inducible promoter were introduced into the wild-type strain carrying an *nrdA'–'lacZ* fusion. Upon overexpression of *nrdHIEF*, the activity of the *nrdA'–'lacZ* fusion decreased five-fold (Figure 6B). However, expression of *nrdEF* alone or *nrdDG* did not lead to repression of the *nrdA'–'lacZ* fusion. These results strongly suggest that the level of dNTPs and not the RNR itself is responsible for the negative regulation of RNR expression.

Discussion

A dual function in the cell cycle for the ATP-DnaA to ADP-DnaA switch

The ability to set into motion a program of gene expression in response to major cellular events is critical for cell function (Volkert and Landini, 2001; Zheng *et al*, 2001; Patten *et al*, 2004). However, the transcriptional response throughout the cell cycle, notably in response to initiation of DNA replication, has been poorly studied in *E. coli* in contrast to eukaryotic systems or to the prokaryote *Caulobacter crescentus* (Lania *et al*, 1999; Martin *et al*, 2004). Here we describe a molecular mechanism which would allow coordination of the synthesis of RNR with the onset of DNA replication. Prior work has shown that the inactivation of DnaA via conversion of the ATP-bound to the ADP-bound form is critical for the prevention of premature reinitiation of replication, thus allowing only a single round of replication per cell cycle (Kato and Katayama, 2001; Camara *et al*, 2003). In the present study, we provide support for a model in which the ATP-DnaA to ADP-DnaA switch occurring with initiation is also required to provide dNTP synthesis for the elongation phase of DNA replication. We suggest that this latter role of DnaA is the more critical of the two for growth. This is indicated by the finding that RNR overexpression suppresses the growth defect observed in an *hda* deletion strain where the molecular switch is defective; the growth defect is largely due to a lack of dNTP synthesis (Figure 3).

We propose a model to explain how loading of the DNA replicase affects the expression of the genes encoding RNR (*nrdAB*) (Figure 7). Prior to initiation, DnaA is largely in its ATP-bound form and represses the *nrdAB* operon, thereby downregulating the pool of dNTPs. However, as a consequence of the initiation of DNA replication, β-subunit is loaded on the replication fork, facilitating Hda-induced hydrolysis of ATP-DnaA to ADP-DnaA. This relieves repression of *nrdAB*, giving the high levels of RNR required for the burst of DNA synthesis. As ATP-DnaA also represses its own promoter, the repression of *dnaA* expression is relieved (although somewhat delayed due to sequestration). The levels of ATP in the cells being much higher than ADP levels, the newly synthesized DnaA will be mainly in its ATP-bound form. ATP-DnaA progressively accumulates in the cell prior to the next round of replication (Kurokawa *et al*, 1999) leading to progressive repression of *nrdAB* transcription.

The following findings provide the basis for our model: (i) three mutations that shift the state of DnaA from mostly ATP-bound to ADP-bound form cause derepression of RNR. Two *dnaA* mutations affect DnaA directly, while the *dnaN* mutation affects DnaA indirectly presumably through its interaction with Hda, (ii) ATP-bound DnaA represses *nrdAB*
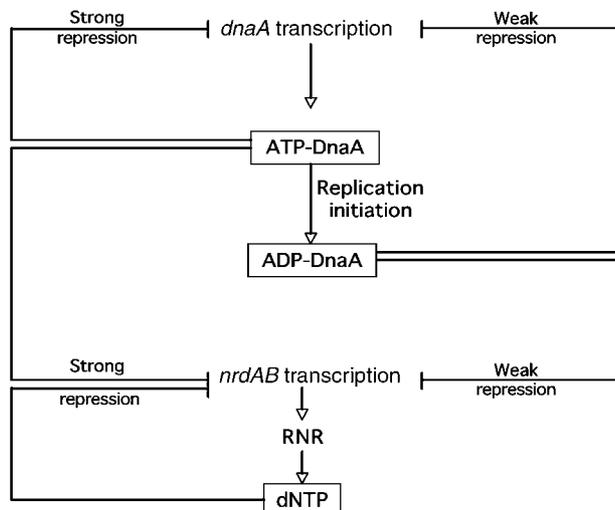
Figure 7 Model for coupling of deoxyribonucleotides (dNTPs) synthesis and DNA replication by the initiation factor DnaA. Newly synthesized DnaA protein is in the ATP-bound form, which is active for DNA replication initiation. ATP-DnaA is also a transcriptional repressor of *dnaA* and *nrdAB* genes. At the end of initiation, ATP-DnaA is hydrolyzed to ADP-DnaA in a β-subunit-dependent manner. The fraction of DnaA protein in the cell in the ADP-bound form increases. ADP-DnaA causes only a weak repression of *nrdAB* operon transcription, which leads to an increase of dNTP synthesis, allowing elongation to occur. ADP-DnaA also causes only weak repression of *dnaA* leading to newly synthesized DnaA. If DNA replication is slow or does not occur, cells experience a time lag between successive replication cycles. During this lag period, ATP-DnaA accumulates in the cell as RIDA would not be functioning because of the absence of DNA-loaded sliding clamps. Binding of ATP-DnaA to the DnaA boxes of the *nrdAB* promoter leads to a strong repression of *nrdAB* transcription and to a cessation of RNR synthesis.

