Stimulation of the λ p_R promoter by *Escherichia coli* SeqA protein requires downstream GATC sequences and involves late stages of transcription initiation

Robert Łyżeń,¹ Grzegorz Węgrzyn,¹ Alicja Węgrzyn²
and Agnieszka Szalewska-Pałasz¹

Correspondence

Agnieszka Szalewska-Pałasz
szalewsk@biotech.univ.gda.pl

Department of Molecular Biology, University of Gdansk¹ and Laboratory of Molecular Biology (affiliated with University of Gdansk), Institute of Biochemistry and Biophysics, Polish Academy of Sciences², Kladki 24, 80-822 Gdansk, Poland

Escherichia coli SeqA protein is a major negative regulator of chromosomal DNA replication acting by sequestration, and thus inactivation, of newly formed *oriC* regions. However, other activities of this protein have been discovered recently, one of which is regulation of transcription. SeqA has been demonstrated to be a specific transcription factor acting at bacteriophage λ promoters p_i , p_{aQ} and p_R . While SeqA-mediated stimulation of p_i and p_{aQ} occurs by facilitating functions of another transcription activator protein, *cII*, a mechanism for stimulation of p_R remains largely unknown. Here, it has been demonstrated that two GATC sequences, located 82 and 105 bp downstream of the p_R transcription start site, are necessary for this stimulation both *in vivo* and *in vitro*. SeqA-mediated activation of p_R was as effective on a linear DNA template as on a supercoiled one, indicating that alterations in DNA topology are not likely to facilitate the SeqA effect. *In vitro* transcription analysis demonstrated that the most important regulatory effect of SeqA in p_R transcription occurs after open complex formation, namely during promoter clearance. SeqA did not influence the appearance and level of abortive transcripts or the pausing during transcription elongation. Interestingly, SeqA is one of few known prokaryotic transcription factors which bind downstream of the regulated promoter and still act as transcription activators.

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INTRODUCTION

The SeqA protein regulates chromosome replication in *Escherichia coli* by sequestration of newly formed *oriC* regions, leading to their inactivation and thus prevention of overinitiation (Lu *et al.*, 1994; von Freiesleben *et al.*, 1994; Slater *et al.*, 1995). This regulation appears to be mediated by binding of SeqA to hemimethylated GATC sequences (Bach & Skarstad, 2005). Nevertheless, it appears that the cellular function of SeqA is not as simple as supposed shortly after its discovery. Subsequent studies (exemplified briefly below) have revealed many additional activities of SeqA which shed new light on its roles in cell biology.

SeqA is an inhibitor of the onset of *E. coli* chromosome replication *in vivo* (Boye *et al.*, 1996) and in the presence of high concentrations of the replication initiator protein DnaA *in vitro* (Wold *et al.*, 1998); however, it may stimulate DNA synthesis at low DnaA concentrations *in vitro* (Wold *et al.*, 1998). Studies on DNA supercoiling and the localization of SeqA in cells suggested that this protein affects organization of the nucleoid (Hiraga *et al.*, 1998; Weitao *et al.*, 1999). It has been observed that in the absence of SeqA, negative supercoiling of DNA increases, and purified

SeqA generates positive DNA supercoils *in vitro*, strongly suggesting that this protein may take part in the organization of the chromosome *in vivo* (Klungsoyr & Skarstad, 2004; Odsbu *et al.*, 2005). Results of experiments in which SeqA was overproduced suggested that the function of this protein in the regulation of replication initiation is linked to chromosome segregation and cell division (Bach *et al.*, 2003). SeqA could be a global cellular regulator, involved in the formation of so-called hyperstructures (Norris *et al.*, 2002; Molina & Skarstad, 2004). Moreover, there is evidence indicating that SeqA is also involved in the protein degradation process, as in the absence of functional SeqA, the mutant DnaA204 protein has been shown to be stabilized (Torheim *et al.*, 2000; Słomińska *et al.*, 2003c).

Perhaps an unexpected finding was discovery that permeability, and possibly other properties of *E. coli* cellular membranes, are significantly altered in a *seqA* mutant relative to wild-type bacteria (Węgrzyn *et al.*, 1999). One possible explanation for this phenomenon could be SeqA-mediated regulation of the expression of certain genes. In fact, subsequent studies have revealed that SeqA is also a specific transcription factor. It can stimulate activities of

certain promoters both *in vivo* and *in vitro*, including p_I , p_{aQ} and p_R of bacteriophage λ , while other promoters, like λ p_E and p_L , are not influenced (Słomińska *et al.*, 2001, 2003a, b).

