Temperature-dependence of the DnaA–DNA interaction and its effect on the autoregulation of dnaA expression

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The DnaA protein is a key factor for the regulation of the timing and synchrony of initiation of bacterial DNA replication. The transcription of the dnaA gene in Escherichia coli is regulated by two promoters, dnaAP1 and dnaAP2. The region between these two promoters contains several DnaA-binding sites that have been shown to play an important role in the negative autoregulation of dnaA expression. The results obtained in the present study using an in vitro and in vivo quantitative analysis of the effect of mutations to the high-affinity DnaA sites reveal an additional effect of positive autoregulation. We investigated the role of transcription autoregulation in the change of dnaA expression as a function of temperature. While negative autoregulation is lost at dnaAP1, the effects of both positive and negative autoregulation are maintained at the dnaAP2 promoter upon lowering the growth temperature. These observations can be explained by the results obtained in vitro showing a difference in the temperature-dependence of DnaA–ATP binding to its high- and low-affinity sites, resulting in a decrease in DnaA–ATP oligomerization at lower temperatures. The results of the present study underline the importance of the role for autoregulation of gene expression in the cellular adaptation to different growth temperatures.

Key words: cell cycle, DNA replication, Escherichia coli, temperature adaptation, transcription regulation.

INTRODUCTION

The DnaA protein is a key regulator of the initiation of DNA replication in most bacterial species, affecting both the timing and synchrony of origin activation [1]. The melting of the DNA at the origin (oriC) and the subsequent assembly of the replication complex depend on the binding of the DnaA protein to its specific sites at oriC [2,3]. In addition DnaA also acts as a transcription factor for a large set of genes, possibly acting as a co-ordinator of the gene expression and DNA replication programmes in response to perturbations in DNA synthesis ([4,5] and references therein). DnaA in the cell is found bound either to ATP or ADP, both forms contributing to the timely formation of an activated origin leading to the recruitment of the DNA replication factors [6]. This process has been shown to take place in sequential stages: the binding of DnaA–ATP or –ADP to high-affinity sites provides a scaffold for formation of a higher-order structure by the filling of the lower affinity sites when the amount of DnaA–ATP in the cell increases (reviewed in [7]). The current model for the regulation of the timing of initiation in Escherichia coli is based mainly on the observation that the timing of initiation of DNA replication can be affected by a change in the amount of DnaA–ATP in the cell by either overexpression or titration of the protein [8–11]. Following initiation of DNA replication two main processes contribute to a decrease in DnaA activity and reduce the amount of protein available for replication initiation in the cell: (i) the increase in the rate of DnaA–ATP hydrolysis mediated by the interaction with the Hda and β-clamp proteins [RIDA (regulatory inactivation of DnaA) process] in a manner dependent on the activity of the replication forks and (ii) the titration of the free protein on sites of different affinities found all along the genome, such as the datA locus [12]. In addition, the binding of SeqA to the dnaA promoter inhibits novel synthesis of the protein following the passage of the replication fork. Before initiation both dnaA gene expression and the recycling of DnaA–ADP into DnaA–ATP [13] contribute to the increase in the amount of DnaA–ATP in the cell. The amount of DnaA–ATP available for DNA binding thus oscillates during the cell cycle to peak at the time of initiation, and the rate at which the amount of DnaA–ATP increases during the cell cycle can affect the timing of initiation [1,14].

The dnaA promoter region (termed dnaAp) contains two promoter sequences, dnaAP1 and dnaAP2. While dnaAP1 provides a basal level of expression, dnaAP2 contains all of the sequence elements usually found in stable RNA promoters, whose growth-rate-dependence and stringent response have been well characterized [15]. Several DnaA-binding sites are found both upstream and overlapping the main promoter elements, resulting in negative autoregulation of transcription by DnaA–ATP [16–20]. This negative autoregulation, combined with the growth-rate-dependence of dnaA expression and DnaA titration on the chromosome, has been proposed to contribute to maintaining a constant concentration of DnaA protein in the cell as a function of growth rate and temperature [21–24]. A study of specific mutations of the DnaA highest affinity binding site at the promoter, however, has failed to show any effect on the amount of DnaA or on the growth rate of the cells [17,25]. It has thus been proposed that autoregulation plays a role mainly in response to specific stresses and changes in the growth environment [26,27]. The quantitative approach used in the present study has permitted for the first time characterization of the changes in DnaA–ATP DNA-binding activity in vitro as a function of temperature and to obtain a direct measurement of the effects that the temperature-dependence of this interaction has on both positive and negative autoregulation of promoter activity in vivo. The results of the present study allow us to propose a role for autoregulation of gene expression in the cellular adaptation to different growth temperatures.

Abbreviations used: DBD, DNA-binding domain; GFP, green fluorescent protein; RIDA, regulatory inactivation of DnaA.

