The mechanism of regulation of bacteriophage $\lambda pR$ promoter activity by Escherichia coli DnaA protein

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SUMMARY

Apart from its function as an initiator of DNA replication, the *Escherichia coli* DnaA protein is also a specific transcription factor. It activates and represses a number of promoters. However, mechanisms of transcription stimulation by DnaA remained unknown.

Bacteriophage λ *pR* promoter is one of the promoters activated by DnaA. It was reported previously that DnaA binds downstream of the *pR* promoter and perhaps interacts with the RNA polymerase β subunit. Here we demonstrate that DnaA positively regulates transcription from *pR* by stimulation of two steps in transcription initiation: RNA polymerase binding to the promoter region and promoter escape. For this transcription activation, two weak DnaA boxes located downstream of *pR* are necessary and sufficient. Such a mechanism of transcription activation and location of the activator binding sites relative to the transcription start point are unusual in prokaryotes. Changes in the distance between the transcription start point and the first DnaA box by 5 and 10 bps, and alterations in the orientation of these boxes did not abolish the stimulation of transcription by DnaA, but the efficiency of the promoter activation was different for various mutations. It seems plausible that formation of higher-order nucleoprotein structures, involving DNA looping, is necessary for effective stimulation of the *pR* promoter. At high concentrations, DnaA is a repressor of *pR* rather than an activator. This repression was found to be due to inhibition of RNA polymerase binding to the promoter region.
INTRODUCTION

For initiation of chromosomal replication, bacteria require a specific nucleotide sequence, oriC, and an initiator protein that recognises the origin region. In most, if not all, bacteria the role of the initiator is played by DnaA protein (for recent reviews see, for example, refs. 1, 2, 3). However, apart from its role as an initiator of DNA replication, the *Escherichia coli* DnaA protein is also a specific transcription factor that regulates expression of many genes (for a review see ref. 4).

DnaA may cause a premature termination of transcription (5), and it can act as either a repressor or activator of transcription initiation. DnaA-mediated transcription termination might be explained simply by binding of this protein to DnaA-binding sites (DnaA boxes) located within a transcribed DNA region and formation of a barrier for RNA polymerase. Similarly, repression of transcription initiation (promoters repressed by DnaA are exemplified by those for dnaA, mioC, rpoH and uvrB genes; for a review see ref. 4) may be ascribed to binding of DnaA to specific sequences near the promoter and inhibition of RNA polymerase binding. However, mechanisms of transcription activation by DnaA seem to be more complicated. DnaA-mediated stimulation of transcription of several *E. coli* genes (nrd, glpD, fliC, polA) was reported (4, 6), but the mechanism of this process remains completely unknown.

Bacteriophage λ *pr* promoter was also shown to be stimulated by DnaA both *in vivo*...
and *in vitro* (7, 8). This stimulation of \( p_R \), which is an immediate early promoter of the phage, seems to play a crucial role in the control of the frequency of DNA replication initiation at \( ori\lambda \) (9, 10) and in the regulation of directionality of this replication (11, 12, 13). This is because transcription from \( p_R \) gives mRNA for synthesis of \( \lambda \) replication proteins, O and P, and also it serves as a process called transcriptional activation of \( ori\lambda \), i.e. transcription proceeding into the region of the *origin* and stimulating initiation of bidirectional \( \lambda \) DNA replication. In fact, certain *dnaA* mutants cannot be transformed by wild-type \( \lambda \) plasmids (i.e. plasmids bearing the bacteriophage \( \lambda \) replication region, and containing \( ori\lambda \) as the only replication start site), and this phenomenon is partially due to impaired transcription from the \( p_R \) promoter (14).

Genetic analysis suggested that DnaA may contact the \( \beta \) subunit of RNA polymerase during activation of the \( p_R \) promoter, as effects of specific point mutations in the *dnaA* gene could be suppressed by certain point mutations in the *rpoB* gene in an allele-specific manner (8). Moreover, it was demonstrated that during activation of \( p_R \) the DnaA protein binds downstream of the promoter (8). In fact, there are several potential weak DnaA boxes located downstream of \( p_R \) (Fig. 1), and electron microscopy studies demonstrated that the DnaA protein binds to these sites (15). Such a binding downstream of a promoter is unusual for prokaryotic transcription activators, but it is rather common in eukaryotic systems.