An intriguing question is how can SeqA, a regulator of DNA replication, control transcription initiation at certain promoters. Lobner-Olsen *et al.* (2003) performed a microarray analysis to show that in a *seqA* mutant the levels of some transcripts were significantly decreased while certain transcripts were evidently more abundant relative to wild-type bacteria. However, they failed to find any correlation between the presence of GATC motifs (SeqA-binding sites) in promoter sequences and transcription activity and suggested that SeqA-mediated regulation of transcription may be indirect. However, subsequent studies have indicated that when larger DNA fragments, encompassing positions –250 to +250 relative to the transcription start site, were analysed, some common features of GATC distribution near the promoters activated by SeqA could be suggested (Strzelczyk *et al.*, 2003). In such cases, two or more GATC motifs are often located downstream of a stimulated promoter. This also applies to promoters whose activities have to date been demonstrated to be stimulated by SeqA *in vitro* (λ p_I , p_{aQ} and p_R) and thus for which a direct stimulatory mechanism can be assumed (Strzelczyk *et al.*, 2003).

In vitro transcription studies indicated that SeqA stimulates transcription from p_I and p_{aQ} promoters by facilitating the function of another activator, the λ cII protein (Słomińska *et al.*, 2003b). The cII gene product is a positive regulator of these promoters and it has been proposed that in the presence of SeqA it may bind to these promoter sequences more efficiently. This is in contrast to p_E , another cII-dependent promoter, which has been demonstrated to be insensitive to SeqA. Interestingly, DNA templates used in *in vitro* transcription assays from p_E did contain GATC sequences upstream, but not downstream of the promoter (Słomińska *et al.*, 2003b).

Contrary to p_I and p_{aQ} , a mechanism for stimulation of p_R activity by SeqA has remained unclear. Although p_R is also stimulated by another transcription activator, DnaA (Szalewska-Pałasz *et al.*, 1998; Glinkowska *et al.*, 2003), it has been demonstrated that there is competition between SeqA and DnaA at p_R rather than cooperation (Słomińska *et al.*, 2003a). Therefore, a mechanism analogous to that observed for p_I and p_{aQ} is not possible for p_R . On the other hand, since SeqA is a global regulator of various crucial cellular processes (see above), including regulation of gene expression, it would appear to be important to elucidate a mechanism for the stimulation of activity of one of these model promoters, p_R , by this protein. Therefore, the aim of this work was to learn about the mode of SeqA-mediated activation of the p_R promoter. Our *in vivo* and *in vitro* studies revealed that two GATC sequences located downstream of the p_R transcription start site are crucial for this activation, which is independent of DNA topology and can occur after open complex formation.

METHODS

Bacterial strains. *E. coli* WAM106 [F^- *araD139* Δ (*argF-lac*)U169 Δ (*his-gnd*) *thi* *rpsL150* *glu* *flbB5301* *relA1* *deoC1* *rbsR*], described by Thomas & Glass (1991) and referred to as *seqA*⁺ (or wild-type, WT), and its Δ *seqA*::Tn10 derivative, BM761 (Słomińska *et al.*, 2001), were used in *in vivo* experiments. The MG1655 wild-type strain and its *dam13*::Tn9 derivative (Słomińska *et al.*, 2001) were employed to obtain methylated and unmethylated plasmid DNAs, respectively.

Plasmids. Plasmid pTAC3734 (Brondsted & Atlung, 1994), bearing a promoterless *lacZ* reporter gene, was used for construction of p_R -*lacZ* fusions. The wild-type λ p_R promoter region (207 bp, from –73 to +135), bearing two GATC sequences located at positions +82 and +105 relative to the transcription start site (named box A and box B, respectively, in this report) was amplified by PCR using primers pR-Sma and pR-Hind2, which introduced *Sma*I and *Hind*III restriction sites, respectively, at the ends of the product (Table 1). This DNA fragment was inserted into the corresponding sites of pTAC3734. The GATC motifs were then changed for CATC by PCR-mediated site-directed mutagenesis, using primers 1mutA1 and 1mutA2 (for mutating box A) and 1mutB1 and 1mutB2 (for mutating box B). Thus, a series of plasmids bearing a wild-type p_R -*lacZ* fusion or its derivatives bearing either mutated box A or box B or both, was obtained.