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Figure 1  Sequence of the E. coli dnaA promoter region (dnaAp) and specific mutations of the DnaA sites

(A) dnaAp contains multiple DnaA-binding sites [17]. The high-affinity sites specific for both DnaA–ATP and DnaA–ADP are indicated by boxes with a black edge. The low-affinity binding sites are represented by boxes with a grey edge, whereas the discriminator region at the +1 start site of dnaAP2 is indicated as a box with a dotted edge. The positions indicated are based on the +1 start site of transcription of dnaAP2, 153 bp upstream of the GTG start codon of dnaA [58]. GATC sites are represented by black stars. Binding sites for Fis are also shown based on [37] and on Virtual Footprint [59]. Two out of the three divergent rpmH promoters upstream of dnaAP1 are included in the 427 bp DNA fragment used in the present study. The main primers used for PCR amplification (PdnAR, PdnaAF, PdnaAF2) are represented as grey arrows (Supplementary Table S1 at http://www.biochemj.org/bj/449/bj4490333add.htm). (B) Sequence of the dnaA promoter. The —35 and −10 regions are underlined by a dotted line. The site-directed mutations in the DnaA-binding sites constructed in the present study are shown. dnaAP1 (mutation of the dnaAP2 minimal medium (8.9 g/l Na₂HPO₄ (PerkinElmer) set with an automatically repeating protocol of + in 96-well plates in a Wallac Victor3 multiwell fluorimeter + E. coli antibiotic selection was maintained with ampicillin at 100 μg/ml, whereas the discriminator region at the +1 start site of dnaAP2 is indicated as a box with a dotted edge. The positions indicated are based on the +1 start site of transcription of dnaAP2, 153 bp upstream of the GTG start codon of dnaA [58]. GATC sites are represented by black stars. Binding sites for Fis are also shown based on [37] and on Virtual Footprint [59]. Two out of the three divergent rpmH promoters upstream of dnaAP1 are included in the 427 bp DNA fragment used in the present study. The main primers used for PCR amplification (PdnaAF, PdnaAR, PdnaAF2) are represented as grey arrows (Supplementary Table S1 at http://www.biochemj.org/bj/449/bj4490333add.htm). (B) Sequence of the dnaA promoter. The —35 and −10 regions are underlined by a dotted line. The site-directed mutations in the DnaA-binding sites constructed in the present study are shown. dnaAP1 (mutation of the dnaAP2 —10 region), A-85G and A-86G; Mbox1, A-85G and A-86G + T-60A and C-41G; Mbox2, A-85G and A-86G + T-37A and C-41G; Mboxb-35, A-85G and A-86G + T-31A.

MATERIALS AND METHODS

Bacterial strain, plasmids and growth conditions

E. coli K-12 strain BW25113 [rmb3 Δ lacZ4787 hsdR514 Δ araBAD567 Δ (rhaBAD)568 rplE-I] was used for the in vivo fluorescent reporter measurements [28]. The E. coli IM109 strain was used for classical cloning (Promega). The E. coli WM2287 strain carrying the pDNA116 plasmid was used for DnaA purification [29]. Plasmid pGEMT-Easy (Promega) was used for classical cloning procedures. The medium-to-low copy plasmid pKK-gfp (GFP is green fluorescent protein) was used for construction of dnaAP–gfp fusions (see [30] for details). Strains were grown routinely in Luria–Bertani medium at 37°C, and antibiotic selection was maintained with ampicillin at 100 μg/ml and/or chloramphenicol at 35 μg/ml. For in vivo measurements of gfp expression in dnaAP–gfp fusions, strains were grown in M9 minimal medium (8.9 g/l Na₂HPO₄, 2H₂O, 3 g/l KH₂PO₄, 1 g/l NH₄Cl, 0.5 g/l NaCl, 0.5 mM CaCl₂, 10 mM MgSO₄, 0.02 mg/ml tryptophan and 0.025 mg/ml thymidine) supplemented with 0.4% glucose and 0.5% casamino acids. pKK-gfp plasmid construction, DnaA protein purification and the DNase I footprinting assay are detailed in the Supplementary Online Data (http://www.biochemj.org/bj/449/bj4490333add.htm) and in [4].

In vivo measurement of PdnaA activity

Measurement of the fluorescence of the GFP protein expressed under the control of the dnaAP or its mutants in the medium-to-low copy pKK plasmid was used to determine promoter activity. The rate of GFP production, divided by the cell density as a function of time [31], provides a real-time measurement of the change in GFP ([dGFP/dt]/A₆₀₀) as a function of time [31]. Cells carrying the different plasmids with the different promoter variants were diluted 1:100000 from an overnight culture in M9 minimal medium supplemented with 0.4% glucose and 0.5% casamino acids and were grown in 96-well plates in a Wallac Victor3 multiwell fluorimeter (PerkinElmer) set with an automatically repeating protocol of shaking and cell density (A₆₀₀) and fluorescence readings. GFP fluorescence was measured at 535 nm after excitation at 485 nm. Measurements were taken every 9 min over 20 h of growth. The data analysis protocol for the plate reader experiments are detailed in the Supplementary Online Data and in [4]. The copy number of pBR-322-derived plasmids, such as pKK used in the present study, is dependent on the growth rate of the cells, decreasing as the growth rate increases ([10] and references therein). In the growth medium used in the present study the additional DnaA sites brought by the plasmid do not have a significant effect on the timing of initiation, as indicated by the lack of change in cell mass (Supplementary Figure S1 at http://www.biochemj.org/bj/449/bj4490333add.htm). This is in agreement with previous results obtained with the same plasmid containing the nrdAB promoter sequence with a similar number of DnaA sites [4].