In spite of determination of some basic rules of DnaA-mediated activation of the \( p_R \) promoter (summarised above), the molecular mechanism of this phenomenon remained unknown. Therefore, the aim of this work was to investigate this mechanism, especially to identify requirements for particular DnaA box(es) and to determine the step(s) of transcription initiation stimulated by the DnaA protein. Moreover, it was demonstrated previously that at
high concentrations DnaA represses the Pr promoter rather than activates (8). Therefore, here the mechanism of this repression was also investigated.

EXPERIMENTAL PROCEDURES

Bacterial strains and basic plasmids

Escherichia coli wild-type strain MG1655 (16) and the H221 strain bearing fadA::Tn10 and polA1 alleles were used. Plasmid pKB2 is a standard λ plasmid bearing the λ replication region and a kanamycin-resistance gene (17). Plasmid pLamber, a hybrid plasmid bearing two replication origins (oriλ and oriColE1-like) was constructed by ligation of the 3054 bp BamHI–SspI fragment of the ColE1-like replicon pBR328 (18) and the 5485 bp BamHI–NruI fragment of pKB2. Plasmid pRM(minus) is a derivative of pUC19 (19) bearing a fragment of phage λ DNA that contains the Pr promoter but not the pM promoter. pRM(minus) was constructed by cloning a PCR fragment, obtained using primers 19 and 15 (Table 1) and pKB2 plasmid DNA as a template, into HindIII–SspI sites of pUC19.

Construction of plasmids bearing mutated DnaA box sequences

The mutant plasmids were constructed by site-directed mutagenesis according to Langer et al. (20). A pair of complementary primers bearing the desired mutation and two
external primers (flanking the whole region described below) were used in separate PCR reactions to obtain two partially overlapping fragments. Those fragments were subsequently mixed in a hybridization buffer (33 mM Tris-acetate pH 7.9, 10 mM Mg-acetate, 66 mM K-acetate, 0.5 mM DTT), heated to 94°C and allowed to cool down slowly. In the next step, hybridized fragments were elongated by T4 DNA polymerase (1 U) in the presence of 200 mM dNTPs, and used for secondary PCR reactions with external primers. Obtained full-length fragments were subsequently cut with appropriate restriction enzymes (NsiI in the case of boxes #1, #2 and #3, and MunI-SspI in the case of boxes #4, #5 and #6) and cloned into the pLamber vector. Primer sequences and changes introduced by mutagenesis are listed in the Tables 1 and 2, respectively. All primers used for introducing mutations have their complementary counterparts (sequences not shown).

**Proteins**

DnaA protein was purified as described by Schaper and Messer (21). RNA polymerase holoezyme was purchased from Epicentre Technologies (Madison, WI).

**Efficiency of transformation**

Transformation of bacterial cells with plasmid DNA was performed by the calcium chloride method according to Sambrook *et al.* (22). 0.1 µg of plasmid DNA was added to excess of competent cells and, after the transformation procedure, the efficiency of transformation was calculated on the basis of number of colonies formed after overnight incubation on plates with a selective medium.

**In vitro transcription**
In vitro transcription experiments were performed as run-off transcription reactions according to Szalewska-PaBasz et al. (8). For preparation of linear templates for in vitro transcription, 311 bp DNA fragments (\(\lambda\) coordinates 37939 to 38250) with wild-type or mutated DnaA-boxes were amplified during PCR reactions using primers 16 and 17 (Table 1) and appropriate plasmids as templates. Reaction products were separated electrophoretically and quantified using the PhosphorImager system (BioRad).

Abortive in vitro transcription assay

The HindIII-NsiI fragment of the pRM(minus) plasmid (1 nM) was incubated with various concentrations of DnaA protein in the M buffer (20 mM HEPES pH 8.0, 5 mM magnesium acetate, 4 mM DTT, 1 mM EDTA, 1 mM ATP, 5 mg/ml BSA, 0.2% TritonX-100, 5% glycerol) for 10 min at 37\(^\circ\)C. Subsequently, 0.5 U (15 nM) of RNA polymerase was added and incubation was continued for another 15 min. Reaction was started by adding nucleotides (0.5 mM ApU, 50 \(\mu\)M GTP, 5 \(\mu\)M UTP, 2 \(\mu\)Ci \([-^{32}\text{P}]\)UTP). After 12 min at 37\(^\circ\)C, the reaction was terminated with a stop solution (7 M urea, 0.1 M EDTA, 0.4% SDS, 40 mM Tris-HCl pH 8.0, 0.05% bromphenol blue, 0.05% xylencyanol). Samples were loaded onto 20% polyacrylamide sequencing gel (bis:acrylamide ratio 59:1), separated electrophoretically and bands were visualized by phosphorimaging.

