

# Transcription regulation of the *Escherichia coli* *pcnB* gene coding for poly(A) polymerase I: roles of ppGpp, DksA and sigma factors

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**Abstract** Poly(A) polymerase I (PAP I), encoded by the *pcnB* gene, is a major enzyme responsible for RNA polyadenylation in *Escherichia coli*, a process involved in the global control of gene expression in this bacterium through influencing the rate of transcript degradation. Recent studies have suggested a complicated regulation of *pcnB* expression, including a complex promoter region, a control at the level of translation initiation and dependence on bacterial growth rate. In this report, studies on transcription regulation of the *pcnB* gene are described. Results of in vivo and in vitro experiments indicated that (a) there are three  $\sigma^{70}$ -dependent (p1, pB, and p2) and two  $\sigma^S$ -dependent (pS1 and pS2) promoters of the *pcnB* gene, (b) guanosine tetraphosphate (ppGpp) and DksA directly inhibit transcription from pB, pS1 and pS2, and (c) pB activity is drastically impaired at the stationary phase of growth. These results indicate that regulation of the *pcnB* gene transcription is a complex process, which involves several factors acting to ensure precise control of PAP I production. Moreover, inhibition of activities of pS1 and pS2 by ppGpp and DksA suggests that regulation of transcription from promoters requiring alternative  $\sigma$  factors by these effectors of the stringent response might occur according to both passive and active models.

**Keywords** *pcnB* gene · RNA polyadenylation · Transcription initiation · Sigma factors · ppGpp · DksA

## Introduction

Activity of bacterial poly(A) polymerase (PAP) was discovered almost 50 years ago (August et al. 1962). This discovery apparently has been overlooked for many years, most probably due to technical problems in demonstrating the presence of poly(A) tails at the ends of short-lived bacterial transcripts. In 1986, Lopilato et al. (1986) reported the presence of the *pcnB* gene in *Escherichia coli*. Mutants in this gene significantly influenced copy number of a ColE1-like plasmid pBR322 and its derivatives; thus, the name *pcnB* (for plasmid copy number) was proposed. Soderbom and Wagner (1998) found effects of the *pcnB* gene product on degradation of CopA, an antisense RNA involved in the regulation of R1 plasmid replication. In the meantime, Cao and Sarkar (1992) discovered that the main *E. coli* PAP I, an enzyme catalyzing RNA polyadenylation at the 3' end, is encoded by *pcnB*.

In contrast to eukaryotic cells, RNA polyadenylation in bacteria usually leads to its faster degradation (Regnier and Arraiano 2000). This was demonstrated for various specific transcripts, whose half-lives increased significantly in *pcnB* mutants (O'Hara et al. 1995; Xu and Cohen 1995; Szalewska-Pałasz et al. 1998; Blum et al. 1999). Since Mohanty and Kushner (2006) demonstrated that transcripts derived from over 90% of open reading frames are polyadenylated in exponentially growing cultures of *E. coli*, it could be suggested that expression of most of bacterial genes may be regulated by polyadenylation-dependent mechanisms. One of the first insights suggesting that this may be the case came from studies on a short transcript of

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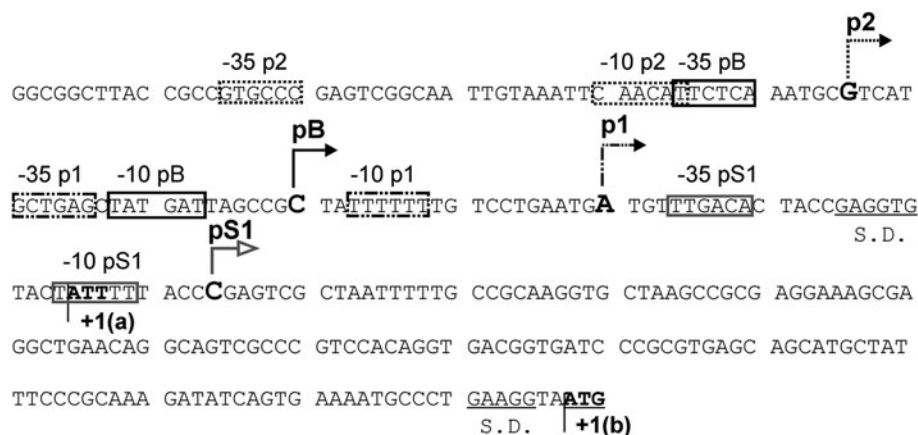
bacteriophage  $\lambda$ , called *oop* RNA, which demonstrated that *oop* RNA is polyadenylated by PAP I (Wrobel et al. 1998) and that this modification results in its decreased stability (Szalewska-Palasz et al. 1998). In fact, *oop* RNA transcripts were shown to be polyadenylated more efficiently in slowly growing cells than in rapidly growing bacteria (Jasiecki and Wegrzyn 2003). Recently, Joanny et al. (2007) demonstrated the poly(A)-dependent regulation of expression of the *glmS* gene in *E. coli*. Although this regulation appears to be indirect and involves the control of stability of a small regulatory RNA encoded by *glmY* (Reichenbach et al. 2008; Urban and Vogel 2008), these results suggest that efficiency of polyadenylation of certain RNAs may be different under various environmental conditions, indicating an important regulatory role for this reaction (Szalewska-Palasz et al. 2007b). Since some other small regulatory RNAs were shown to be regulated by polyadenylation (Soderbom and Wagner 1998; Szalewska-Palasz et al. 1998; Wrobel et al. 1998; Viegas et al. 2007), one might speculate that this is a major mechanism for controlling gene expression by this process. Furthermore, PAP I is phosphorylated in *E. coli*, which significantly influences the activity of this enzyme (Jasiecki and Wegrzyn 2006a). PAP I is located in both the cytoplasm and cytoplasmic membrane (Jasiecki and Wegrzyn 2005), thus any factors affecting specific localization of molecules of the *pcnB* gene product may indirectly cause alterations in expression of other genes.

Since RNA polyadenylation appears to be a global regulatory process in the physiological control of gene expression in bacteria and of replication of extrachromosomal genetic elements (plasmids and phages) (for a review, see Szalewska-Palasz et al. 2007b), discovery of mechanisms controlling production of PAP I is necessary for both

understanding these crucial cellular processes and possibly employing them in genetic engineering and biotechnology.

In this light, perhaps surprisingly, regulation of expression of the *pcnB* gene in *E. coli* is relatively poorly understood. Liu and Parkinson (1989), on the basis of nucleotide sequence analysis, proposed a location of the putative *pcnB* gene promoter (named pS1 in this report) (Fig. 1). Subsequent studies indicated, however, that this region is located downstream of the actual translation start codon (a non-canonical initiation codon AUU), and another transcription start site (called pB) was discovered by Binns and Masters (2002) (Fig. 1). It appeared that regulation of the *pcnB* gene expression occurs at the stage of translation initiation (Binns and Masters 2002); however, subsequent studies strongly suggested that transcription control may contribute significantly to the modulation of PAP I production (Jasiecki and Wegrzyn 2003). In fact, it was demonstrated that apart from the pB promoter (Binns and Masters 2002), there are two other promoters, designated p1 and p2 (Fig. 1), that might potentially influence *pcnB* expression (Jasiecki and Wegrzyn 2006b).

The discovery of the dependence of *pcnB* expression on bacterial growth rate (Jasiecki and Wegrzyn 2003) might suggest that factors responsible for modulation of transcription in response to various nutritional conditions could be involved in the control of PAP I production. In fact, the stringent control, a bacterial response to amino acid starvation and many other nutritional and environmental stresses, is a global regulatory system that ensures optimal energy usage under unfavorable growth conditions (for recent reviews, see Potrykus and Cashel 2008; Wu and Xie 2009). Molecules recognized as alarmons of this control system have been considered to belong to the most far-reaching effectors, whose major role is an immediate response to



**Fig. 1** Nucleotide sequence of the *pcnB* promoter region. The previously reported (or proposed) transcription start sites from particular promoters (pS1, p1, pB and p2) are marked, with first transcribed nucleotides indicated by *large bold letters* and *arrows* showing the

direction of transcription, and  $-35$  and  $-10$  boxes are shown in *frames*. The Shine–Dalgarno (S.D.) sequences are *underlined*, and translation initiation codons (ATT and ATG, called +1(a) and +1(b), respectively) are indicated by *bold letters*

rapidly changing environmental conditions (Szalewska-Palasz et al. 2007b).