RESULTS

Temperature-dependence of DnaA–ATP binding to its own promoter

We used a quantitative DNase I footprinting assay in order to measure the binding affinity of DnaA–ATP to the dnaAP2 promoter region. Previous work had shown that the dnaAP2 promoter region contains both high- and low-affinity sites, and that DnaA binding to the low-affinity sites is dependent on its ATP-bound state (Figure 1) [17,20]. The box 1 site matches exactly the consensus sequence (TT₄/TNCACA), whereas box 2 differs at one position and box 4 at two positions [32]. Box 4 was defined by Hansen et al. [32] and overlaps with the box A dnaA–ATP site as defined by Speck et al. [17]. Finally, Speck et al. [17] also identified two additional sites, box b and box c, overlapping the dnaAP2 core promoter region.

The DNase I footprinting experiments show that DnaA–ATP binds to the dnaAP promoter protecting the DNA from —73 to —25 of dnaAP2, and resulting in one strong hypersensitive site at the upstream edge of box 1, indicative of DNA bending at this site (Figure 2A). The sequence between box 1 and box 2 is poorly cleaved by DNase I because of its A T-rich content, thus it is not possible to quantify protection in this region where a possible low-affinity site has been proposed [32]. Quantitative
The measurement of DnaA binding to the lower affinity sites is less precise owing to the higher amounts of protein required and the subsequent non-specific interactions that take place on the DNA at higher DnaA concentrations. Thus the results obtained for the −10 site only allow us to estimate a lower limit for the value of the $K_d$. Nevertheless, we can see that protection extends downstream to include the −10 region of dnaAP2 at concentrations that are at least 5-fold greater than those required for protection of the higher affinity sites (Figure 2A and Table 1). In addition, there is less co-operativity of binding to the lower affinity sites, as can be observed from the shallower slope of the binding curves (Figure 3). These results are in agreement with previous studies [17, 20] and show that, as the DnaA–ATP concentration is increased, binding to the box 1 site is followed by the co-operative formation of a higher-order oligomeric structure spanning the adjacent sites and, at higher concentration, by the protection of the core promoter sequences.

Previous work had proposed that the increase in dnaA expression at low temperatures was a result of the loss of DnaA-binding affinity for the DNA and thus a decrease in the extent of negative autoregulation [22]. In order to measure the temperature-dependence of the activity of DnaA–ATP binding to the DNA, we repeated the DNase I footprinting measurements at 30°C and 20°C (Figure 2A). Quantification of these experiments showed that, as the temperature is decreased, the binding affinity of DnaA–ATP for box 1, box 2 and box 4 sites remains more or less constant between 37 and 30°C and then decreases between 30 and 20°C (Figure 2B and Table 1, and Supplementary Figure S2). Furthermore, the temperature-dependence of the different sites is not the same, resulting in an increased difference between the dissociation constants of box 1 and box 2, and box 4 and box b as the temperature is decreased. Finally, the hypersensitive site and the protection of the upstream region including the dnaAP1 promoter are almost completely lost as the temperature is decreased to 20°C (Figure 2A).

**Mutation of DnaA sites in the dnaAP2 promoter region affects DnaA binding to both high- and low-affinity sites**

In order to characterize the effect of DnaA binding to its own promoter’s activity we first measured the effects of specific mutations of its sites in vitro and subsequently carried out in vivo experiments to determine whether these effects found a correlation in the level of promoter activity in the cell. We introduced specific mutations at most of these sites in order to substitute the bases known to define the DnaA-binding consensus sequence (Figure 1B) and measured the effect that these substitutions have on DnaA–ATP binding in vitro by DNase I footprinting. The effect of the mutations of the different binding sites can be described by a qualitative analysis of DnaA–ATP titrations obtained by DNase I footprinting. Figure 4 clearly shows that the mutation of the box 1 site or both box 1- and box 2-binding sites (Mbox1 and Mbox1_2) results in DNA protection appearing at higher DnaA–ATP concentrations. The 2 bp substitutions to box 1 (Figure 1B) strongly affect the protection by DnaA of the other binding sites, and addition of the box 2 mutation, in the context of the mutated box 1 (Mbox1_2), results in a further decrease in affinity for box 1 compared with Mbox1, in agreement with the presence of co-operativity in DnaA binding stabilizing the interactions of DnaA–ATP with the weaker sites. The single-site mutation of the box b sequence (Mboxb-35) on the other hand does not influence DnaA binding to the promoter; DnaA–ATP binding in this region is probably stabilized by protein–protein interactions.

**Promoter activity in vivo is influenced both in a positive and a negative fashion by mutation of the DnaA sites**

In order to determine the effect of DnaA binding on the promoter activity in vivo, we have inserted the different mutated sequences described above in a translational fusion upstream of the sg_gfp gene on the pBR322-derived pKK plasmid (Supplementary Online Data). We measured the fluorescence and absorbance of the cell culture as a function of time in a temperature-controlled 96-well plate reader, obtaining the rate of GFP synthesis and the rate of growth as a function of the growth curve (Figure 5). Under these conditions the bacterial culture grows exponentially without reaching balanced growth before entering stationary phase. The main advantage of this technique is that it provides a direct quantitative measure of the rate of change of GFP synthesis, which is proportional to the activity of the promoter, as a function of the growth conditions. The changes in both the rates of GFP...
The graphs represent the fractional saturation of the DnaA sites as a function of DnaA–ATP concentration. The values obtained from the non-linear least-square fitting of the data (dissociation constant ($K_d$) and Hill coefficient ($n$)) are summarized in Table 1. Results are shown from at least three independent experiments.