Gel retardation assay

Reactions were performed in the M buffer (20 mM HEPES pH 8.0, 5 mM magnesium acetate, 4 mM DTT, 1 mM EDTA, 1 mM ATP, 5 mg/ml BSA, 0.2% TritonX-100, 5% glycerol, 50\(\mu\)g/ml poly dI-dC). 50 ng of a PCR fragment obtained using primers 16 and 17 (Table 1) were mixed with DnaA protein (at indicated concentrations), incubated for 10 min
at 37°C and separated electrophoretically on a 4% polyacrylamide gel (acrylamide:bis-
acrylamide 29:1), in 0.5 x TBE buffer at 4°C. Bands were visualized by SYBR Green
staining and analyzed using FluoroImager (Molecular Dynamics).

**DNaseI footprinting**

PCR fragments, obtained using 5'-32P-labeled primers 16 and 17 (Table 1) and
bacteriophage » DNA, were used as a template (100 cps/reaction). The template was
incubated with DnaA protein (at indicated concentrations) for 10 min at 37°C in the binding
buffer, composed of 40 mM Hepes pH 7.6, 50 mM potassium glutamate, 5 mM magnesium
acetate, 1 mM ATP, 0.5 mg/ml BSA, 5% glycerol, 0.5 mM DTT, 10 mM MgCl2 and 5 mM
CaCl2. Then, 1 U (30 nM) or 0.2 U (6 nM) of RNA polymerase was added (where indicated)
and incubation was carried on for another 15 minutes. DNaseI was added to a final
concentration 0.4 mU/¼l and after 2 min incubation at 37°C the reaction was quenched with
an equal volume of a stop solution (1% SDS, 200 mM NaCl, 20 mM EDTA pH 8.0),
extracted with phenol-chlorophorm (1:1 v/v) and precipitated with 2 volumes of 96% ethanol.
Samples were centrifuged, dried and resuspended in 4 ¼l of H2O. Then, 4 ¼l of the loading
buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) was
added, samples were loaded onto a 8% polyacrylamide sequencing gel containing 8 M urea.
After separation, the gel was dried and bands were visualized by phosphorimaging.

**KMnO4 footprinting**

1 ¼g of pKB2 plasmid DNA was incubated for 20 min at 37°C in the binding buffer
(see DNase I footprinting) with 1 U of RNA polymerase. Then, heparin was added to a final
concentration of 100 $\mu$g/ml and the samples were incubated for another 10 min with various concentrations of DnaA protein (10, 20, 50, 100 and 200 nM). KMnO$_4$ solution was added to a final concentration of 8 mM, and after another 2 min at 37°C the reaction was quenched with 2-betamercaptoethanol. Samples were extracted with phenol and precipitated with 2 volumes of 96% ethanol. Following suspension of the DNA pellet in water, NaOH was added to a final concentration of 10 mM, and the DNA solution was incubated at 80°C for 2 min, cooled and primer extension was performed using 5'-$^{32}$P-labeled primer 18 (10 pmol) (Table 1). The reaction mixture, containing 50 mM Tris-HCl pH 7.2, 10 mM MgSO$_4$, 2 mM DTT, was incubated at 50°C for 3 min to allow primer hybridization. Following addition of dNTPs (5 mM each) and 1 U of the Klenow fragment of DNA polymerase I, the chain elongation was carried out at 50°C. After 10 min the reactions were quenched with 1/3 volume of the stop solution (4 mM ammonium acetate, 20 mM EDTA). DNA was precipitated and separated electrophoretically on 8% polyacrylamide-urea sequencing gel.

**Estimation of promoter escape efficiency**

Reactions were performed in a total volume of 80 $\mu$l, in the M buffer (20 mM Hepes pH 8.0, 5 mM magnesium acetate, 4 mM DTT, 1 mM EDTA, 1 mM ATP, 5 mg/ml BSA, 0.2% Triton X-100, 5% glycerol) using 1 mM *HindIII*-NsiI fragment of pRM(minus) plasmid DNA as a template. The template was incubated with RNA polymerase 0.5 U (30 nM) for 20 min at 37°C, and then heparin was added to a final concentration of 60 $\mu$g/ml, together with DnaA (35 nM). The reaction was started by addition of a nucleotide mix (100 $\mu$M ATP, CTP and GTP (each), 10 $\mu$M UTP and 10 $\mu$Ci/reaction ±-$^{32}$P]UTP). Samples (12 $\mu$l each) were withdrawn at 0.25, 0.5, 1, 2, 4 and 8 min after the onset of the reaction, and
the reaction was quenched with a stop buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylencyanol). Simultaneously, identical control reactions without the DnaA protein were always performed. Samples were separated by electrophoresis in 6% polyacrylamide gel containing 8 M urea. Phosphorimaging and densitometry were performed subsequently using the BioRad phosphorimager system.