There are two major effectors of the stringent response. The first one is a couple of two specific nucleotides, guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp), collectively called (p)ppGpp and rapidly produced in response to variety of physico-chemical and nutritional stresses (for reviews, see Magnusson et al. 2005; Potrykus and Cashel 2008). Since in *E. coli* ppGpp appears to be more abundant and of higher physiological importance than pppGpp, it is often mentioned as the main stringent response factor. In fact, ppGpp can directly and indirectly regulate expression of variety of genes, important for survival of bacterial cells. It interacts with RNA polymerase (Artsimovitch et al. 2004); however, its exact binding site is still controversial (Vrentas et al. 2008), and it does not introduce any long-lasting conformational alterations to this enzyme (Szalewska-Palasz 2008). The second effector of the stringent response is the DksA protein, which was found as an indispensable factor for ppGpp-mediated effects on ribosomal promoters in vivo (Paul et al. 2004). This protein interacts with RNA polymerase (Perederina et al. 2004), and it was suggested that DksA is a co-factor of the stringent response, enhancing both negative and positive effects of ppGpp on activities of promoters (Paul et al. 2005). Nevertheless, recent reports indicated that roles of ppGpp and DksA in transcription regulation can be independent and even opposing (Magnusson et al. 2007; Lyzen et al. 2009; Merrikh et al. 2009).

The major mechanism of direct ppGpp-mediated transcription regulation was proposed to be a decrease of stability of the promoter-RNA polymerase open complexes (Barker et al. 2001a, b). According to this “active model”, promoters that form unstable open complexes are inhibited by ppGpp, while those forming relatively stable complexes might be either stimulated by this nucleotide or insensitive to its presence. Although it was demonstrated that a promoter forming an extremely stable open complex with RNA polymerase can also be negatively regulated by ppGpp both in vivo and in vitro (Potrykus et al. 2002), the model assuming that ppGpp negatively regulates only promoters which form unstable open complexes is generally accepted as the best explanation of impairment of transcription from promoters dependent on the major  $\sigma$  factor in *E. coli*,  $\sigma^{70}$  (Srivatsan and Wang 2008).

Another mechanism is proposed to explain effects of ppGpp and DksA on transcription from promoters dependent on alternative  $\sigma$  factors. The stringent response effectors have been reported as positive regulators of various promoters recognized by RNA polymerase holoenzymes containing  $\sigma$  subunits known to operate under specific environmental or physiological conditions, e.g.  $\sigma^{54}$  ( $\sigma^N$ ),  $\sigma^{38}$  ( $\sigma^S$ ) and  $\sigma^{24}$  ( $\sigma^E$ ) (for a review, see Srivatsan and

Wang 2008). Thus, the “passive model” of ppGpp- and DksA-mediated transcription regulation was proposed (summarized by Srivatsan and Wang 2008). According to this model, stimulation of transcription by ppGpp and DksA under stress conditions is a global consequence of strong negative effects of these effectors on functions of stringently regulated powerful  $\sigma^{70}$ -dependent promoters. Namely, dramatic down-regulation of transcription from rRNA promoters leads to a significant increase in cellular availability of the core of RNA polymerase for association with alternative  $\sigma$  factors. Such a type of regulation has been experimentally supported mainly for  $\sigma^N$ -dependent promoters (Bernardo et al. 2006, 2009; Szalewska-Palasz et al. 2007a); nevertheless, it is generally accepted for promoters recognized by other alternative  $\sigma$  factors, especially in the light of several examples of ppGpp- and DksA-mediated stimulation of transcription from  $\sigma^S$ -dependent promoters (reviewed by Srivatsan and Wang 2008). On the other hand,  $\sigma^{28}$ -dependent promoters can be inhibited rather than stimulated by ppGpp and DksA (Lemke et al. 2009), indicating that the above-presented mechanism must be more complicated.

In this report, we present results of our studies on the control of *pcnB* gene transcription. Our experiments indicated that this control is complex, and involves activities of several factors, including  $\sigma^{70}$  and  $\sigma^S$  subunits of RNA polymerase, ppGpp and DksA.

## Materials and methods

### Bacterial strains, plasmids and growth conditions

*E. coli* K-12 strain MG1655 (Xiao et al. 1991) and its *rpoD800* (Grossman et al. 1983), *relA spoT* (ppGpp<sup>0</sup>) (Szalewska-Palasz et al. 2007a), *dksA::kan* (DksA<sup>0</sup>) (Kang and Craig 1990) and  $\Delta pcnB::kan$  (*pcnB*) (Wrobel et al. 1998) derivatives were used. The *relA spoT dksA* (ppGpp<sup>0</sup> DksA<sup>0</sup>) mutant was constructed by P1vir transduction of the  $\Delta dksA::tet$  allele from TE8114 (Brown et al. 2002) into the above-described *relA spoT* strain. Due to frequent accumulation of suppressor mutations by ppGpp<sup>0</sup> and DksA<sup>0</sup> strains, bacteria used for experiments were checked for specific phenotypes, like amino acid auxotrophy. The *rpoS* mutant was constructed by P1vir transduction of the *rpoS359::Tn10* from RH90 (Lange and Hengge-Aronis 1991) into MG1655. The *rpoS relA spoT* strain was constructed by P1vir transduction of *rpoS359::Tn10* into ppGpp<sup>0</sup>.

Plasmid pTE103 (Elliott and Geiduschek 1984) was employed. For construction of the plasmid pTE103-F4-fanti, a PCR fragment, flanked by primers Fuzja 4 (5' AGA ATT CTC ATT CAT CGC CGT GAT G) and fanti (5'-AGG

ATC CAA ATT AGC GAC TCG GGT) (Jasiecki and Wegrzyn 2006b) was cloned into *Bam*HI and *Eco*RI sites of pTE103. This construct was verified by DNA sequencing.

Bacteria were cultured either in LB medium (Sambrook et al. 1989) or in a minimal medium MM (Jasiecki and Wegrzyn 2003) supplemented with various carbon sources: 0.2% glucose (MMGlu), 0.2% glycerol (MMGly), 0.6% sodium succinate (MMSuc) or 0.8% sodium acetate (MMAce).

### Proteins and ppGpp

DksA protein, and RNA polymerase core and various  $\sigma$  factors were purified according to previously reported procedures (Gamer et al. 1992; Janaszak et al. 2007; Szalewska-Palasz et al. 2007a). The  $\Delta$ HTH-PspF protein was purified as already described (Jovanovic et al. 1999). His-tagged proteins were purified by Ni-affinity chromatography using the BioLogic LP chromatography system (Bio-Rad). ppGpp was purified as described previously (Lyzen et al. 2009). The  $\sigma^S$  and  $\sigma^{54}$  factors were provided by Dr. Anna Janaszak (Medical University of Gdańsk, Poland). The  $\sigma^E$  factor was provided by Ewa Stec (University of Gdańsk, Poland). The *E. coli* RNA polymerase core, the  $E\sigma^{70}$  holoenzyme, and the DksA protein were purified as described previously by Lyzen et al. (2009).

### RNA isolation

Total RNA was isolated from either exponentially growing bacterial cultures (samples were withdrawn at  $A_{575}$  of 0.4) or cultures being at the stationary phase (at the beginning of the plateau value of  $A_{575}$  in the bacterial growth curve). 10 ml of the culture was centrifuged (3,000g, 10 min) and RNA was isolated from bacterial cells using the Total RNA kit (A&A Biotechnology, Gdynia, Poland), according to manufacturer's instruction. Quality of RNA samples was verified electrophoretically and spectrophotometrically.