For construction of supercoiled templates for *in vitro* transcription, plasmid pTE103 (Elliot & Geiduschek, 1984) was used as a vector. This plasmid bears the sequences of strong terminators located downstream of the cloning sites. The p_R promoter fragments (wild-type and those harbouring mutations) were transferred from the pTAC3734-derived plasmids into pTE103 by insertion of the *Sma*I–*Hind*III fragments bearing the p_R region to corresponding sites of the recipient. Thus, a series of plasmids analogous to that described above, was obtained.

All molecular cloning procedures were performed according to Sambrook & Russell (2001). All constructs were verified by DNA sequencing.

Proteins. SeqA protein, purified as described by Skarstad *et al.* (2000) was provided by Dr Kirsten Skarstad (Institute for Cancer Research, Oslo, Norway). The σ^{70} -containing RNA polymerase holoenzyme was purchased from Epicenter Technologies.

Measurement of β -galactosidase activity in *E. coli* cells. β -Galactosidase activity in bacterial cells was measured according to a previously described method (Miller, 1972). Since multicopy gene fusions (located on plasmids) were used, the results were normalized per amount of plasmid DNA in cells; plasmid copy number in *E. coli* was estimated by isolation of plasmid DNA from a known number of cells, linearization with restriction endonuclease, separation during agarose gel electrophoresis, staining with ethidium

Table 1. Oligonucleotides used in this study

Oligonucleotide	Sequence (5'–3')
pR-Sma	CCC GGG TCA CCG CAA GGG ATA AAT A
pR-Hind2	CGA AGC TTT CGG CCT GCA TGA ATG G
1mutA1	CCA AGA CAG CTA AAC ATC TCG GCG
1mutA2	CGC CGA GAT GTT TAG CTG TCT TGG
1mutB1	CAA AGC GCC ATC AAC AAG GCC ATT C
1mutB2	GAA TGG CCT TGT TGA TGG CGC TTT G

bromide and densitometric analysis of the bands relative to known amounts of plasmid DNA separated on the same gel, as described by Węgrzyn *et al.* (1996).

In vitro transcription assay. Either linear or supercoiled DNA templates were used for the reactions. Unmethylated linear templates were obtained by PCR using pTAC3734-derived plasmids and pR-Sma and pR-Hind2 primers (Table 1). Fully methylated linear templates were obtained by treatment of the PCR products (2 µg) with 10 units Dam methylase (New England BioLabs) for 1 h at 37 °C. Methylated and unmethylated supercoiled templates were obtained by isolation of pTE103-derived plasmids from *dam*⁺ or *dam13*::Tn9 bacteria, respectively, and purification of supercoiled plasmid DNA by ultracentrifugation in a CsCl-ethidium bromide density gradient (Sambrook & Russell, 2001).

The *in vitro* multi-round transcription reactions were performed in a total volume of 25 µl in buffer M (20 mM HEPES, pH 8.0, 5 mM magnesium acetate, 4 mM DTT, 1 mM EDTA, 1 mM ATP, 5 mg BSA ml⁻¹, 0.2% Triton X-100, 5% glycerol). Template DNA (12.5 nM) and the indicated amounts of the SeqA protein were used. The binding reaction of SeqA was carried out for 10 min at 37 °C, then RNA polymerase was added (to a final concentration of 50 nM) and incubation was continued for the next 10 min. After the addition of nucleotides {CTP and GTP up to a final concentration of 150 µM, ATP up to 1 mM, UTP up to 15 µM and [α -³²P]UTP up to 1 µCi ml⁻¹ (3.7 × 10⁴ Bq)}, the samples were incubated at 37 °C for 12 min. Following the addition of heparin (100 µg ml⁻¹) and further incubation for 5 min, the reaction was halted by addition of 5 µl stop buffer (150 mM EDTA, 1.05 M NaCl, 7 M urea, 10% glycerol, 0.0375% xylene cyanol, 0.0375% bromophenol blue). The samples were separated by electrophoresis in a 4.5% polyacrylamide gel containing 7 M urea in the TBE buffer (Sambrook & Russell, 2001) at 350 V. The gel was dried and RNA bands were visualized and quantified using the PhosphorImager system (Bio-Rad).

For single-round transcription, heparin was added to the NTP mixture to a final concentration of 100 µg ml⁻¹.