RESULTS

Requirement for DnaA boxes in DnaA-mediated stimulation of the $p_R$ promoter in vivo

To investigate which of the weak DnaA-binding sequences located near the $p_R$ promoter are required for its stimulation, we used a simple in vivo test of transformation of bacteria with plasmid DNA. It was established previously that impaired stimulation of $p_R$ by DnaA results in a failure of plasmids derived from bacteriophage $\lambda$ (so called $\lambda$ plasmids) to replicate in E. coli cells, thus such bacteria cannot be transformed by $\lambda$ plasmids carrying an antibiotic-resistance gene (14, 17, 23). A double-origin plasmid, carrying replication regions of $\lambda$ and a ColE1-like plasmid, was used. In such a construct, particular DnaA boxes in the $\lambda$ replication region were scrambled by site-directed mutagenesis to obtain a series of plasmids, each bearing one DnaA box inactivated. Since apart from box #3 (see Fig. 1), all other boxes are located in $\text{cro}$, $\text{cII}$ or $\text{O}$ genes, the sequences were changed in such a way that the most important bases in the boxes were changed to obtain a region unable to bind DnaA protein (according to ref. 20), whereas all codons were replaced by codons determining the same amino acids. Therefore, wild-type Cro, CII and O proteins were synthesized from all constructs. Details of primers used for construction of the mutants and a list of changes in the DnaA box sequences are shown in Tables 1 and 2, respectively.
ColE1-like plasmids cannot replicate in *E. coli* strains deficient in *polA* gene function (24). Therefore, transformation of the *polA1* mutant by the double-origin, λ-ColE1 hybrid plasmid could be efficient only when oriλ was active. In such an experimental system, transformation was effective in the case of all constructs except those with either box #1 or box #2 scrambled (Fig. 2). These results suggest that oriλ is inactive in the absence of box #1 or box #2, most probably due to impaired DnaA-mediated stimulation of transcriptional activation of the origin.

Since boxes #1 and #2 seem to be necessary for activation of *pR* by DnaA, we have changed the orientation of each of these boxes. These changes did not influence the efficiency of transformation of the *polA1* host (Fig. 2). Similar results, i.e. efficient transformation, were obtained when box #1 was moved either 5 or 10 bp away from the *pR* transcription start site (Fig. 2).

**Effects of changes in DnaA boxes on *in vitro* transcription from the *pR* promoter**

Since the transformation assay provided only indirect suggestions about the requirement of particular DnaA boxes for DnaA-mediated stimulation of *pR*, *in vitro* transcription assays were performed using templates containing different configurations of DnaA boxes, as described in the preceding paragraph.

Efficient activation of *pR* was observed at relatively low DnaA concentrations, when wild-type template was used (Fig. 3). In the same experiment, repression of the promoter was detected at high DnaA concentrations, in accordance to previously published results (8). However, when either box #1 or box #2 were scrambled, neither activation nor repression of the *pR* promoter was observed (Fig. 4A). These results demonstrate directly that box #1 and
box #2 are necessary for DnaA-mediated regulation of $p_R$ activity. DnaA efficiently activated (at low concentrations) and repressed (at high concentrations) the $p_R$ promoter in the absence of other DnaA boxes. This holds as well for reactions in which a short template completely devoid of boxes #3, #4, #5 and #6 was used (data not shown). When an even shorter template was used, containing only one DnaA box (box #1), we observed no DnaA-mediated activation and repression of $p_R$ (data not shown). Therefore, boxes #1 and #2 are both necessary and sufficient for this regulation.

Efficient stimulation of $p_R$ by DnaA was also observed when box #1 or box #2 was inverted (Fig. 4B). However, inversion of box #1 resulted in maximal activation of $p_R$ at higher DnaA concentrations relative to the wild-type template, and inversion of box #2 resulted in a lower efficiency of the activation (Fig. 4B).

Increasing the distance between the transcription start site and box #1 (18 bp from site +1 to the center of the box in the wild-type template) by 5 bp (a half of a helical turn) had no significant effect on DnaA-mediated regulation of $p_R$ activity (Fig. 4C). However, further increase of this distance by another 5 bp (i.e. 10 bp, about one helical turn, relative to the wild-type position) resulted in significant decrease in the efficiency of the promoter stimulation (Fig. 4C).