### Primer extension analysis

The primer extension reactions were performed essentially as described by Janaszak et al. (2007). Briefly, either 50  $\mu$ g of total RNA or 1.5  $\mu$ g of specific *pcnB* mRNA (generated by in vitro transcription), was mixed with 0.5 pmol of  $^{32}$ P-labeled primer. Different primers were used to detect various transcripts: Pr11 (5'-CAG CCT CGC TTT CCT CGC GGC TTA GC) (Jasiecki and Wegrzyn 2006b), *pcnTR2* (5'-ACT GTC TCA GTG AAC TCA TCC AT), PS1.rev (5'-TAC ATT ACC TTC AGG GCA TTT TCA), PS2.rev (5'-TTG CTC GAC GCT GAA ATC CTG CCA), *OmpA* (5'-GCA CCA GTG TAC CAG GTG TTA TC), *ompArev* (5'-CGG TGA AGG ATT TAA CCG TG), Rsd.1

(5'-ATA TCG TGA CGC CGC TGC TGT TGT), Rsd.rev (5'-TGA CGC GCT CCG TCA GGT TAT CGA G), Fim.B1 (5'-ACT GCG CTC CAT GAA ATA GCC AT), FimB.rev (5'ACG TTG CCA TAC AAA CGC CAT GCT). The mixture was incubated at 85°C for 20 min. Following addition of the M-MuLV buffer (Fermentas, Vilnius, Lithuania), a primer was hybridized to RNA at optimal temperature for 60 min. Then, samples were immediately put into ice-bath, and 1  $\mu$ l of 25 mM solution of four dNTPs, 1  $\mu$ l of RevertAid M-MuLV Reverse Transcriptase (Fermentas) and RNase inhibitor (1 unit per  $\mu$ l) were added. The reaction was conducted for 60 min at 42°C, and stopped by addition of 5  $\mu$ l of the denaturation solution and incubation at 95°C for 5 min. The reaction products were separated during electrophoresis in 7% polyacrylamide gel with 8 M urea; products of the sequencing reaction, with the use of the same primer, were separated in the same gel. The bands were visualized by autoradiography and analyzed using the Quantity One software (Bio-Rad).

Each primer extension experiment was repeated at least three times to assess reproducibility.

### In vitro transcription

The in vitro transcription reactions in the presence of ppGpp and/or DksA were performed as described previously (Lyzen et al. 2009), with minor modifications. The following templates were used: for *pcnB* promoters, plasmid pTE103-F4-fanti; for measurement of  $p_R$  activity, pTE103-derived plasmid described by Lyzen et al. (2009); and for studies on  $p_L$  activity, plasmid pVI901 (Szalewska-Palasz et al. 2007a). Plasmid DNA was purified by CsCl density gradient ultracentrifugation and chromatography, using P-30 columns (Bio-Rad). Reactions were conducted in the final volume of 20  $\mu$ l, in the transcription buffer (50 mM Tris-HCl pH 8, 10 mM MgCl<sub>2</sub>, 10 mM  $\beta$ -mercaptoethanol, 150 mM KCl, 10  $\mu$ g/ml BSA). Following preliminary experiments, in which optimal concentrations of the template and RNA polymerase were estimated, further experiments were performed with either 4 or 8 nM template and either 30 or 60 nM RNA polymerase holoenzyme. DNA template, RNA polymerase holoenzyme and indicated amounts of ppGpp and/or DksA were incubated for 10 min at 37°C. Following addition of a nucleotide mix (to final concentrations: 1 mM ATP, 150  $\mu$ M GTP, 150  $\mu$ M CTP, 15  $\mu$ M UTP, and 1  $\mu$ Ci [ $\alpha$ - $^{32}$ P]UTP, 3,000 Ci/mmol) the mixture was incubated for 12 min at 37°C. Then, heparin was added (to 0.1 mg/ml) and the incubation was continued for 5 min. The reaction was stopped by addition of 5  $\mu$ l of the denaturation solution (150 mM EDTA, 1.05 M NaCl, 7 M urea, 10% glycerol, 0.0375% xylene cyanol, 0.0375% bromophenol blue) and incubation for 5 min at 95°C. The reaction products were separated by electrophoresis in a

4% polyacrylamide gel containing 8 M urea in the TBE buffer (Sambrook et al. 1989), and visualized by autoradiography using PhosphorImager (Bio-Rad). The results were quantified densitometrically, employing Quantity One software (Bio-Rad).

In vitro transcription with RNA polymerase holoenzymes containing different  $\sigma$  factors:  $\sigma^{70}$ ,  $\sigma^{54}$ ,  $\sigma^{32}$ ,  $\sigma^E$  or  $\sigma^S$  ( $E\sigma^{70}$ ,  $E\sigma^{54}$ ,  $E\sigma^{32}$ ,  $E\sigma^E$  or  $E\sigma^S$ , respectively) was performed in either the TN buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM  $MgCl_2$ , 1 mM DTT, 0.1 mM EDTA), for  $E\sigma^{70}$ ,  $E\sigma^{32}$ ,  $E\sigma^E$  and  $E\sigma^S$ , or the STA buffer (25 mM Tris-acetate pH 8.0, 8 mM magnesium acetate, 10 mM KCl, 1 mM DTT, 3.5% PEG 6000), for  $E\sigma^{54}$ , in the presence of BSA (100  $\mu$ g/ml) at 37°C. Before the transcription reactions,  $\sigma$  factors were preincubated with the RNA polymerase core enzyme (at molar ratio 1:4) for 10 min at 37°C. The final volume of the reaction samples was 20  $\mu$ l, and contained 8 nM template and 30 nM appropriate RNA polymerase holoenzyme. When  $E\sigma^{54}$  was used, an unspecific activator protein  $\Delta$ HTH-PspF, which can stimulate transcription from  $\sigma^{54}$ -dependent promoters independently of the DNA sequence (Jovanovic et al. 1999; Janaszak et al. 2007; Wigneshweraraj et al. 2003), was added to 1  $\mu$ M together with 2.5 mM ATP, and incubation was continued for 10 min (in experiments with other holoenzymes, this stage was omitted). Following addition of nucleotide mixture (as described above), the reaction was conducted for 12 min, and after addition of heparin, for another 5 min. The reaction was stopped by addition of the denaturation solution and separated electrophoretically (as described above).

When the in vitro transcription reaction products were used subsequently for primer extension experiments, unlabeled UTP (150  $\mu$ M) was added instead of [ $\alpha$ - $^{32}$ P]UTP, and the reaction volume was 100  $\mu$ l. Furthermore, the RNA products were purified using the phenol-chloroform extraction and ethanol precipitation (Sambrook et al. 1989).

#### DNase I footprinting

Primer Pr10 (5'-AGC ACC TTG CGG CAA AAA TTA GCG AC) (Jasiecki and Wegrzyn 2006b) was end-labeled with [ $\gamma$ - $^{32}$ P]ATP and T4 polynucleotide kinase (Fermentas). DNA fragments were PCR amplified using  $^{32}$ P end-labeled Pr10 primer and unlabeled Przedpcn primer (5'-AGA ATC ATG CGC CTG CGT TGC). DNase I footprinting reactions were performed as described previously (Janaszak et al. 2007), in a total volume of 10  $\mu$ l of the TN buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM  $MgCl_2$ , 1 mM DTT, 0.1 mM EDTA). The labeled DNA fragments (at final concentration of 10 nM) were incubated for 15 min at 30°C with  $E\sigma^{70}$ . DNase I ( $5 \times 10^{-4}$  units) was added, and after incubation for 1 min at 37°C the reaction was stopped by the addition of EDTA to the final concentration