Table 1  Temperature-dependence of the dissociation constant and co-operativity for DnaA–ATP binding to the different sites on the dnaA promoter

<table>
<thead>
<tr>
<th>Region</th>
<th>$K_d$ (nM)</th>
<th>Hill coefficient</th>
<th>$K_d$ (nM)</th>
<th>Hill coefficient</th>
<th>$K_d$ (nM)</th>
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<tr>
<td>Box 1</td>
<td>23 ± 2</td>
<td>4 ± 1</td>
<td>21 ± 1</td>
<td>5 ± 2</td>
<td>29 ± 2</td>
<td>4.5 ± 1</td>
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<tr>
<td>Box 2</td>
<td>29 ± 1</td>
<td>4.2 ± 0.7</td>
<td>30 ± 2</td>
<td>3.3 ± 0.7</td>
<td>52 ± 4</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>Box 4</td>
<td>29 ± 3</td>
<td>5 ± 2</td>
<td>29 ± 4</td>
<td>5 ± 3</td>
<td>53 ± 6</td>
<td>2.0 ± 0.6</td>
</tr>
<tr>
<td>Box b</td>
<td>30 ± 3</td>
<td>3.6 ± 0.8</td>
<td>41 ± 6</td>
<td>3 ± 1</td>
<td>52 ± 4</td>
<td>7.4 ± 2</td>
</tr>
<tr>
<td>Box c</td>
<td>$\geq 161$ ± 93</td>
<td>1.6 ± 0.8</td>
<td>$\geq 119$ ± 30</td>
<td>2 ± 1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-10 Region</td>
<td>$\geq 118$ ± 34</td>
<td>1.8 ± 0.6</td>
<td>$\geq 162$ ± 61</td>
<td>2 ± 1</td>
<td>$\geq 132$ ± 31</td>
<td>2 ± 2</td>
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synthesis and cell growth can be measured thanks to the high temporal resolution obtained by the use of the plate reader. The plate-reader-based assay has been used in the past to obtain a quantitative measure of the kinetics of transcription activation, of the effect of specific mutations on transcription activity and the relative promoter activities in a library of E. coli promoters ([4,34] and references therein).

First, we have measured the contribution of the dnaAP1 and dnaAP2 promoters to the overall level of expression at 37°C (Figure 5A). The rate of GFP production from the wild-type and mutant promoters increases from the beginning of the growth curve and it begins to decrease only after the growth rate of the bacteria slows down. A second peak in promoter activity is observed prior to the entry into stationary phase, and expression from both promoters stops once cell growth stops. During exponential growth the main contribution to gene expression comes from the dnaAP2 promoter, as expected from its stable RNA promoter-like characteristics. The dnaAP1 promoter, instead, is induced mainly upon entry into stationary phase. This increase in expression is independent of possible changes in the plasmid copy number per cell since it is dependent on the promoter sequence present on the same plasmid. It could be explained by the previously observed increase in the amount of ppGpp in the transition from exponential to stationary phase, resulting
in the inhibition of expression of stable RNA-like promoters, such as dnaAP2, and thus in the increase in the amount of RNA polymerase available in the cell for transcription of weaker promoters [35].

We then measured the effect of the mutations of the DnaA-binding sites on the rate of expression from both the dnaAP1 and dnaAP2 promoters (Figures 5B and 6). The results show that the expression from dnaAP2 was either increased or decreased depending on the site of the mutation (Figures 5B and 5C). Mutation of the high-affinity box 1 sequence (Mbox1) resulted in an increase in GFP synthesis from dnaAP2 throughout the growth curve. When the mutation to box 2 was added to the box 1 mutant (Mbox1_2), however, a decrease in expression resulted (Figure 5B). Mutation of box 2 alone (Mbox2) also resulted in a decrease in the level of expression compared with the wild-type dnaAP2, indicating that DnaA binding to these sites could lead to activation of transcription (Figure 5B). The Mboxb-35 mutation resulted in a 3-fold increase in promoter activity (Figure 5C). The results obtained in vitro, however, showed that Mboxb-35 did not have any effect on DnaA binding. This mutation slightly improves the already weak −35 sequence for RNA polymerase (from AGATCT to TGATCT, the consensus sequence being TTGACA). Addition of the box 1 and box 2 mutations to Mboxb-35 resulted in a significant decrease in expression confirming the presence of positive autoregulation (Figure 5C). Previous work had shown that DnaA−ATP represses expression from both dnaAP1 and dnaAP2 [17, 20]. We therefore measured the effect of Mbox1 on the activity of dnaAP1 and found a significant increase in expression rate at 37 °C, whereas the presence of Mbox2 had no effect on dnaAP1 activity (Figure 6D and Supplementary Figure S3A at http://www.biochemj.org/bj/449/bj4490333add.htm).