**An ideal box #1 decreases the efficiency of transcription activation of $p_R$ by DnaA**

All the DnaA boxes located between $p_R$ and ori $\lambda$ have weak affinity to DnaA (8, 15). Therefore, it was interesting to investigate the effects of replacing the wild-type box #1 with the DnaA box consensus sequence (ideal DnaA box), known to bind the DnaA protein
strongly.

The λ-CoIE1 hybrid plasmid bearing the ideal DnaA box centred at position +18 transformed the polA1 host efficiently (Fig. 2). In in vitro transcription using the DNA template with the ‘ideal’ box #1, some DnaA-mediated stimulation of transcription was observed (Fig. 4D). However, this stimulation was of low efficiency and occurred at very low DnaA concentrations.

**DnaA affects abortive transcription from pR similarly to normal transcription**

We asked which step of transcription from the pR promoter is affected by DnaA. Since one of the DnaA boxes necessary for DnaA-mediated regulation of this transcription is located 18 bp downstream of pR, it was likely that transcription initiation is regulated by DnaA. According to this prediction, we found that effects of DnaA on abortive transcription from pR are similar to those observed in standard in vitro transcription reactions (Fig. 5). Therefore, we conclude that DnaA affects initiation of transcription at pR.

**DnaA stimulates binding of RNA polymerase to the pR promoter region**

To investigate the mechanism of DnaA-mediated activation of transcription initiation at the pR promoter, each stage of this process was investigated in vitro. The first step in transcription is binding of RNA polymerase to a promoter. Protein-DNA interaction can be studied by footprinting experiments. However, it is worth noting that interactions of DnaA molecules with weak DnaA boxes (especially at relatively low DnaA concentrations which are similar to those causing stimulation of pR) are unstable. They could not be detected by
footprinting (15), but could by gel retardation (Fig. 6). Nevertheless, if DnaA stimulates
binding of RNA polymerase to the $p_R$ promoter region, one might expect a more efficient
protection of a promoter DNA fragment by RNA polymerase in the presence of DnaA than in
the absence of this protein.

When DNase I footprinting experiments were performed using relatively high RNA
polymerase concentrations, efficient protection of the $p_R$ promoter region was observed, and
addition of DnaA protein did not enhance this signal (Fig. 7 and data not shown). However,
when the concentration of RNA polymerase was decreased to such a value that little
protection of the $p_R$ region was observed under standard reaction conditions, addition of low
amounts of DnaA resulted in an appearance of the $p_R$ region protection similar to that
observed at high RNA polymerase concentrations (Fig. 7). Higher DnaA concentrations
inhibited RNA polymerase binding, which is compatible with the results measuring the
transcript levels from the $p_R$ promoter. We conclude that DnaA recruits RNA polymerase to
the $p_R$ promoter, especially when both proteins are present at relatively low concentrations,
which may resemble in vivo conditions.

**Promoter escape, but not isomerization, is stimulated by DnaA**

Using in vitro KMnO$_4$ footprinting technique, we found no significant changes in the
kinetics of isomerization (formation of the open complex from the closed complex) at $p_R$ in
response to the presence of various DnaA concentrations (data not shown). Therefore, we
propose that the isomerization step is not affected by DnaA during stimulation of transcription
from the $p_R$ promoter.
In contrast to the isomerization, we found that the process of promoter clearance (promoter escape) is more efficient in the presence of low DnaA concentrations than in the absence of DnaA (Fig. 8). The effect was not dramatic but significant. Therefore, we conclude that DnaA activates transcription from the $p_R$ promoter by a double mechanism, i.e. by stimulation of RNA polymerase binding to the promoter region and by facilitating promoter clearance (promoter escape).

**Excess of DnaA inhibits RNA polymerase binding to the $p_R$ promoter region**

As demonstrated previously, DnaA protein stimulates the $p_R$ promoter at low concentrations, whereas activity of this promoter is impaired at higher DnaA concentrations (8; Figs. 3-5 in this article). We found that at moderately high concentrations of DnaA, interactions of RNA polymerase with the $p_R$ promoter region are significantly less efficient than in the absence of DnaA (Fig. 9). No protection by DnaA alone was observed at the weak DnaA boxes (Fig 8). Therefore, we conclude that DnaA at high concentrations inhibits binding of RNA polymerase to $p_R$. This inhibition is sufficient to account for DnaA-mediated repression of transcription from this promoter.