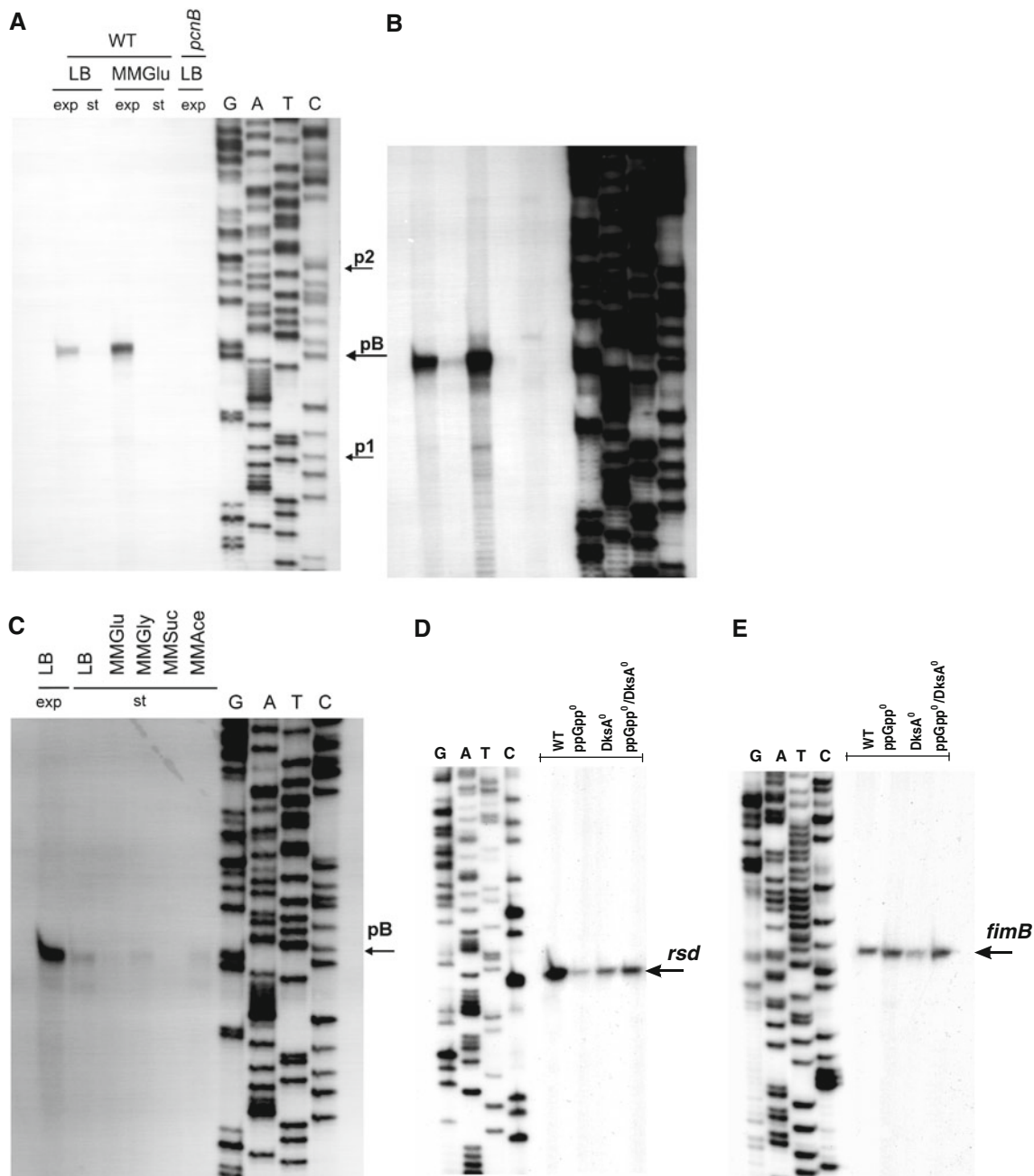
of 50 nM, followed by heating for 5 min at 95°C in the urea loading dye. The reaction products were separated by electrophoresis in a 7% polyacrylamide gel containing 8 M urea, together with the products of DNA sequencing reactions performed using the Pr10 primer and the *fmol* DNA Cycle Sequencing System (Promega). Each footprinting experiment was repeated at least three times to assess reproducibility.

## Results

pB is the major promoter of the *pcnB* gene

Previous studies indicated that there are at least three promoters located upstream of the *pcnB* coding sequence in *E. coli* MG1655 genome, called p1, pB and p2 (see Fig. 1; Jasiecki and Wegrzyn 2006b). These promoters were found by primer extension experiments, in which relatively gentle reaction conditions were used (Jasiecki and Wegrzyn 2006b). Such conditions were allowed to obtain comparable strengths of signals from all these promoters. To distinguish actual activities of p1, pB and p2, we have performed primer extension experiments using significantly more stringent reaction conditions that should cause impairment of signals caused by annealing of the primer to mRNA molecules which occur in low abundance in cells. Relative to the previously reported conditions (Jasiecki and Wegrzyn 2006b), the following changes were made in the protocol used in this work: (a) higher amount of total RNA (50  $\mu$ g vs. 10–500 ng in the previously reported study) and lower amount of the primer (0.5 vs. 15–20 pm) were used, (b) the denaturation step was longer (20 vs. 5 min) and performed at higher temperature (80 vs. 65°C), and (c) the annealing step was added [incubation for 1 h at the temperature equal to  $T_m$  of the primer was introduced rather than starting the primer extension reaction immediately after cooling the mixture (composed of RNA and primer molecules) to 42°C, and addition of other components of the reaction].

Under these experimental conditions (i.e. under the “stringent” conditions), a highly predominant signal corresponded to the product of the pB promoter activity (Fig. 2a). The p1- and p2-derived transcripts could also be detected, but only after significantly longer exposure of the electrophoretic gels with labeled reaction products (Fig. 2b). Such a picture of pB was observed when RNA was isolated from bacteria growing in a rich medium (LB), as well as in a minimal medium (MMGlu), but the weaker promoter signals were detected only in samples from the minimal medium (Fig. 2). This is important as previously described experiments were performed either only in LB (when pB was discovered; Binns and Masters 2002) or only in MMGlu (when p1 and p2 were discovered;



**Fig. 2** Transcription from the *pcnB* (a–c), *rsd* (d) and *fimB* (e) promoter regions under various growth conditions of bacterial cultures. In experiments shown in a and b, wild-type (WT) *E. coli* or the *pcnB* ( $\Delta pcnB::kan$ ) mutant was grown in LB or MMGlu medium, to exponential (exp) or stationary (st) phase of growth at 37°C. Primer extension experiments were performed with primer Pr11 as described in “Materials and methods”, and the products of the reactions were separated during polyacrylamide gel electrophoresis, with the products of the sequencing reaction (performed using the same primer) run at the same gel (lanes G, A, T and C). The position of the products of primer extension reactions, corresponding to transcripts originating from the pB promoter, is shown in a. Positions previously reported as p1 and p2 transcription start sites are also marked by arrows. Apart from results of the experiment performed using the standard procedure (shown in a), results of a significantly longer exposition of the same gel during

autoradiography are demonstrated (b) to show products corresponding to activities of other promoters. Analogous experiments, but with *E. coli* cells cultured at the exponential phase in LB medium (LB exp) and at the stationary phase (st) in various media (LB, MMGlu, MMGly, MMSuc, MMAce) are shown in c. In control experiments, levels of the *rsd* (d) and *fimB* (e) transcripts in *E. coli* wt cells (WT), and ppGpp<sup>0</sup> (*relA spoT* double mutant), DksA<sup>0</sup> (*dksA::kan* mutant) and ppGpp<sup>0</sup> DksA<sup>0</sup> (*relA spoT dksA* triple mutant) derivatives cultured at the stationary phase (d) or the exponential phase (e) in LB medium. Primer extension experiments were performed with Rsd.rev and FimB.rev primers, and the products of the reactions were separated during polyacrylamide gel electrophoresis, with the products of the sequencing reaction (performed using the same primer) run at the same gel (lanes G, A, T and C)

Jasiecki and Wegrzyn 2006b), thus, suggesting the source of ostensible discrepancies between the results. Nevertheless, it appears that pB is the major promoter for *pcnB* expression under various growth conditions of *E. coli* cultures.

#### Activity of the pB promoter in the stationary phase of growth

Although the pB promoter was active in bacteria cultured in both rich (LB) and minimal (MMGlu) media during the exponential growth (Fig. 2a), we found that its activity is drastically reduced at the stationary phase of growth (Fig. 2c). This impairment of the pB activity was independent of the growth medium, as similar results were observed in LB and MMGlu, as well as in other minimal media, in which various carbon sources were employed (Fig. 2c).

Since we observed a lack or drastically decreased level of particular signals on gels in samples derived from stationary phase bacterial cultures (Fig. 2), one might argue that this effect could result from a putative general process occurring under specific experimental conditions and causing either impaired transcription or enhanced RNA degradation. However, in control experiments, we were able to detect other transcripts, specific for *rsd* (Fig. 2d) or *fimB* (Fig. 2e) genes which were demonstrated previously to occur in starved *E. coli* cells (Jishage and Ishihama 1999) or to be differentially regulated by ppGpp and DksA (Aberg et al. 2008), respectively, in the same samples which were employed for analyses of *pcnB* transcription. These experiments served also as internal controls for RNA preparation quality.

#### ppGpp and DksA inhibit transcription from pB

Under conditions of nutritional starvation, which occur also in bacteria being in the stationary phase of growth, the level of ppGpp, a mediator of the stringent response of bacterial metabolism to starvation conditions, increases significantly (for a recent review, see Potrykus and Cashel 2008). It was demonstrated that ppGpp interacts directly with RNA polymerase. Recent studies indicated that the DksA protein plays an important role in modulation of the ppGpp action on transcription efficiency from various promoters (for reviews, see Szalewska-Palasz et al. 2007b; Potrykus and Cashel 2008). Therefore, we have estimated levels of pB-derived transcripts in wild-type cells as well as in the *relA spoT* mutant (unable to produce ppGpp, called ppGpp<sup>0</sup>), *dksA* mutant (called DksA<sup>0</sup>), and the *relA spoT dksA* strain (called ppGpp<sup>0</sup> DksA<sup>0</sup>).