transcription more effectively than ADP-bound DnaA, and the *dnaA* mutants are recessive to wild-type *dnaA* with respect to RNR synthesis, (iii) overexpression of *nrdAB* suppresses the growth defect of an *hda* deletion strain in which the switch from ATP- to ADP-DnaA cannot occur, (iv) overexpression of *nrdAB* leads to high mutation rates, (v) high levels of dNTPs strongly repress *nrdAB* transcription in a DnaA-dependent manner.

Excess dNTP's trigger repression of nrdAB transcription by a newly identified regulatory mechanism

We also uncovered an additional regulatory mechanism for RNR synthesis in which overproduction of RNR leads to repression of *nrdAB* transcription (Figure 6). Why does *E. coli* exhibit such complicated regulation of dNTP synthesis and not simply maintain excess dNTPs throughout the cell cycle. It could be simply that overproduction of dNTP is wasteful. However, in *Saccharomyces cerevisiae*, an excess of dNTPs in cells results in an increased mutation rate (Kunz and Kohalmi, 1991; Chabes *et al*, 2003). Our results show that this is also the case in *E. coli*.

This regulation is likely mediated through the accumulation of dNTP's in the cell, and not by NrdAB itself, as indicated by the finding that when dNTP's are produced from a different RNR (NrdEF), repression still takes place (Figure 6A and B). The findings that this regulation is lost in the $dnaA_{A345S}$ and $dnaA_{T174P}$ mutants, but not in the $dnaN_{G157C}$ mutant, suggest that DnaA is the transcriptional

factor involved in this regulation (Figure 6A). The effect on DnaA could be due to binding of a dNTP, consistent with previous findings that DnaA binds dATP (Sekimizu *et al*, 1987). Since overexpression of RNR leads to abnormally high dNTPs, one could imagine that direct binding of dATP to DnaA leads DnaA to adopt a conformation similar to ATP-bound DnaA. DnaA in this conformation could be responsible for *nrdAB* repression by binding the ATP-DnaA-binding sites located in the *nrdAB* promoter. It appears that the utilization of initiation factor DnaA to tightly coordinate production of dNTP and replication allows the cell to precisely regulate the amount of dNTP in both a positive and negative manner so that enough dNTPs are synthesized, but no more.

Coordination of RNR synthesis and DNA replication in other organisms

Notably, regulation of DNA replication initiation is mechanistically similar in all organisms, using a nucleotide switch of the initiator protein. For example, in *S. cerevisiae*, considerable evidence supports a model in which ORC, an ATP-binding protein and the functional equivalent of DnaA, is inactivated for initiation of DNA replication by ATP hydrolysis soon after initiation (Klemm *et al*, 1997; Bell and Dutta, 2002). Comparison of the ATP regulation of these factors suggests that ATP binding and hydrolysis act as a molecular switch that couples key events during initiation of replication in prokaryotic and eukaryotic cells.

While it is currently not known, it is tempting to speculate that transcriptional activation of RNR genes in eukaryotic cells during the cell cycle (Engstrom *et al*, 1985) is also dependent on the nucleotide-state of ORC.