Order of addition in *in vitro* transcription assays. The wild-type supercoiled DNA template was used in all these assays. The buffer, temperature and nucleotide concentration were as described for the assay of *in vitro* multi-round transcription. Pre-incubation of the template with either SeqA (final concn 1 µM) or RNA polymerase (50 nM) for 10 min was followed by addition of the second protein (alternatively, both proteins were pre-incubated in the absence of DNA). The transcription reaction was initiated by addition of nucleotides and heparin (100 µg ml⁻¹) at various times (including time 0) after the addition of the second protein. The mixture was incubated for 12 min and then the reaction was stopped, and analysis of samples was performed as described for the assay of *in vitro* multi-round transcription.

Estimation of promoter escape efficiency. Reactions were performed in a total volume of 80 µl in M buffer (see above) using the wild-type supercoiled template. The template was incubated with RNA polymerase (50 nM) for 20 min at 37 °C, and then heparin was added to a final concentration of 100 µg ml⁻¹, either together with SeqA (1 µM) or without this protein. The reaction was started by the addition of a nucleotide mix (see description of *in vitro* multi-round transcription assay). Samples (12 µl each) were withdrawn at 0, 0.5, 1, 2 and 4 min after the onset of the reaction and the reaction was quenched with stop buffer. Samples were separated by electrophoresis and analysed as described for the assay of *in vitro* multi-round transcription.

RESULTS

Two downstream GATC motifs are required for stimulation of *p_R* by SeqA

Since SeqA exerts most, if not all, its functions by binding to GATC motifs, and because a correlation between the presence of such motifs and a transcription factor activity of this protein has been demonstrated (Słomińska *et al.*, 2001, 2003a; Strzelczyk *et al.*, 2003), we tested the dependence of SeqA-mediated stimulation of *p_R* on the presence and position of GATC sequences. Previous studies indicated that maximal activation by SeqA could be achieved when using DNA templates bearing only proximal GATC motifs located downstream of *p_R* (Słomińska *et al.*, 2001, 2003a). Therefore, a series of *p_R-lacZ* fusions was constructed, containing the *p_R* promoter region with two GATC sequences (named A and B) located at positions +85 and +102 (with respect to the first nucleotide of the GATC box), which were either wild-type or mutated. In the latter cases, the G was replaced with C in box A, box B or both. This mutation abolished SeqA binding to the specific sequences (Fujikawa *et al.*, 2004).

In vivo *p_R* promoter activity (assessed as β -galactosidase reporter activity in cells containing the fusions) indicated that scrambling of one of the GATC motifs (either A or B) or both resulted in significant impairment of SeqA-mediated stimulation of *p_R* transcription. The effects were more dramatic in the case of disruption of box A and both boxes (Fig. 1); when only box A was present, SeqA retained some

Fig. 1. Stimulation of the activity of the *p_R* promoter by SeqA *in vivo*. Activities of β -galactosidase were measured in *E. coli* WAM106 (*seqA*⁺) and its Δ *seqA*::Tn10 derivative, harbouring a wild-type *p_R-lacZ* fusion (WT) or its mutant derivatives, bearing G to C change(s) in the GATC box A, box B or both boxes. The values measured in the Δ *seqA*::Tn10 mutant were calculated per single copy of the fusion and subtracted from corresponding values estimated in the *seqA*⁺ host. The value calculated by this method for the wild-type fusion (corresponding to 7540 ± 994 Miller units) was assumed to be 100% stimulation; other values presented are relative to this value. The results are mean values from three measurements with bars representing SD.

stimulatory effect on p_R . These results suggest that both GATC motifs, that serve as SeqA-binding sites, are required for p_R stimulation.

The results obtained in *in vivo* studies were subsequently confirmed in *in vitro* transcription assays using a linear DNA template, analogous to experiments described previously in which stimulation of the p_R promoter by SeqA was demonstrated for the first time (Słomińska *et al.*, 2001). In these experiments, we used either fully methylated or unmethylated DNA templates. It was demonstrated previously that SeqA can activate p_R located on both hemimethylated and fully methylated templates (with equal efficiency), but not on unmethylated DNA (Słomińska *et al.*, 2001). We found that the maximal level of p_R activation by SeqA was about two- to threefold when activator protein was present at optimal concentration (1 μ M SeqA for 50 nM RNA polymerase and 12.5 nM template) and when a wild-type fully methylated DNA template was used, which was in agreement with previous observations (Słomińska *et al.*, 2001). The *in vitro* activation was directly dependent on the presence of intact GATC sequences (Fig. 2). Mutations in these motifs notably impaired the activation by SeqA (box B) or abolished it completely (box A or both). As expected, the activation was strictly dependent on methylation of the template: when unmethylated template was used, no significant activation was observed, irrespective of the status of GATC motifs (Fig. 2). A similar effect was observed for single-round transcription assays (data not shown).