Since expression of the *ompA* gene is maintained at constant levels in *relA* strains (Durfee et al. 2008), we have estimated abundance of *ompA* transcripts (Fig. 3a) in the tested samples to normalize results according to this internal

control. Increased levels of transcripts derived from pB were observed in exponentially growing bacteria devoid of ppGpp, DksA or both (Fig. 3b). However, even more dramatic changes in abundance of these transcripts could be observed when RNA samples from stationary phase cells were analyzed. The signal in the sample from the *dksA* mutant was significantly higher than that from the wild-type strain, and levels of the studied transcript derived from both ppGpp<sup>0</sup> and ppGpp<sup>0</sup> DksA<sup>0</sup> strains were comparable to those estimated for exponentially growing wild-type cells (Fig. 3c). Therefore, we suggest that a combined action of ppGpp and DksA is responsible for dramatically decreased transcription from the pB promoter during stationary phase of bacterial culture growth.

Results of the in vivo experiments, suggesting inhibition of pB activity by ppGpp and DksA, were confirmed in the in vitro transcription reactions, in which production of pB-initiated transcripts in the presence or absence of ppGpp, DksA or both, was tested. DksA weakly inhibited this transcription, while significantly more pronounced impairment of pB activity was caused by ppGpp (Fig. 4). Importantly, a dramatic decrease in the level of pB-derived transcripts was observed when both DksA and ppGpp were present in the reaction mixture (Fig. 4). In control experiments, we have demonstrated that effects of ppGpp and DksA on the activity of the  $\lambda p_R$  promoter were very different from those found for pB (Fig. 4), indicating specificity of the effects on pB and expected activity of purified reaction compounds.

#### $\sigma^{70}$ - and $\sigma^S$ -dependent transcription of *pcnB*

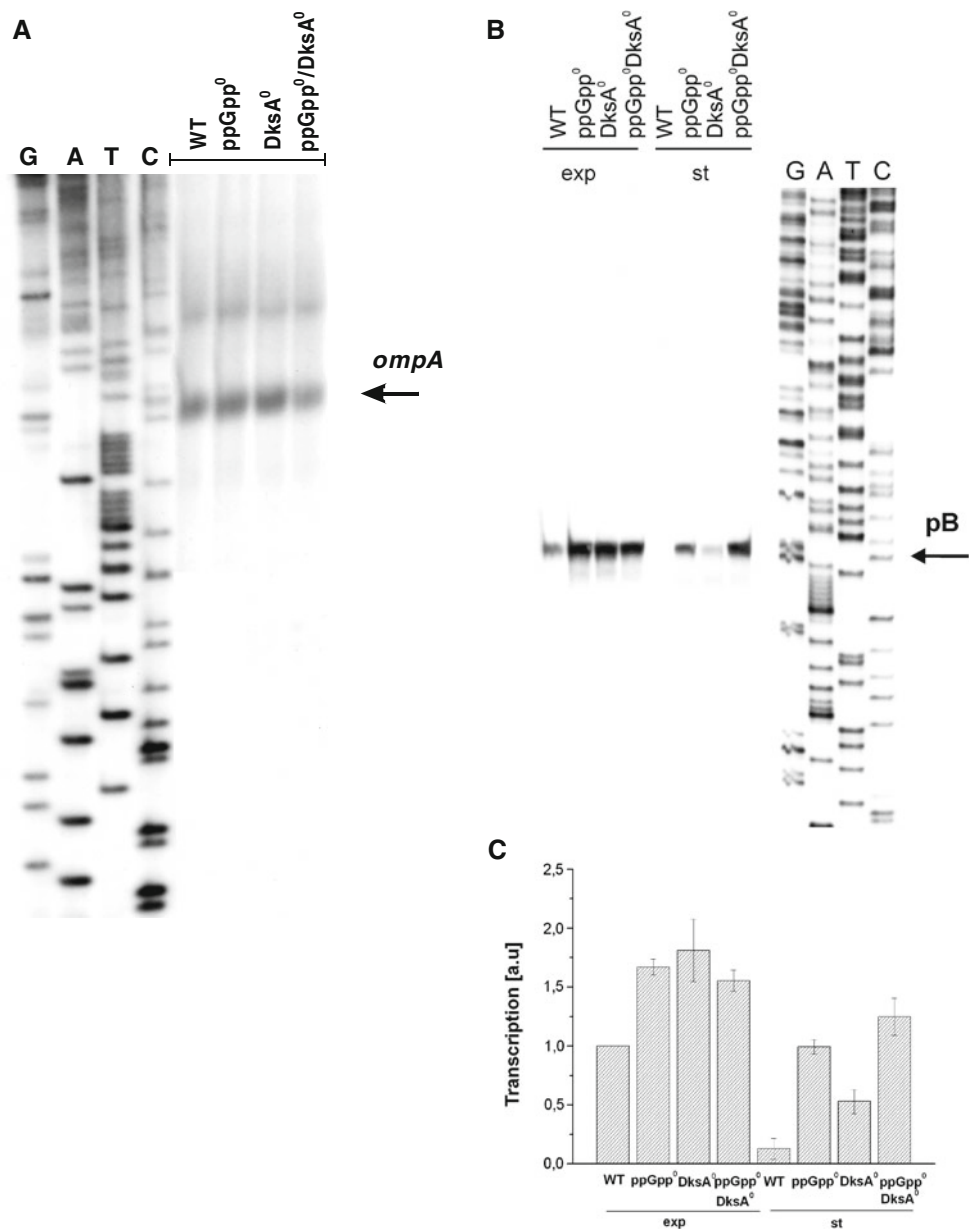
The pB promoter sequence, proposed by Binns and Masters (2002), may correspond to the sequence recognized by RNA polymerase holoenzyme containing the  $\sigma^{70}$  subunit ( $E\sigma^{70}$ ) (Fig. 1). However, since some *E. coli*  $\sigma$  factors may be responsible for recognizing similar promoter sequences, we tested whether pB is a  $\sigma^{70}$ -dependent promoter indeed.

The *rpoD800* mutation results in production of the  $\sigma^{70}$  subunit whose activity is impaired at elevated temperatures (Liebke et al. 1980; Lowe et al. 1981). We found that abundance of the pB-derived transcripts increased in wild-type bacteria shortly (15 min) after the transfer of cultures from 37 to 45°C, while the signals were comparable in the *rpoD800* mutant before and after the temperature shift (Fig. 5).

DNA footprinting experiments, performed with  $E\sigma^{70}$  and the template encompassing the pB promoter region, indicated that the RNA polymerase holoenzyme binds to this DNA fragment, though apparently the promoter was not fully occupied with the protein, which may support previous suggestions (Binns and Masters 2002; Jasiecki and Wegrzyn 2006b) that pB is not a strong promoter (Fig. 6).

The results described above strongly suggested that pB is a  $E\sigma^{70}$ -dependent promoter. However, to verify this

**Fig. 3** Effects of ppGpp and DksA on the *ompA* gene expression (a) and the pB promoter activity (b, c). Wild-type (WT) *E. coli* or ppGpp<sup>0</sup> (*relA spoT* double mutant), DksA<sup>0</sup> (*dksA::kan* mutant) and ppGpp<sup>0</sup> DksA<sup>0</sup> (*relA spoT dksA* triple mutant) strains were cultured in the LB medium at 37°C to exponential (a and exp in b) or stationary (st in b) phase of growth. Primer extension experiments were performed as described in “Materials and methods”, and the products of the reactions were separated during polyacrylamide gel electrophoresis, with the products of the sequencing reaction (performed using the same primer) run at the same gel (lanes G, A, T and C). a and b show representative autoradiograms (positions of the products of primer extension reactions, corresponding to transcripts originating from *ompA* and pB promoters, are shown). c demonstrates quantification (by densitometric analysis) of the results exemplified in b (mean values from three independent experiments are shown in c with error bars indicating SD)