The change in autoregulation as a function of temperature in vivo reflects the differences in temperature-dependence of DnaA–DNA binding observed in vitro

In order to determine the role of positive and negative autoregulation in the temperature-dependence of dnaA expression we also measured the level of expression of the dnaA promoters and their mutants at 30 and 25 °C (Supplementary Figures S4 and S5 at http://www.biochemj.org/bj/449/bj4490333add.htm). We then calculated the ratio of the expression rates for the different mutants compared with the wild-type in exponential phase (Figure 6). In Figure 6(A) one can see that the contribution of dnaAP1 to the total amount of expression increases as the temperature is lowered. This observation can be explained by a loss of negative autoregulation by DnaA, as indicated by the loss of the effect of Mbox1 on the activity of dnaAP1 (Figure 6D). The effects of autoregulation on the activity of the dnaAP2 promoter, however, do not follow the same trend (Figure 6B). Upon mutation of the box 1 site (Mbox1) the increase in promoter activity in vivo becomes significantly different from 1 at 30 and 25 °C. A similar increase in the effect due to the mutation to the box 2 site is also observed, indicating that at lower temperatures there is also an increase in positive autoregulation. These results are consistent with an increase in the amount of active DnaA−ATP throughout the cell cycle as the temperature is lowered. This increase, however, does not suffice to maintain negative autoregulation of dnaAP1 which requires extended oligomerization of DnaA−ATP, in agreement with the in vitro footprinting results showing a loss of dnaAP1 protection as the temperature is lowered (Figure 5A). An additional implication of these results is that dnaAP1 transcription is not repressed by DnaA−ATP bound on the sites downstream of

Figure 5  GFP expression rate from dnaAp-sg_gfp reporter plasmids and change in A600 measured in living cells

BW25113 cells carrying wild-type and mutated dnaA promoters fused to sg_gfp in the pKK plasmid were grown at 37 °C on a 96-well plate reader in M9 minimal medium supplemented with 0.4 % glucose and 0.5 % casamino acids (1.8 ± 0.1 doublings/h). [(dGFP/dt)/A600] is a measure of the promoter activity as a function of time (left-hand y-axis, arbitrary units). A600 is a simultaneous measure of the growth of the bacterial population as a function of time (right-hand y-axis, broken line). Error bars represent the variation between three different samples in one experiment. (A) Comparison of the level of expression from the wild-type construct (dnaAP1 + dnaAP2) or from either the dnaAP1 or dnaAP2 promoter alone. (B) Effect of the mutations to the box 1 (Mbox1), box 2 (Mbox2) or both sites (Mbox1_2) on the rate of expression of the dnaAP2 promoter. (C) Effect of the box b mutation (Mboxb-35) on the activity of the dnaAP2 promoter and effect of mutations to box 1 and box 2 in the context of the Mboxb-35 mutation (Mbox1_2_b-35). WT, wild-type.
its initiation site and overlapping the dnaAP2 promoter. Finally, Figure 6(C) shows the change in promoter activity due to the Mboxb-35 mutation and of the mutations to box 1 and 2 in the context of the Mboxb-35 mutant. The decrease in promoter activity due to the Mbox1_2 mutation is maintained independently of temperature also in this context.

**DISCUSSION**

**Positive autoregulation of dnaA expression**

The results described in the present paper show that both positive and negative autoregulation of dnaA expression can take place at the dnaAP2 promoter from the same set of DnaA-binding sites. This is possible thanks to the formation of DnaA–ATP complexes of different sizes with significantly different affinities for promoter DNA. The sequential assembly of DnaA on its own promoter had been previously observed [17,20], in the present study it is associated with distinct transcription regulation activities.

The observation of the effects of both positive and negative transcription regulation by DnaA in vivo is consistent with the oscillation in the amount of DnaA–ATP available as a function of the DNA replication cycle (reviewed in [36]) that could result in activation and repression of the reporter gene constructs to take place at different times during the cell cycle. A similar regulatory mechanism was also previously observed in a study of the regulation of nrdAB expression, coding for the main aerobic ribonucleotide reductase enzyme in *E. coli* [4]. Although earlier studies had focused on the role of DnaA as an autorepressor [16–20], the results of the present study reveal a significant effect of positive autoregulation. Positive autoregulation of dnaA2 transcription may play an important role to counteract the effect of possible repressors, such as Fis [37] or in the presence of negative effectors of transcription, such as ppGpp and DksA, known to decrease the activity of promoters such as dnaAP2 containing a GC-rich discriminator region [15,35].

In the absence of autoregulation the intrinsic activity of the promoter could in theory suffice to maintain the required average amount of DnaA protein per cell [25,27]. Negative autoregulation has been shown to dampen the noise inherent in gene expression and result in a more uniform distribution of protein amounts among the cells of a population. In addition, by setting an upper limit to the in vivo concentration of the transcription factor, it allows for higher promoter activity, resulting in faster kinetics of transcription factor accumulation [38]. Co-operativity in positive autoregulation on the other hand has been shown to result in a delay in gene expression followed by the rapid induction of transcription activity once the transcription factor has reached the concentration required to bind to its own promoter [39]. The sum of positive and negative autoregulation at dnaAP2, therefore, can result in the synthesis of the amount of DnaA–ATP required for the adaptation of the cell to changes in growth or in response to stress to take place within a smaller fraction of the cell cycle. This may be especially important during rapid growth at shorter cell-doubling times, and thus result in a more stable regulation of initiation of DNA replication. Finally, our recent mathematical modelling study of this system indicates that negative autoregulation can result in maintaining a more uniform amplitude of the cell-cycle oscillation of the DnaA–ATP/DNA ratio in cells at different growth rates [24]. We are currently testing these hypotheses.
Autoregulation and the temperature-dependence of DnaA–DNA interactions

The change in bacterial growth rate as a function of temperature is mainly due to the change in the rate of protein synthesis [40]. Thus although several cellular parameters are dependent on temperature, such as the melting of the origin [41], the melting of the transcription bubble by RNA polymerase [42,43] and the rate of RNA synthesis during transcription elongation [44], the concentration of most proteins remains constant, especially in the range between 20 and 40°C [45,46]. dnaA has been previously shown to be one of the genes whose product instead increases upon a shift to colder temperatures [22]. In that study it was proposed that more DnaA per cell was required in these conditions because the protein may be less active, resulting in decreased autorepression and thus an increased protein concentration. In the case of the dnaAP1 promoter this is exactly what we observe. In this context, the effect of the box 1 mutation (dnaAP1_Mbox1) shows that, as the temperature is lowered, the amount of negative autoregulation of dnaAP1 decreases (Figure 6D), and at the same time the relative activity of dnaAP1 compared with wild-type dnaAp increases (Figure 6A).