The power of genetics for discovery of novel regulatory networks

The findings presented here were made possible by the characterization of *dnaA* and *dnaN* mutants detected as suppressor mutations that restored sufficient RNR activity to a strain missing the glutaredoxin and thioredoxin reductants of the enzyme. The present work reveals additional and unexpected complexity in the cell cycle regulatory mechanisms leading to DNA replication. Our results show how analysis of suppressors of mutants affecting central physiological pathways can lead to the revelation of intricately evolved networks for such pathways even in such a well-characterized organism as *E. coli*. In addition, the *dnaA* mutations can be quite useful in further studies as they represent the first viable mutations in *dnaA* that cause rapid accumulation of ADP-DnaA and confer an DnaA ATP-binding defect and/or constitutive ATPase activity.

Materials and methods

Bacterial strains and growth conditions

Table I Strains and plasmids used in this work

Strains/plasmids	Relevant genotype	Reference
Plasmids		
pDSW204	*ori* pBR, amp^R, IPTG inducible	Weiss *et al* (1999)
pDSW206	*ori* pBR, amp^R, IPTG inducible	Weiss *et al* (1999)
pBAD18kan	*ori* pBR, kan^R, arabinose inducible	Guzman *et al* (1995)
pBAD43	*ori* pSC101, spc^R, arabinose inducible	Guzman *et al* (1995)
pSMG7	pDSW204-*nrdAB*	Ortenberg *et al* (2004)
pSMG8	pDSW204-*nrdEF*	This work
pSMG9	pDSW204-*nrdHIEF*	This work
pSMG10	pDSW204-*nrdDG*	This work
pSMG11	pDSW204kan	This work
pSMG12	pDSW204kan-*nrdAB*	This work
pSMG13	pDSW204kan-*nrdEF*	This work
pSMG14	pDSW204kan-*nrdHIEF*	This work
pSMG15	pDSW204kan-*nrdDG*	This work
pSMG16	pDSW206-*dnaA*	This work
pSMG17	pDSW206-*dnaA$_{A345S}$*	This work
pSMG18	pDSW206-*dnaA$_{T174P}$*	This work
pSMG19	pBAD43-*hda*	This work
pZL411	pET19b-His-*dnaA*	Li and Crooke (1999)
pHisDnaA$_{A345S}$	pET19b-His-*dnaA$_{A345S}$*	This work
pHisDnaA$_{T174P}$	pET19b-His-*dnaA$_{T174P}$*	This work
pTB101	pBS*oriC*	Baker and Kornberg (1988)
M13mpRE85		Fuller *et al* (1981)
Strains		
DHB4[a]	Δ(*ara–leu*)7697 *araD139* Δ*lacX74 galE galK rpsL phoR* Δ(*phoA*)*Pvu*II Δ*malF3 thi* contains a mutation in *dnaA* leading to a P to S change in DnaA amino acid 18 relative to other wild-type strains (*dnaA$_{P18S}$*)	Boyd *et al* (1987)
SMG218	DHB4 *dnaA*…MiniTn10Cm^R	This work
SMG217	DHB4 *dnaA$_{A345S}$*…MiniTn10Cm^R	This work
SMG219	DHB4 *dnaN$_{G157C}$*…MiniTn10Cm^R	This work
SMG275	DHB4 *dnaA$_{T174P}$*…MiniTn10Cm^R	This work
MG1655	Wild type	Jensen (1993)
SMG378	MG1655 *dnaA$_{T174P}$*… MiniTn10Cm^R	This work
SMG379	MG1655 *dnaA$_{A345S}$*…MiniTn10Cm^R	This work
SMG380	MG1655 *dnaN$_{G157C}$*…MiniTn10Cm^R	This work
SMG329	DHB4 Δ(λ*attL-lom*)::*bla nrdA'–'lacZ*	This work
SMG334	SMG329 *dnaA$_{A345S}$*…MiniTn10Cm^R	This work
SMG337	SMG329 *dnaA$_{T174P}$*…MiniTn10Cm^R	This work
SMG340	SMG329 *dnaN$_{G157C}$*…MiniTn10Cm^R	This work
SMG300	DHB4 pDSW204	This work
SMG301	DHB4 pDSW204-*nrdAB*	This work
SMG318	SMG301 Δ*hda*::tet^R	This work
BLR(DE3)pLysS	F$^-$ *ompT hsdS$_B$(r$_B^-$m$_B^-$) gal dcm* Δ*(srl-recA)306*::*Tn10*(Tet^R) λ(DE3) [pLysS(Cam^R)]	Novagen

[a]Variant of DHB4 that had been cured of the F′ plasmid was used throughout this study.