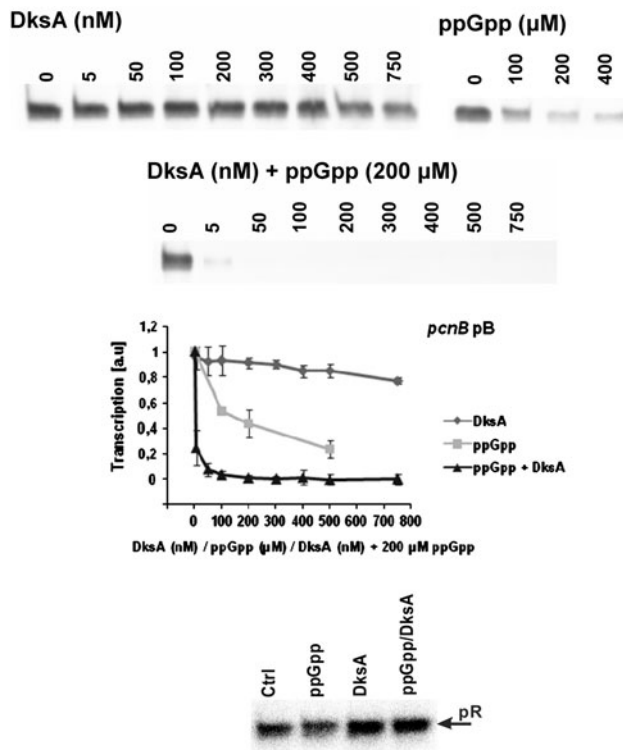
hypothesis, we have performed in vitro transcription reactions with the DNA template containing the pB promoter sequence and RNA polymerase holoenzymes containing different  $\sigma$  subunits, namely:  $\sigma^{70}$ ,  $\sigma^{54}$ ,  $\sigma^{32}$ ,  $\sigma^S$ , and  $\sigma^E$ . To identify precisely products of these reactions, the obtained transcripts were subjected to primer extension reactions. As demonstrated in Fig. 7, specific products were obtained when  $E\sigma^{70}$  or  $E\sigma^S$  was used in the in vitro transcription reaction. No products could be detected in experiments with  $E\sigma^{54}$ ,  $E\sigma^{32}$ , and  $E\sigma^E$  (data not shown). Interestingly, it appears that apart from pB, which is a  $\sigma^{70}$ -dependent promoter but can also be weakly recognized by  $E\sigma^S$  (Fig. 7), there is another promoter, whose activity is weak in the presence of  $E\sigma^{70}$ , but which is active in the presence of  $E\sigma^S$  (Fig. 7). The transcription start site of this promoter, which

we named pS1, corresponds exactly to the localization of the putative promoter of the *pcnB* gene, proposed previously (Liu and Parkinson 1989) (compare Figs. 1 and 7). That proposal was subsequently suggested to be erroneous (Jasiecki and Wegrzyn 2006b), in the light of the work by Binns and Masters (2002). Nevertheless, our results (Fig. 7) indicated that pS1 may be a functional promoter, but recognized considerably more effectively by  $E\sigma^S$  than by  $E\sigma^{70}$ .

Two  $\sigma^S$ -dependent promoters are located in the region upstream of the *pcnB* gene

By using the in vitro transcription system allowing to obtain longer transcripts, we found that in the region of the *pcnB* gene there are two promoters (rather than only one)



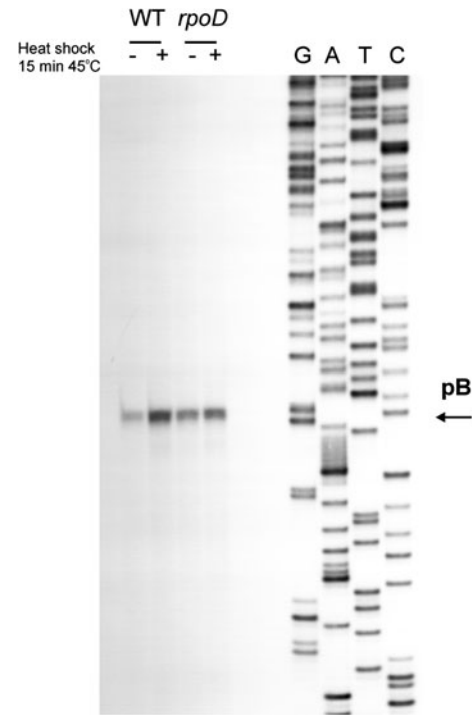


**Fig. 4** Effects of DksA and ppGpp on in vitro transcription from the pB promoter. Representative results are shown on autoradiograms, and the summary of the results (mean values from three experiments with error bars indicating SD) is shown at the diagram (the presence of following factors in the reaction mixture is shown: diamonds DksA, squares ppGpp, triangles DksA and ppGpp). In control experiments, analogous reactions were performed with the  $\lambda p_R$  promoter (the bottom panel), with either no additional factors (Ctrl), ppGpp (200  $\mu$ M), DksA (400 nM) or ppGpp and DksA (200  $\mu$ M and 400 nM, respectively)

whose activities are evident in the presence of  $E\sigma^S$  (Fig. 8a). Apart from the  $\sigma^{70}$ -dependent transcription signal from pB, and the two  $\sigma^S$ -dependent signals (pS1 and pS2), no other transcription initiation signals could be detected in this region with the use of all other tested  $\sigma$  factors (Fig. 8a).

The region of transcription initiation from the second  $\sigma^S$ -dependent promoter for *pcnB*, called pS2, was determined in primer extension experiments, in which RNAs obtained in in vitro transcription experiments were employed as templates (Fig. 8b). Note that there are perhaps at least two alternative start sites from pS2, located in the region between positions  $-263$  and  $-265$  relative to the translation start codon ATT.

The regions of the pS1 and pS2 promoters are shown in Fig. 8c. While putative  $-10$  and  $-35$  boxes can be easily predicted for pS1, in the case of pS2, it is possible to predict only  $-10$  element (TAAACT), whereas there is no obvious  $-35$  box. On the other hand, both pS1 and pS2 sequences bear the C residue in the neighborhood of the  $-10$  element.

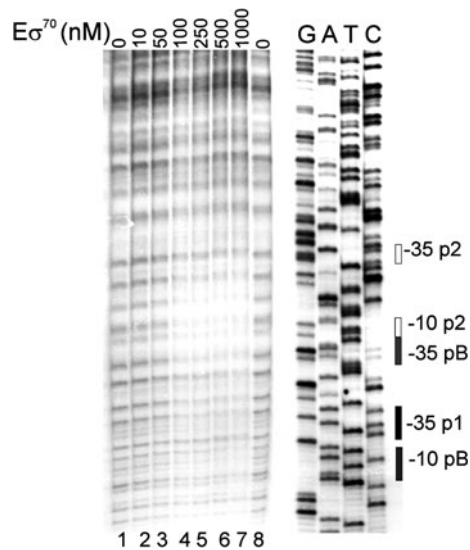


**Fig. 5** Effects of heat shock on levels of pB-derived transcripts in *E. coli* wild-type (WT) bacteria and the *rpoD800* (*rpoD*) mutant. Bacteria were cultured to  $A_{575}$  of 0.4 (exponential phase) and then one half of the culture was transferred to  $45^\circ\text{C}$  (+) while the second half remained at  $37^\circ\text{C}$  (–). Following further cultivation for 15 min, RNA was isolated and primer extension experiments were performed as described in “Materials and methods”. The position of the products of primer extension reactions, corresponding to transcripts originating from the pB promoter, is shown

Such a residue is known to be crucial for selective recognition of promoters dependent on  $E\sigma^S$  (Lacour et al. 2003).

#### Activity of the $\sigma^S$ -dependent promoters of the *pcnB* gene in vivo

We asked whether the  $\sigma^S$ -dependent promoters detected in in vitro experiments are active in vivo. To address this question, primer extension experiments were performed. Wild-type bacteria as well *rpoS* and ppGpp<sup>0</sup> mutants were cultured in LB broth, and samples were withdrawn at the exponential and stationary phases of growth. Following RNA isolation, the primer extension experiments revealed no detectable signal from the pS1 promoter in exponentially growing cells, as expected. Surprisingly, also no such a signal could be observed in samples from stationary phase wild-type bacteria (Fig. 9). However, a signal corresponding to the pS1-initiated transcription was evident in the ppGpp<sup>0</sup> mutant. This transcription was  $\sigma^S$ -dependent as it was totally impaired in the *rpoS* mutant, irrespective of the presence or absence of ppGpp (Fig. 9).