The activity of dnaAP2, instead, does not change in the same way in response to the mutation of the DnaA-binding sites as a function of temperature. Two different aspects need to be taken into consideration. The first is that promoters containing a GC-rich discriminator region at the site of DNA melting by RNA into consideration. The first is that promoters containing a GC-function of temperature. Two different aspects need to be taken way in response to the mutation of the DnaA-binding sites as a DNA interactions and DnaA co-operativity shown in the present DNA complexes exhibits a non-linear temperature-dependence [56]. Negative autoregulation can thus play an important role in maintaining a linear response in the regulation of downstream genes [57] upon changes in environmental variables, such as temperature, affecting the activity of a DNA-binding protein.

Figure 7 Summary of the mechanism of autoregulation of dnaA expression

(A) At low concentrations of DnaA–ATP (grey ovals), DnaA binding to the high-affinity sites box 1 and box 2 (filled black boxes) results in activation of transcription. (B) As the amount of DnaA–ATP in the cell increases, oligomerization of DnaA–ATP on the dnaAP2 promoter region results, at first, in repression of transcription of dnaAP2 and then in repression of dnaAP1. (C) The broken line indicates that the extent of dnaAP1 repression depends on the temperature, reflecting the temperature-dependence of DnaA–ATP binding to the lower affinity sites. study. Moreover, the change in autoregulation as a function of temperature can help to explain the cold-sensitive phenotype of certain DnaA and Hda mutants [52–54]. These mutants either result in a decrease in the activity of the RIDA process or in a DnaA protein that is always in an ATP-bound-like conformation. The loss of negative autoregulation of dnaAP1 as the temperature is decreased leads to a higher increase in the proportion of DnaA–ATP to total DnaA in these mutants compared with the wild-type, and thus in overinitiation of DNA replication and stalled replication forks.

Finally, over 40% of E. coli’s transcription factors are negatively autoregulated [55] and the formation of many protein–DNA complexes exhibits a non-linear temperature-dependence [56]. Negative autoregulation can thus play an important role in maintaining a linear response in the regulation of downstream genes [57] upon changes in environmental variables, such as temperature, affecting the activity of a DNA-binding protein.

AUTHOR CONTRIBUTION

Chiara Saggioro designed and performed experiments, and analysed data. Anne Olliver designed and performed experiments. Bianca Sclavi supervised the project, analysed data and wrote the paper. All authors discussed the results and implications, and commented on the paper at all stages.

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REFERENCES


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SUPPLEMENTARY ONLINE DATA
Temperature-dependence of the DnaA–DNA interaction and its effect on the autoregulation of dnaA expression

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MATERIALS AND METHODS

Plasmid construction

The dnaA promoter sequence (dnaAp) was amplified by PCR using E. coli BW25113 chromosomal DNA as a template, and PdnaAR and PdnaAF primers that introduce XbaI and XmnI restriction sites at either end (Table S1). This dnaAp sequence was then cloned in the pKK-sg_gfp plasmid in order to obtain fusions where the fluorescent protein GFP is expressed under the control of the dnaA promoter after transformation in the BW25113 strain [1]. The copy number of this kind of plasmid is dependent on the growth rate of the cells, thus in rich medium, such as the one used in the present study, the extra copies of the DNA sites brought by the plasmid have a smaller effect on the initiation mass than at slower growth rates ([2] and references therein) (Figure S1). Accordingly, control experiments carried out for our previous work on the mrdAB promoter, containing a very similar arrangement of DnaA sites as the DnaA promoter region, did not reveal a significant effect of DnaA titration by the presence of the plasmid [1].

Mutagenesis

Site-directed mutagenesis was carried out as described previously [1] using the PfuUltra High-Fidelity DNA Polymerase (Stratagene). Nine different mutated dnaAp promoters were created. Base position is relative to the +1 of dnaAP2 promoter transcription start site: pGEM-dnaAP1 (A-7G and A-9G), pGEM-dnaAP2 (A-85G and A-86G), pGEM-Mbox1 (A-85G and A-86G + T-60A and C-64G), pGEM-Mbox2 (A-85G and A-86G + T-37A and C-41G), pGEM-Mbox1_2 (combination of Mbox1 and Mbox2 mutations), pGEM-Mboxb-35 (A-85G and A-86G + T-31A), pGEM-Mbox1_2_b (combination of Mbox1, Mbox2, Mboxb mutations), pGEM-dnaAP1Mbox1 and pGEM-dnaAP1Mbox2 (Figure 1B of the main text).

DnaA protein purification

The DnaA protein was purified as described previously [1,3]. The activity of the protein from different preparations was calibrated using the titration binding curves at 37°C.