Plasmid construction

The *nrdEF, nrdHIEF* and *nrdDG* coding sequences were amplified by PCR using primers that introduce an *Eco*RI and a *Hin*dIII restriction site. The product was digested and ligated into the same sites of the pDSW204 (Weiss *et al*, 1999) to create plasmid pSMG8, pSMG9 and pSMG10. The DNA fragment containing the kanamycin-resistance gene and its promoter region was extracted from the pBAD18kan (Guzman *et al*, 1995) by digestion with *Bsa*I and *Ahd*I. This product was ligated into the same sites of pDSW204, pSMG7, pSMG8, pSMG9 and pSMG10 to create plasmids pSMG11, pSMG12, pSMG13, pSMG14 and pSMG15.

The *dnaA*-coding sequence was amplified by PCR using DHB4, SMG275 and SMG217 chromosomal DNA as template and primers that introduce an *Nco*I and a *Hin*dIII restriction site. The products were digested and ligated into the same sites of pDSW206 plasmid (Weiss *et al*, 1999). The resulting plasmids, pSMG16, pSMG17 and pSMG18, contain, respectively, *dnaA*, *dnaA$_{T174P}$* and *dnaA$_{A345S}$* gene with their own Shine Delgarno sequence under control of *trc* promoter.

The *dnaA*-coding sequence was amplified by PCR using SMG217 and SMG275 chromosomal DNA as template with primers that introduce an *Nde*I and a *Bam*HI restriction site. The digested products were ligated into the same sites of pZL411 plasmid to replace the wild-type *dnaA* (Li and Crooke, 1999). The resulting plasmids were designated pHisDnaA$_{A345S}$ and pHisDnaA$_{T174P}$, respectively.

Genetic and molecular biology procedures

Standard techniques were used for cloning and analysis of DNA, PCR, electroporation, transformation and P1 transduction (Sambrook *et al*, 1989; Miller, 1992).

Construction of strains containing a nrdA′–′lacZ fusion

To obtain a translational fusion of the N-terminal part of ribonucleotide reductase α-subunit, R1 (encoded by *nrdA* gene), with β-galactosidase, a DNA fragment was amplified by PCR using primers that hybridize to a position 778 nucleotides upstream of the translation start site of the *nrdA* gene and contain a *Hin*dIII site and a primer that anneals within the *nrdA*-coding region and contains a *Bam*HI site. The digested product was ligated into the same sites of pNG102 plasmid (Ritz *et al*, 2000) to give plasmid pSMG5. The construct expresses a hybrid protein with the first 13 amino acids of R1 subunit fused to β-galactosidase. The fusion was then integrated into the chromosome of DHB4 by using specialized transduction with lambda phage InCh (Boyd *et al*, 2000) to give SMG329. β-Galactosidase activity assays in liquid media were performed as described (Miller, 1992).

Analytical procedure

Proteins were resolved by SDS–PAGE and stained with Coomassie brilliant blue. For Western blotting, proteins were transferred to

a nitrocellulose membrane after electrophoresis; the ECL-Western blotting system (Amersham Pharmacia) was used for detection.

Purification of His-tagged DnaA

Histidine-tagged wild-type and mutant DnaA$_{A345S}$ and DnaA$_{T174P}$ proteins were expressed, respectively, from pZL411, pHisDnaA$_{A345S}$, and pHisDnaA$_{T174P}$ plasmids in strain BLR(DE3)pLysS and purification was performed as previously described (Li and Crooke, 1999).

ATP binding

ATP binding was assessed as previously described (Sekimizu *et al*, 1987). Briefly, histidine-tagged DnaA proteins were incubated with 1 μM [α-^{32}P]ATP at 0°C for 15 min. Samples were filtered through nitrocellulose filters and retained nucleotides measured by liquid scintillation counting.