**DISCUSSION**

DnaA protein is a replication initiator protein, but also a transcription factor (4). However, mechanisms of its function in stimulation and inhibition of transcription remained unclear. It was speculated, simply on the basis of location of potential DnaA-binding sequences relative to promoters, that DnaA-mediated repression of transcription may be due to inhibition of RNA polymerase binding to a promoter region. Results presented in this
report indicate that such speculations were substantiated. Excess of DnaA efficiently impairs binding of RNA polymerase to the $p_R$ promoter region.

Significantly more problematic, and more difficult to predict, was the mechanism of stimulation of transcription by DnaA. Here we demonstrate that the mechanism of DnaA-mediated activation of bacteriophage $\lambda$ $p_R$ promoter is complicated and unusual. First, DnaA binds downstream of the activated promoter, which is uncommon in bacteria. Second, DnaA stimulates both binding of RNA polymerase to the promoter region and promoter clearance. This is also peculiar as most transcription activators either recruit RNA polymerase to a particular promoter or stimulate the isomerization process (i.e. formation of the open complex) (25, 26). Heparin could potentially interact with DnaA and then affect the interpretation of the results of promoter clearance experiments. But even in such a case, we would underestimate effects of DnaA rather than overestimate them. In addition, we did experiments estimating promoter clearance efficiency without heparin, and results analogous to those depicted in Fig. 8 were obtained (data not shown). Furthermore, when in vitro run-off transcription experiments were performed in the presence and absence of heparin, stimulation of the $p_R$ promoter activity was observed exactly at the same DnaA concentrations in both types of experiments (Figs. 3 and 4, and data not shown). These results strongly suggest that interaction of heparin with DnaA is weak, if any, and it is not significant for DnaA-mediated transcription activation.

Two weak DnaA boxes located downstream of the $p_R$ promoter are necessary and sufficient for transcription stimulation. It seems that weak interaction of DnaA with these regions is important, as replacement of the most proximal weak DnaA box with the consensus DnaA box sequence (‘ideal’ DnaA box) resulted in significant impairment of the promoter
activation. Interestingly, λ plasmids bearing the ideal DnaA box #1 could transform *E. coli* cells efficiently. Clearly, the transformation assay is a less sensitive test to assess the transcription activator function of DnaA than *in vitro* transcription experiments. Moreover, a competition between DnaA boxes located on *E. coli* chromosome and plasmid DNA for DnaA protein binding may be significantly more complicated *in vivo* than in our *in vitro* assays, where only several DnaA-binding sequences were present on one DNA molecule. Therefore, precise prediction of amounts of DnaA protein available for binding to the box #1 and box #2 in bacterial cells is impossible at this stage of research. Finally, one cannot exclude an influence of DNA topology on the efficiency of DnaA-mediated stimulation of $p_R$ activity, especially at various concentrations of this protein. In this light, it is worth noting that in our *in vitro* assays we used linear DNA templates whereas plasmids employed in the transformation assay occur in cells as circular, superhelically twisted molecules. Although efficiency of DnaA-mediated stimulation of $p_R$ observed *in vivo* during gene fusion analysis is generally similar to that measured in *in vitro* transcription assays at optimal DnaA concentration (8), this parameter might vary in details between linear and circular DNA templates.

The requirement for two weak DnaA boxes to activate $p_R$ may be explained by the finding that for binding of DnaA protein to each particular weak DnaA box the presence of at least two DnaA-binding sequences is absolutely necessary (1, 2, 27). These boxes may be separated even by a few hundred bp (27), and in fact, the boxes #1 and #2 (Fig. 1) are separated by about 200 bp. Interestingly, increase in the distance between the transcription start site and the proximal DnaA box from 18 to 23 bp (i.e. by a half of the helical turn) did not affect DnaA-mediated regulation of $p_R$. Further increase in this distance made the
activation less effective, which indicates that the distance from the promoter to the proximal DnaA box, rather than a precise location of the bound DnaA protein at either side of the DNA helix relative to a promoter-bound RNA polymerase, is crucial. On the other hand, inversion of one of the two DnaA boxes had some effects on the transcription stimulation, suggesting that proper arrangement of DnaA protein molecules may, nevertheless, play a role in the activation of $p_R$.

Our previous electron microscopic studies revealed that DnaA boxes #1 and #2 are not the preferential DnaA-binding sites on the $\lambda$ DNA fragment encompassing the replication region, though some binding to those regions was unambiguously documented (14). In addition, using a DNA template of different length, an unambiguous DnaA binding to box #1 and to box #2 was observed in electron microscopic experiments, provided that both boxes were present (27). This corroborates the demonstration here that these DnaA boxes are necessary and sufficient for DnaA-mediated stimulation of the $p_R$ promoter activity.