DNase I footprinting assay

The DNase I protection pattern of the dnaA promoter region by DnaA was obtained by in vitro DNase I footprinting on a linear DNA template [a 292 bp PCR fragment using primers PdnaAF2 and PdnaAR (Table S1)]. Primer PdnaAF2 was 5’ labelled with [γ-32P]ATP (GE Healthcare) and T4 polynucleotide kinase (Fermentas). It was purified with the Bio-Spin 6 Columns (Bio-Rad Laboratories) according to the manufacturer’s instructions.

Binding reactions were carried out with 1 nM linear DNA, and 0–205 nM DnaA–ATP (dialysed as described previously [1,3]) in 10 μl of binding buffer [1 mM DTT (dithiothreitol), 0.5 mg/ml Ac-BSA (acylated BSA), 1 mM Hepes (pH 7.6), 2.5 mM K glutonate and 0.25 mM MgCl2] and 3 mM ATP (Sigma–Aldrich). Samples were incubated for 20 min at 37, 30 or 20°C. The DNA was then cut by the addition of 1 μl of 4 μg/ml DNase I diluted in a digestion buffer [25 mM Tris/HC1 (pH 7), 5 mM MgCl2, 1 mM CaCl2, 2 mM DTT and 100 mM KCl] for 30 s at 37°C. At 30°C 6 μg/ml DNase I for 30 s and at 20°C 8 μg/ml DNase I for 45 s were used respectively. The pH of Hepes buffer changes from 7.31 to 7.55 from 37 to 20°C. The reaction was stopped by the addition of 10 μl of a gel loading buffer (90% formamide, 0.025% Bromophenol Blue, 0.025% Xylene Cyanol and 0.04 mg/ml calf thymus DNA). The samples were incubated for 5 min at 95°C and loaded on to 9% acrylamide gels in denaturing conditions [8 M of urea in 1×TBE (Tris borate EDTA) buffer]. A G + A-specific DNA-sequencing reaction was realized for each DNase I experiment in order to localize the protected regions [4]. After electrophoresis, gels were dried and autoradiographed with a PhosphorImager screen (GE Healthcare).

Quantification of DnaA–ATP binding

The intensities of the bands in the gels were quantified using ImageQuant 5.0 software (GE Healthcare). The band intensities

Table S1 Oligonucleotides used in the present study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PdnaAF</td>
<td>CTAGTCCTAGACTGCAATCGGCAAGACACTG</td>
</tr>
<tr>
<td>PdnaAR</td>
<td>CTAGCCCGGGGGTGAGATTATACGGCCTGA</td>
</tr>
<tr>
<td>PdnaAF2</td>
<td>GACGGACCAGCCTGTCTGCC</td>
</tr>
<tr>
<td>Construction of dnaAP2</td>
<td>TCGGGCGAGCATCTTTCAAGCCGGATGCTGAAAGTC</td>
</tr>
<tr>
<td>PdnaAR1mutf2</td>
<td>GACITCCTCGAAGCTCGCAAGATCTGCACG</td>
</tr>
<tr>
<td>PdnaAR1mutf2</td>
<td>TGTGCAGATTACGAGCGATGGCTTC</td>
</tr>
<tr>
<td>P2off F</td>
<td>GAGACCGGCGATCTGCCAAGAATCCTGAA</td>
</tr>
<tr>
<td>P2off R</td>
<td>GAGITCGCAGGAAATCCTGCCGAGAATCTGC</td>
</tr>
<tr>
<td>Construction of Mbox1</td>
<td>GAGITCTCGAAGATCTGCCGAGAATCTGC</td>
</tr>
<tr>
<td>Pbox1AF2</td>
<td>CAGATTTTCCCGATTTA</td>
</tr>
<tr>
<td>Pbox1AR2</td>
<td>TAGCCTAAACTCGCGCAAGATCTCGAAGAATCTGC</td>
</tr>
<tr>
<td>Construction of Mbox2</td>
<td>CGCGGCGGATCTGCCAAGAATCCTGAA</td>
</tr>
<tr>
<td>Pbox2AF</td>
<td>TCGCGGCAGGATCTGCCAAGAATCCTGAA</td>
</tr>
<tr>
<td>Pbox2AR</td>
<td>CTAGTCCTAGACTGCAATCGGCAAGACACTG</td>
</tr>
<tr>
<td>Construction of Mboxb-35</td>
<td>CTAGTCCTAGACTGCAATCGGCAAGACACTG</td>
</tr>
<tr>
<td>Pboxb35F</td>
<td>TGTCGAGACCGAGAATCTGCAGAGAATCTGC</td>
</tr>
<tr>
<td>Pboxb35R</td>
<td>TCGCGGCAGGATCTGCCAAGAATCCTGAA</td>
</tr>
</tbody>
</table>

1 To whom correspondence should be addressed (email sclavi@lbpa.ens-cachan.fr).
were normalized by the intensity of a reference band that does not change as a function of DnaA concentration and relative to the intensity of the band in the absence of protein [5]. Non-linear least-square curve fitting of the data was carried out using the following binding equation (eqn S1):

$$y = L + (U - L) \times \left(\frac{K^n \times x^n}{1 + K^n \times x^n}\right)$$  \hspace{1cm} (S1)

Where \(y\) is the fractional saturation of the site, \(x\) is the protein concentration (in nanomolar), \(L\) is the lower \(y\) value, \(U\) is the upper \(y\) value, \(K\) is an affinity constant and \(n\) is the Hill coefficient using the Origin software (OriginLab). The values obtained for \(U\) and \(L\) were used to normalize the data from 1 to 0 in order to combine the results of independent experiments, as shown in the plots in Figure 3 of the main text and Figure S2. This value is inversely proportional to the fractional saturation of the site by the protein. The results of at least three independent experiments were combined and fit again to obtain the values for the dissociation constant (\(K_d\)), the Hill coefficient and their respective S.E.M. values shown in Table 1 and Figure 2B of the main text.