Both *in vivo* and *in vitro* studies indicated that box A is indispensable for SeqA-mediated stimulation of p_R , while optimal activation is achieved in presence of both GATC

boxes. Box A, when alone, was able to mediate moderate activation, 20–30 % relative to wild-type.

SeqA-mediated stimulation of p_R is independent of DNA topology

Since SeqA can affect DNA topology (see Introduction) and the activity of various promoters may depend on template supercoiling (for review, see Muskhelishvili & Travers, 2003), we constructed a supercoiled DNA template for *in vitro* transcription assays. This was achieved by introducing the analogous DNA fragment that was used in previous assays (bearing the p_R region with wild-type GATC sequences and with mutated box A, box B or both) into a pTE103 plasmid vector (Elliot & Geiduschek, 1984). This vector bears strong termination signals downstream of the cloning site, and thus can be employed in studies of promoter activity *in vitro* using a supercoiled template. The constructed plasmids were introduced into a *dam*⁺ host and an otherwise isogenic *dam* mutant, and either methylated or unmethylated templates were obtained following isolation of plasmids from these hosts, respectively.

We found that in the *in vitro* multi-round transcription experiments the efficiency of SeqA-mediated activation of p_R transcription on the methylated template bearing the wild-type promoter region was comparable to that observed in reactions with a linear template (compare Fig. 3 and Fig. 2, WT). Therefore, we conclude that template supercoiling has little or no influence on stimulation of p_R by SeqA. Similar to experiments with linear templates, this stimulation was abolished in the absence of methylation and was dependent on the presence of intact GATC motifs,

Fig. 2. *In vitro* transcription from the p_R promoter located on linear templates, either fully methylated (●) or unmethylated (○), containing wild-type GATC motifs (WT) or mutant derivatives, bearing G to C change(s) in the GATC box A, box B or both boxes, with the indicated amounts of the SeqA protein used in the reaction mixture. Following 10 min pre-incubation of SeqA with a template, RNA polymerase was added and incubation was continued for the next 10 min. Then, transcription was started by the addition of the nucleotide mix and the reaction was continued for 12.5 min. The value obtained for each template in an experiment without SeqA was assumed to be 1 and other values are relative to this value. The results are mean values from three experiments (in each case SD was below 10 %).

confirming that SeqA exerts its stimulatory role through binding to at least one of two GATC motifs on supercoiled templates (Fig. 3).

Stage(s) of transcription initiation from p_R affected by SeqA

Transcriptional activators can act at various stages of transcription. The common regulatory step is transcription initiation; however, other stages can also be affected. To address the question what is the molecular mechanism underlying SeqA-mediated stimulation of p_R transcription, we performed a series of *in vitro* transcription experiments under various conditions. All experiments described herein were performed using the methylated, supercoiled, wild-type DNA template.

In the *in vitro* experiments described previously, SeqA was usually preincubated with the DNA template, allowing its binding to the appropriate GATC sequences and thus we observed transcription from p_R after the set time of the reaction. In subsequent experiments we measured time-dependent accumulation of the full-length transcript after addition of RNA polymerase. Briefly, after pre-incubation of the template with SeqA (or with a buffer in the control experiments) for 10 min, and addition of RNA polymerase holoenzyme, aliquots were withdrawn at various times (including time 0) and the reaction was started with the addition of NTPs, together with heparin, and stopped after

12.5 min. The reaction products were separated electrophoretically and the amounts of full-length products were analysed. In these types of experiment we observed about twofold higher amounts of p_R -derived transcripts in the presence of SeqA than in its absence (in agreement with the level of activation observed previously); however, in both cases, increased amounts of transcripts appeared after similar times of incubation of the template with RNA polymerase (Fig. 4). Therefore, we concluded that SeqA did not shorten the time necessary for achieving maximal transcription output.