In conclusion, results presented in this report led us to propose the molecular mechanism of DnaA-mediated stimulation of $p_R$ activity. We suggest that the activation of the $p_R$ promoter by DnaA occurs at two steps. First, binding of DnaA to the two weak DnaA boxes stimulates binding of RNA polymerase. In the second step, RNA polymerase clearance is enhanced. The two boxes are separated by about 200 bp, and binding is likely to be cooperative, as suggested previously (27), and by the rules derived from the binding of DnaA protein (1, 2, 28). This may include formation of a higher-order nucleoprotein structure, mediated by DNA looping. Formation of such a structure could be necessary for proper arrangement of DnaA molecules and their interactions with RNA polymerase. Such interactions (particularly with the $\beta$ subunit) were suggested previously (8) and might be
responsible for recruitment of RNA polymerase to the \( p_R \) promoter region. Moreover, formation of the higher-order nucleoprotein structure might result in a partial denaturation of the DNA helix near \( p_R \), which could stimulate promoter clearance.

We suggest that it is important during \( \lambda \) development that activation of \( p_R \) occurs by low DnaA concentrations at low-affinity DnaA boxes. In this way, early \( \lambda \) DNA replication, which proceeds according to the \( \theta \) mode, is stimulated by DnaA, and when DnaA becomes limiting the switch to \( \sigma \)-mode of replication may occur (12).

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FIGURE LEGENDS

Fig. 1. Location of relaxed (weak) DnaA boxes (open rectangles) in the replication region of bacteriophage $\lambda$ DNA. Positions of the $p_R$ promoter and ori$\lambda$ (composed of O-boxes and an AT-rich region) are shown. Two non-canonical DnaA-binding sequences (black rectangles) were found experimentally (15) near the $p_{oop}$ promoter. A scale (in base pairs) is provided in the upper part of the scheme.

Fig. 2. Transformation of the polA1 mutant (strain H221) with derivatives of the hybrid plasmid (pLamber) bearing two replication origins: ori$\lambda$ and oriColE1-like. The relaxed (weak) DnaA boxes are numbered according to their locations relative to the $p_R$ promoter. Wild-type boxes are represented by open rectangles. Scrambled boxes are crossed. Orientations of particular boxes are marked by arrows. An “ideal” DnaA box is shown as a filled rectangle. + denotes transformation efficiency $10^4 - 10^5$ transformants per 1 $\mu$g of plasmid DNA. denotes transformation efficiency < 10 transformants per 1 $\mu$g of plasmid DNA (no transformants were detected in these experiments).

Fig. 3. Effects of DnaA protein on in vitro transcription from the $p_R$ promoter. Indicated amounts of the DnaA protein (in nM) were added to the reaction mixture as described in Experimental procedures. Following electrophoresis, RNA bands were visualized using a PhosphorImager (BioRad).

Fig. 4. Effects of scrambling of box #1 and box #2 (panel A), inversion of box #1 and box #2
(panel B), changes of location of box #1 (panel C) and replacement of the weak box #1 by an ‘ideal’ (strong) DnaA box (panel D) on DnaA-mediated regulation of in vitro transcription from the $p_R$ promoter. The reactions were performed as described in Experimental procedures using the wild-type template (circles), and following mutated templates: panel A, the template with scrambled box #1 (triangles) and the template with scrambled box #2 (squares); panel B: the template with inverted box #1 (triangles) and the template with inverted box #2 (squares); panel C: templates with box #1 moved 5 bp (triangles) or 10 bp (squares) downstream from the promoter; panel D: the template bearing an ‘ideal’ DnaA box instead of the original box #1 (triangles).

Fig. 5. Effects of DnaA protein on abortive in vitro transcription from the $p_R$ promoter. The experiments were performed as described in Experimental procedures.

Fig. 6. Gel mobility shift analysis of DnaA binding to DNA fragment containing the $p_R$ promoter and DnaA boxes #1 and #2. Gel retardation experiments were performed as described in Experimental procedures using indicated amounts of DnaA (in nM). Lower bands represent unbound DNA fragments, and upper bands depict retarded DNA.