### Analysis of the data from the plate reader experiments

A MatLab program was written in order to automate the analysis of the data obtained from the measurement of bacteria growing in 96-well plates in the fluorimeter. The raw data of the measurement of the absorbance and fluorescence as a function of time were smoothed using spline interpolation and the background was subtracted. The background absorbance was obtained from wells containing only the growth medium. The background fluorescence measurements were obtained from cells containing the same plasmid used for our report strains, pKK-tg_gfp, which contains the gene for the GFP, but no promoter sequence upstream of the gene. The curves from several control wells were averaged and subtracted from the data of the different reporter strains. The curves of the absorbance measurement were shifted in the time (\(x\)) axis so that all the curves reached 20\% of their maximal absorbance at 600 nm (\(A_{600}\)) at the same time point. The curves of the fluorescence measurements were consequently shifted by the same amount. The promoter activity was calculated by taking the time derivative of GFP divided by the \(A_{600}\) \([dGFP/dt]/A_{600}\) and the growth rate was calculated by taking the time derivative of the absorbance at 600 nm \((dA_{600}/dt)\).
Figure S1  Cell size as a function of promoter sequence on the pKK plasmid

The top panel shows images of the cells taken with a phase-contrast 60× objective. The cells were grown to exponential phase in M9 minimal medium supplemented with 0.4 % glucose and 0.5 % casamino acids at 37°C. (A) pKK plasmid with no promoter. (B) pKK plasmid with dnaAP2. (C) pKK plasmid with Mbox1-2 mutations. (D) Distributions of cell lengths in the images obtained by microscopy measured from the images using ImageJ (http://rsbweb.nih.gov/ij/). The boxes cover from 25 to 75 % of the dataset and the small square indicates the mean of the distribution. The three samples are not significantly different ($P > 0.05$).
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Figure S2 Quantification of DnaA–ATP binding to DnaA sites on the dnaA promoter region on linear DNA at 30 °C (A) and 20 °C (B)

The graphs represent the fractional saturation of the DnaA sites as a function of DnaA–ATP concentration. Values obtained from the non-linear least-square fitting of the data (dissociation constant \(K_d\) and Hill coefficient) are summarized in Table 1 of the main text. Results are shown from at least three independent experiments.
Figure S3  GFP expression rate from dnaAP1-sg_gfp reporter plasmids and change in A600 measured in living cells

(A) Effect of the mutations to box 1 (Mbox1) and box2 (Mbox2) sites on the rate of expression of dnaAP1. GFP expression rate from dnaAP1-sg_gfp reporter plasmids and its mutants and change in A600 measured in living cells. BW25113 cells carrying wild-type and mutated dnaA promoters fused to sg_gfp in the pKK plasmid were grown at 37°C on 96-well plates in M9 minimal medium supplemented with 0.4% glucose and 0.5% casamino acids (1.8 ± 0.1 doublings/h). A600 is a simultaneous measure of growth of the bacterial population as a function of time (right-hand y-axis, broken line). [(dGFP/dt)/A600] is a measure of the promoter activity as a function of time (left-hand y-axis, arbitrary units). Error bars represent the variation between three different samples in one experiment. (B) Effect of mutating both dnaAP1 and dnaAP2 on GFP expression (dnaAP1dnaAP2), compared with the construct where only dnaAP2 has been mutated (dnaAP1) at 30°C.

Figure S4  GFP expression rate from dnaAP-sg_gfp reporter plasmids and change in A600 measured in living cells at 30°C

BW25113 cells carrying wild-type (WT) and mutated dnaA promoters fused to sg_gfp in the pKK plasmid were grown at 30°C on 96-well plates in M9 minimal medium supplemented with 0.4% glucose and 0.5% casamino acids (1.0 ± 0.1 doublings/h). (A) Comparison of the level of expression from the wild-type, dnaAP1 and dnaAP2 promoters. (B) Effect of the mutations to the box 1 (Mbox1), box 2 (Mbox2) or both sites (Mbox1_2) on the rate of expression of dnaAP2. (C) Comparison of the effect of the box b mutation with dnaAP2 (Mboxb-35) and of the effect of mutations to box 1 and box 2 in the context of the box b mutation (Mbox1_2_b-35). WT, wild-type.
Figure S5  GFP expression rate from dnaAP-sg-gfp reporter plasmids and change in $A_{600}$ measured in living cells at 25°C

GFP expression rate from dnaAP-sg-gfp reporter plasmids and change in $A_{600}$ measured in living cells at 25°C. BW25113 cells were grown at 25°C in M9 minimal medium supplemented with 0.4% glucose and 0.5% casamino acids ($0.5 \pm 0.1$ doublings/h). For separate descriptions of (A–C), please see the legend to Figure S4.

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