**Fig. 6** Interaction of  $E\sigma^{70}$  with the promoter region of the *pcnB* gene as assessed by DNase I footprinting. A  $^{32}\text{P}$ -labeled 0.3 kb DNA fragment was incubated with 0 nM (negative control, lanes 1 and 8), 10 nM (lane 2), 50 nM (lane 3), 100 nM (lane 4), 250 nM (lane 5), 500 nM (lane 6) or 1,000 nM (lane 7)  $E\sigma^{70}$ . The footprinting experiments were performed as described in “Materials and methods”. The  $-10$  and  $-35$  sequences of p1, pB and p2 are indicated

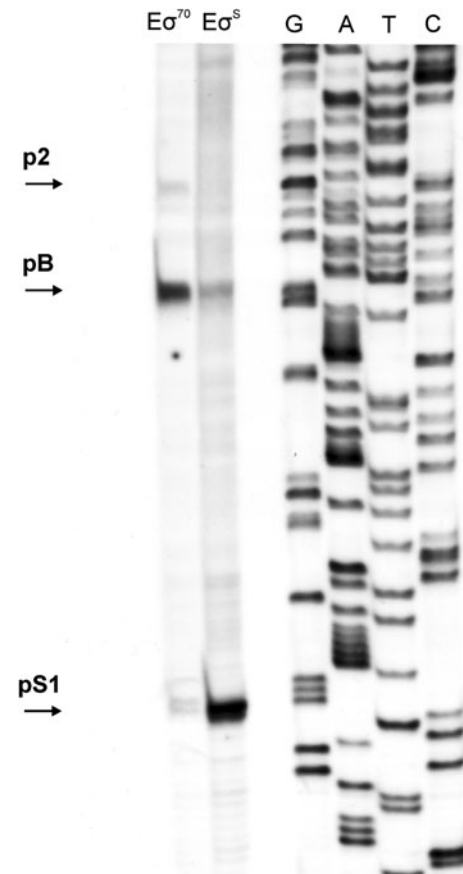
Results analogous to those described above were obtained when the pS2 promoter was tested. Its activity could be detected only in the stationary phase of bacterial growth in the absence of ppGpp and in the presence of  $\sigma^S$  activity (Fig. 10).

The pS1 and pS2 promoters are negatively regulated by ppGpp in vivo

Since pS1 and pS2 activities appeared to be negatively regulated by ppGpp, we have tested their responses to lack of either one or two major effectors of the stringent response, ppGpp and DksA, in bacteria from stationary phase of growth. Both promoters were inactive in the presence of ppGpp, irrespective of the activity of DksA (Fig. 11). These results suggest that ppGpp is the main negative regulator of pS1 and pS2 in vivo.

The pS1 and pS2 promoters are negatively regulated by ppGpp and DksA in vitro

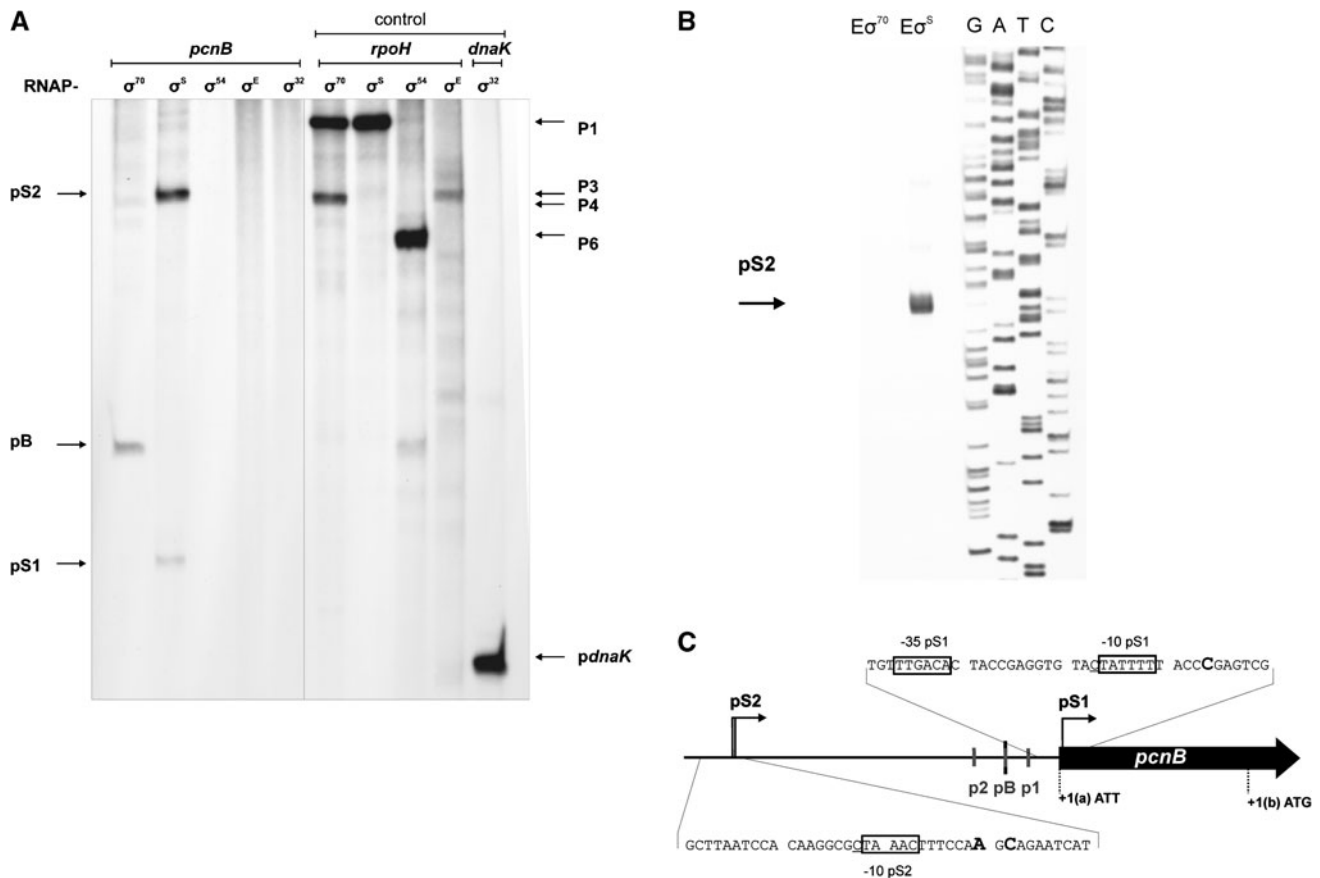
To test whether the ppGpp-mediated negative regulation of activities of pS1 and pS2 in vivo is direct or indirect, we have performed in vitro transcription experiments. Again, both promoters responded in the same way to the presence of ppGpp and/or DksA in the reaction mixture. The DksA protein alone had little, if any, effect on transcription from



**Fig. 7** Effects of the presence of different ( $\sigma^{70}$  or  $\sigma^S$ ) sigma factors on in vitro transcription from *pcnB* promoters. Primer extension experiments were performed with primer pcnTR2 (as described in Fig. 2) using templates obtained in the in vitro transcription reactions, and either  $E\sigma^{70}$  or  $E\sigma^S$ . Positions corresponding to transcripts derived from pS1, pB and p2 promoters are marked by arrows

both tested promoters (Fig. 12a–d). A marked and concentration-dependent impairment of pS1 and pS2 activities could be detected in the presence of ppGpp. These results indicate that the effects of this nucleotide on transcription from these two promoters are direct. Although DksA alone was not able to inhibit activities of pS1 and pS2 significantly, this protein enhanced the inhibitory effects of ppGpp, when present together with this nucleotide in the reaction mixture (Fig. 12a–d).

To test whether the effects of ppGpp and DksA are specific to pS1 and pS2, we have performed control experiments with a template containing a promoter demonstrated previously to be unaffected by these factors. The  $\lambda p_L$  promoter appeared to be a good candidate for such a control. Indeed, we found no significant effects of ppGpp and/or DksA on pL-initiated transcription (Fig. 12e–g). Therefore, we conclude that there is a specific negative regulation of pS1 and pS2 by ppGpp and DksA.