DnaA intrinsic ATPase activity

ATP hydrolysis was assessed as previously described (Li and Crooke, 1999). Briefly, histidine-tagged DnaA proteins were incubated with 1 μM [α-^{32}P]ATP at 0°C for 15 min. The resulting ATP-DnaA was incubated at 38°C with pBS*oriC* (0.1 μg) for various times. Samples were filtered through nitrocellulose membranes. DnaA-bound ATP and ADP were extracted from the filters with 1 M formic acid and spotted onto thin layer PEI Cellulose plates. ADP and ATP were separated and radioactivity was measured using a Molecular Dynamics Storm 840 imaging system.

DNA replication in a crude cell-free extract

In vitro replication of *oriC*-plasmid M13mpRE85 in a crude enzyme fraction was performed as described (Fuller *et al*, 1981; Crooke, 1995). Histidine-tagged DnaA protein was incubated at 30°C for 20 min with a crude DnaA-free protein fraction prepared from WM433 (*dnaA204*(Ts)) strain and with a DNA synthesis reaction containing 0.1 mM each dATP, dGTP, dCTP and [α-^{32}P]TTP and 200 ng M13mpRE85 replicative form I DNA. Nucleotides incorporated into acid-insoluble material were collected on GF/C filters and measured by liquid scintillation counting.

DNA replication with defined components

In vitro replication of pBS*oriC*, dependent on purified replication proteins, was performed as described (Crooke, 1995). Histidine-tagged DnaA protein was mixed at 0°C with 0.1 mM each of dATP, dGTP, dCTP and [α-^{32}P]TTP and 200 ng pBS*oriC*; 450 ng SSB; 10 ng HU; 400 ng gyrase A subunit; 180 ng of gyrase B subunit; 150 ng DnaB/DnaC complex; 12 ng DnaG primase; 500 ng DNA polymerase III*; 25 ng of DNA polymerase III holoenzyme β-subunit and the indicated amounts of DnaA proteins. The mixtures were incubated at 30°C for 20 min. Nucleotides incorporated into acid-insoluble material were collected on GF/C filters and measured by liquid scintillation counting.

Flow cytometry analysis

Flow cytometry analysis was performed as described (Torheim *et al*, 2000). The average DNA content per cell was determined as average Hoechst fluorescence per cell. The average DNA per mass was found by dividing average DNA content by average cell mass.

In vitro transcription

The *in vitro* transcription assay was performed as described (Speck *et al*, 1999). A 608 bp fragment amplified by PCR using DHB4 chromosomal DNA as template containing the nrdA promoter as well as a 381 bp *Fok*I–*Cla*I restriction fragment that contains both dnaA promoters, dnaAp1 and dnaAp2 (Speck *et al*, 1999), were used as a linear template for single-round run-off transcription assays. These were carried out at 37°C in transcription buffer pp60 (50 mM HEPES–KOH (pH 7.6 at 1 M), 2.5 mM magnesium acetate, 20% (v/v) glycerol, 0.007% (v/v) Triton X-100, 0.3 mM EDTA and 7 mM DTT) in a total volume of 20 μl. The DNA template (10 ng) was preincubated for 5 min with 200 ng of DnaA protein. Subsequently, RNA polymerase was added to a concentration of 66 nM, followed by another 5 min incubation. The transcription reactions were initiated by addition of [α-^{32}P]UTP (7.5 μCi, 3000 Ci/mmol), unlabelled UTP (60 μM), ATP (200 μM), CTP and GTP (each at 400 μM), and heparin (200 μg/ml). Mixtures were incubated for 5 min and then the reaction was terminated by addition of stop solution (50 mM EDTA containing 300 μg/ml of yeast tRNA). After the reaction, RNA was precipitated with ethanol and resuspended in 6 μl of loading buffer (95% (v/v) formamide, 20 mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol). After incubation at 96°C for 5 min, the samples were loaded and electrophoresed on a 6% (w/v) polyacrylamide sequencing gels. Gels were visualized by phosphorimager.

Acknowledgements

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