Fig. 7. Effects of low levels of the DnaA protein on RNA polymerase binding to the $p_R$ promoter. DNase I footprinting experiments were performed as described in Experimental procedures using indicated amounts of RNA polymerase (in nM) and DnaA protein (in nM). The region of the $p_R$ promoter is marked.
Fig. 8. Effects of DnaA protein on the $p_R$ promoter clearance (escape) \textit{in vitro}. The experiments were performed as described in Materials and Methods in the absence of DnaA (circles) and in the presence of this protein (35 nM; triangles).

Fig. 9. Effects of high levels of the DnaA protein on RNA polymerase binding to the $p_R$ promoter. DNase I footprinting experiments were performed as described in Experimental procedures using constant amount of RNA polymerase and different amounts of the DnaA protein (in nM). – denotes that a protein was omitted in the reaction. + denotes 1 U (30 nM) of RNA polymerase. The region of the $p_R$ promoter is marked.
Table 1. Primers used for mutagenesis, and for producing templates for *in vitro* transcription and footprinting assays

| No. | Primer sequence | Primer function/purpose |
|-----|-----------------|------------------------|
| 1   | 5'-GTACTAAGGAGGTGAGATGGAACAACGCATA | Scrambling of box #1 |
| 2   | 5'-GTACTAAGGAGTTATCCACAAAAACGCATAAA | Changing box #1 into an ‘ideal’ DnaA box |
| 3   | 5'-GTACTAAGGAGCCATAACAACAAACGCATAAC | Inverting of box #1 |
| 4   | 5'-TGCATGTACTAAGGAGGCATCGTTGTATGGAACAAC | Inserting 5 additional bp between transcription start point at pR and box #1 |
| 5   | 5'-TGCATGTACTAAGGAGGCATCCGTTGGTTGTATGGAACAAC | Inserting 10 additional bp between transcription start point at pR and box #1 |
| 6   | 5'-GGAAGCCTTACGCAGAGGATGAAAGCCCTTC | Scrambling of box #2 |
| 7   | 5'-GGAAGCCTTATCTTCGCAATGGAAGCCCTTC | Inverting of box #2 |
| 8   | 5'-CATAAATAACCCGCCAATATATATCTCCAGCCC | Scrambling of box #3 |
| 9   | 5'-CGCAACGGGCGCCCTGACGAGAATTCG | Scrambling of box #4 |
| 10  | 5'-CAGATCAGCCGATGAAACGGGACTGG | Scrambling of box #5 |
| 11  | 5'-CTTGCCATTCTTAGAAAACCTATGGG | Scrambling of box #6 |
| 12  | 5'-TGAATTCTCTGCGATGAAAGGG | Upper external primer for boxes 1, 2 and 3 |
| 13  | 5'-ATTCAAGGCCTCTGGTGG | Lower external primer for boxes 1, 2 and 3 |
| 14  | 5'-CCCGCTTTACACATCTCCAGCCC | Upper external primer for boxes 4, 5 and 6 |
| 15  | 5'-CCCTGTTTTGAGGATAGCAATCCCC | Lower external primer for boxes 4, 5 and 6 |
| DnaA box          | Wild-type sequence (in λ DNA) | Mutated sequence | Notes                              |
|-------------------|------------------------------|------------------|-----------------------------------|
| Consensus (stringent) | TTÅ/TTNCACA                  | -                | Templates’ amplification          |
| Consensus (relaxed)     | T/C T/C A/T/C T A/C C A/G     | -                | KMnO₄ footprinting                |
| Box #1 (scrambled)      | CCATACAAC                    | CCATCTCACC       | Constructing of plasmid pRM(minus) |
| Box #1 (‘ideal’)        | CCATACAAC                    | TTATCCACA        |                                    |
| Box #2 (scrambled)      | TCTTCCGCA                    | TCCTCTGCG        |                                    |
| Box #3 (scrambled)      | TCTTACACA                    | AACTATATC        |                                    |
| Box #4 (scrambled)      | CTCTACGAA                    | CGCTTCGGA        |                                    |
| Box #5 (scrambled)      | TCTTCCACC                    | GTTTCCATC        |                                    |
| Box #6 (scrambled)      | TTTTACGCA                    | TTTTCCCTAA       |                                    |

Table 2. Sequences of wild-type and mutated DnaA boxes present in plasmids used for transformation efficiency tests and as templates for *in vitro* transcription.
DnaA (nM): 0 5 10 40 50 100 200 400
RNAP:  -  +  +  +  +  +  +  +  +  +  -  -  
DnaA:   -  5  10  25  50  100  200  400  25  50
The mechanism of regulation of bacteriophage lambda pR promoter activity by Escherichia coli DnaA protein
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