Although the distant location of GATC sequences from the transcription start point made this assumption quite unlikely, one cannot exclude that SeqA may facilitate binding of RNA polymerase to the promoter region by direct protein-protein interaction. To check this possibility, we performed an *in vitro* transcription experiment where SeqA and RNA polymerase were allowed to make contact during the initial preincubation without the template. The addition of DNA was considered as time zero; from that point on the samples were withdrawn and the reaction proceeded as described above. A lack of a more rapid appearance of

Fig. 3. *In vitro* transcription from the p_R promoter located on supercoiled templates, either methylated (open columns) or unmethylated (grey columns), containing wild-type GATC motifs (WT) or mutant derivatives, bearing G to C change(s) in the GATC box A, box B or both boxes, with 20 ng SeqA protein in the reaction mixture (optimal for stimulatory effect). A value of 1 corresponds to the amount of p_R -derived transcripts measured in reactions with the wild-type methylated template in the absence of SeqA. The results are means of three independent experiments with SD indicated. Insert: autoradiograph of the transcription from p_R with SeqA (0, 10, 20 ng from left to right) on methylated (Met) or unmethylated (Unmet) supercoiled template.

Fig. 4. *In vitro* single-round transcription from the p_R promoter located on the wild-type supercoiled template, in the absence of SeqA (○) or the presence of 20 ng SeqA (●). Pre-incubation of the template with SeqA was followed by addition of 50 nM RNA polymerase. The transcription reaction was initiated by the addition of nucleotides and heparin at the indicated times after the addition of RNA polymerase. The reaction was continued for 12.5 min. A value of 1 corresponds to the amount of p_R -derived transcripts measured in the reaction in the absence of SeqA at time zero with respect to the addition of RNA polymerase. The experiments were repeated three times and a high level of reproducibility was obtained; a representative experiment is shown.

maximal amounts of transcripts in the presence of SeqA, along with evident stimulatory effects of the protein on transcription efficiency, was also observed in this experiment (data not shown).

A regulatory protein can also act at the stage of promoter escape during transcription. To estimate p_R promoter escape efficiency, RNA polymerase was allowed to bind the template (20 min preincubation) and then heparin was added (to prevent reinitiation) together with SeqA (or buffer in control experiments). The reaction was started by the addition of NTPs and samples were withdrawn at various time points after the onset of the reaction. We found that although SeqA did not shorten the time from the start of the reaction to production of the first full-length transcripts, the kinetics of the process appeared to be faster and formation of transcription products was significantly more efficient in the presence of SeqA protein (Fig. 5). Note that SeqA was added after the stages of RNA polymerase binding to the template and open complex formation.

The apparent effect of SeqA on the later stages of transcription prompted us to test for a possible influence of SeqA on abortive transcription and pausing. Release of short transcripts during the non-productive process, which is known to happen at the p_R promoter (Kubori &

Shimamoto, 1996), was studied in an *in vitro* transcription assay designed as for promoter escape, where the samples were withdrawn at times shortly after the start of the reaction and the products were analysed on 25 % sequencing gels. No effect of SeqA on the appearance of abortive transcripts was observed (data now shown). Moreover, this experimental design allowed us to determine the influence of SeqA on pausing during transcription from p_R . The analysis of transcript products appearing during transcription revealed that SeqA does not alter the presence or relative strength of pause sites (data not shown).

DISCUSSION

SeqA has been shown to be a protein with multiple cellular functions besides regulation of *E. coli* chromosome replication. Apart from SeqA-regulated genes located on the bacterial chromosome (Lobner-Olsen *et al.*, 2003), certain promoters of bacteriophage λ (p_L , p_{aQ} and p_R) have also been shown to be stimulated by SeqA (Słomińska *et al.*, 2001, 2003a, b) with significant effects on the development of λ (Węgrzyn *et al.*, 1999; Słomińska *et al.*, 2001, 2003a, b; Węgrzyn, 2006).

The function of SeqA in the regulation of replication is dependent on a hemimethylated state of DNA, while in transcription activation, SeqA is able to activate p_R when DNA is fully methylated (Figs 2 and 3; Słomińska *et al.*, 2001, 2003a). SeqA exerts its effect when bound to specific GATC sequences. Two such motifs, located at 82 and 105 bp downstream of the transcription start site, are crucial for SeqA-mediated stimulation of p_R both *in vivo* and *in vitro* (Figs 1–3). Specific binding of SeqA to this λ DNA region has been demonstrated previously (Słomińska *et al.*, 2001, 2003a). The optimal SeqA-mediated stimulation of p_R activity was achieved when both of the GATC sequences were present and intact. However, box A, proximal to the promoter, seems to be more important for this effect, possibly indicating that the action of SeqA is facilitated by the site located closer to the promoter.

The downstream location of the activator-binding site raises questions about the possibility of a roadblock for RNA polymerase. For DnaA, acting from the +18 position in p_R transcription, binding has been shown to be relatively weak (Szalewska-Pałasz *et al.*, 1998; Glinkowska *et al.*, 2003). Moreover, it has also been shown that an excess of both DnaA (Szalewska-Pałasz *et al.*, 1998) and SeqA (this work, data not shown) inhibit p_R activity.

Taking into the account the distance apart and sizes of the proteins, direct interaction of SeqA with RNA polymerase is not easy to predict unless a significant DNA bend occurs (not likely due to the short distance), thus indicating a more indirect mode of action. As we demonstrated that SeqA-mediated activation of p_R is independent of DNA topology (compare Figs 2 WT and 3) it is unlikely that SeqA acts through facilitating DNA destabilization near the activated

Fig. 5. Effect of SeqA on p_R promoter clearance (escape) *in vitro*. The wild-type supercoiled template (final concn 12.5 nM) was incubated with RNA polymerase (25 nM) for 20 min and then heparin was added, either together with 20 ng SeqA (●) or without SeqA (○). The reaction was started by the addition of the nucleotide mix. Samples were withdrawn at the indicated times after the onset of the reaction and the reaction was quenched with stop buffer. A value of 1 was assumed to be the maximal level of the transcript measured in reactions with SeqA. The results are mean values from three experiments with SD indicated.

promoter, since template supercoiling is crucial in this process (Sheridan *et al.*, 1998).

In vitro analysis determined that the presence of SeqA did not shorten the time necessary for achieving the maximal transcriptional output (Fig. 4). Twice the initial level of SeqA-mediated transcription (Fig. 4, time zero) may indicate the possible action of SeqA on the initial stages. However, this phenomenon may be a result of other steps in the transcription process affected by the presence of SeqA (which would be observed as an increase in transcriptional output). Besides, it is unlikely that SeqA could stimulate transcription by open complex stabilization because for p_R this is not a rate-limiting step (McKane & Gussin, 2000). Measurement of the efficiency of promoter escape demonstrated notably more efficient transcription from p_R in the presence of SeqA compared to the maximal activation noted in a standard *in vitro* transcription assay (compare Figs 3 and 5). Hence, we conclude that SeqA stimulates p_R promoter activity at the stage(s) following formation of the open complex, possibly by facilitating promoter clearance. However, the precise mechanism by which this protein performs such an action, perhaps not contacting RNA polymerase directly, remains to be elucidated.

In the light of the finding that SeqA exerts its regulatory role in the later stages of transcription, one could assume that the regulation could be exerted by affecting the efficiency of productive elongation. However, the presence of SeqA resulted in no notable differences in the amount or time-dependent pattern of such products. Similarly, we could not see any differences in the appearance of pause sites or pausing efficiency in the experiments with or without SeqA protein. Thus, we suggest that SeqA is unlikely to promote its stimulatory function on p_R by influencing abortive transcription or pausing of RNA polymerase. The possibility that SeqA affects transcription termination at t_{R1} , a terminator downstream of p_R in the λ genome, can be ruled out as λ DNA fragments present in the templates used in this work did not contain t_{R1} , but stimulatory effects on p_R -initiated transcription were still evident.

In summary, we have demonstrated that SeqA-mediated stimulation of p_R transcription is independent of DNA supercoiling, requires two GATC motifs located downstream of the transcription start site and is dependent on template methylation. The major regulatory effect of SeqA in p_R transcription occurs after the formation of open complexes in transcription initiation, e.g. at promoter clearance. Our findings place SeqA in the group of prokaryotic positive regulators of transcription which bind downstream of the transcription start site. Such a feature, found frequently in eukaryotic promoters, is very rare in prokaryotes and is exemplified only in specific cases of DnaA (Szalewska-Pałasz *et al.*, 1998; Glinkowska *et al.*, 2003), Ler (Sperandio *et al.*, 2000) and some activators from the AraC family (Rns, CfaR, VirF, AggR and CsvR) (Martin & Rosner, 2001; Munson *et al.*, 2001).

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