**Fig. 8** In vitro transcription from the region of the *pcnB* gene (**a**) and mapping of the pS2 transcription start sites (**b**). In experiments shown in **a**, *E. coli* RNA polymerase holoenzymes bearing different  $\sigma$  factors (marked above particular lanes) were used in the reactions performed as described in “Materials and methods”. Positions of transcripts derived from pB, pS1 and pS2 promoters are indicated. In the control experiment, activities of all holoenzymes were demonstrated by employing DNA templates containing promoters specific for vari-

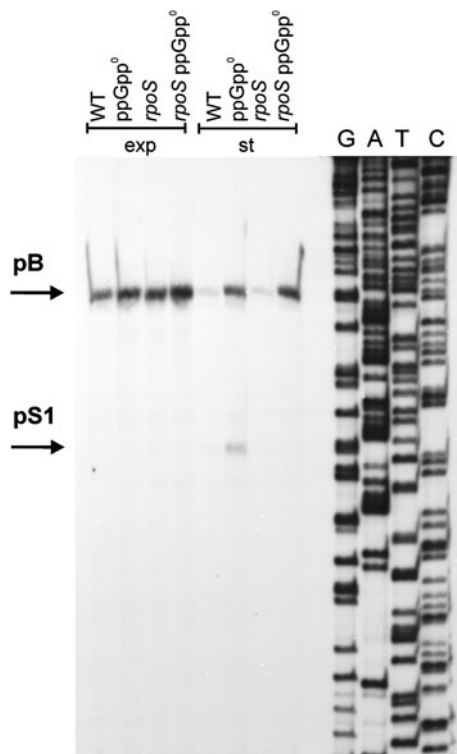
ous  $\sigma$  factors (templates with the *rpoH* gene promoter region and the *dnaK* gene promoter region, described by Janaszak et al. 2007, were used); positions of bands corresponding for transcripts originating from particular promoters are indicated. In experiments shown in **b**, primer extension experiments with primer PS2.rev were performed using the products of in vitro transcription reactions as templates. In **c**, proposed localization of pS1 and pS2 promoters in the region upstream of the coding sequence of the *pcnB* gene is shown

## Discussion

Since polyadenylation of bacterial RNA plays a role in the regulation of expression of as many as 90% of genes (for a review, see Regnier and Hajnsdorf 2009), it is clear that determination of mechanisms leading to different efficiency of adding poly(A) tails at the 3' ends of transcripts is required to understand this specific control process. On the other hand, regulation of RNA polyadenylation efficiency in *E. coli* is relatively poorly understood. It was demonstrated that both production of PAP I and polyadenylation reaction are inversely correlated to bacterial growth rate (Jasiecki and Wegrzyn 2003). However, only basic information on expression of the *pcnB* gene, coding for PAP I, was available to date. It appeared that there are at least three promoters located upstream of the *pcnB* coding sequence (Jasiecki and Wegrzyn 2006b) and that synthesis of PAP I may also be regulated at the level of translation

initiation (Binns and Masters 2002). Moreover, PAP I was found to be a substrate for phosphorylation, and this modification may influence the enzyme activity (Jasiecki and Wegrzyn 2006a).

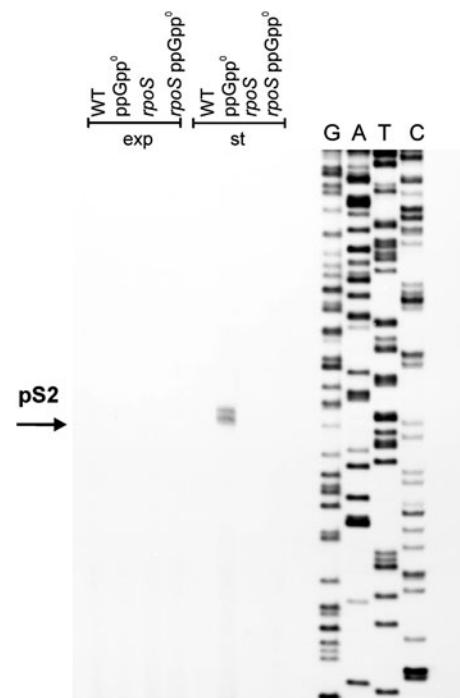
Results demonstrated in this report indicated that among all *pcnB* promoters, pB is the major one in bacteria growing in both rich and minimal media. We also conclude that p1 and p2 may have only minor roles in the control of *pcnB* expression, though they are still active promoters (compare Figs. 2a and 7). The differences in relative activities of pB, p1 and p2 reported in three papers, this one and published previously (Binns and Masters 2002; Jasiecki and Wegrzyn 2006b), may be due to various growth conditions used in different studies (rich vs. minimal media) and various conditions (“stringent” vs. “relaxed”) of primer extension reactions. One should also note that activities of the fusions of pB, p1 and p2 with *lacZ*, reported previously (Jasiecki and Wegrzyn 2006b), reflected collective activities of



**Fig. 9** Activity of the pS1 promoter in vivo and its dependence on the *rpoS* gene function. Wild-type (WT) *E. coli* or ppGpp<sup>0</sup> (*relA spoT* double mutant), *rpoS* and ppGpp<sup>0</sup> *rpoS* (*relA spoT rpoS* triple mutant) strains were cultured in the LB medium at 37°C to exponential (exp) or stationary (st) phase of growth. Primer extension experiments were performed with primer PS1.rev as described in “Materials and methods”, and the products of the reactions were separated during polyacrylamide gel electrophoresis, with the products of the sequencing reaction (performed using the same primer) run at the same gel (lanes G, A, T and C)

different promoters, rather than those of individual promoters, due to close proximity of their sequences. Moreover, these fusions contained only short fragments of sequences located beyond  $-10$  and  $-35$  regions of tested promoters; thus, they might lack important regulatory sequences. This may explain why only weak activity of pB could be detected when a fusion containing only  $-10$  and  $-35$  boxes of this promoter was tested in bacteria grown in minimal media (Jasiecki and Wegrzyn 2006b).

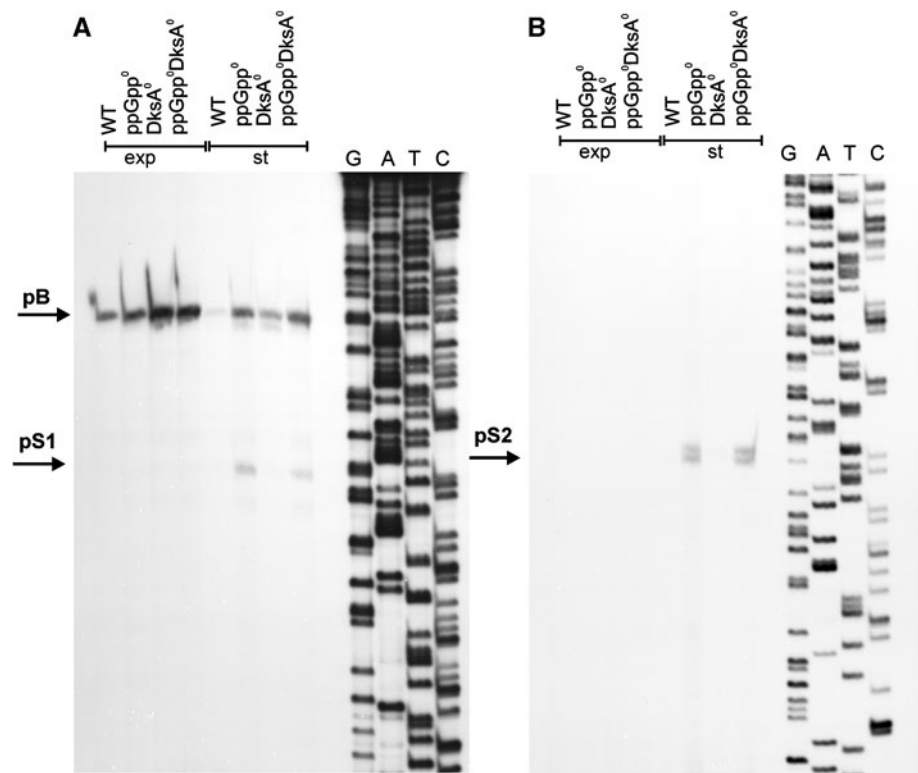
Interestingly, transcription of the *pcnB* gene was found to be drastically inhibited at the stationary phase of growth. The stringent control alarmone, ppGpp, and the DksA protein are responsible for this inhibition as mutations in genes that lead to the absence of ppGpp and DksA caused restoration of transcription from pB in cells from the stationary phase to the level estimated in exponentially growing wild-type bacteria. Moreover, pB activity was also impaired by ppGpp and DksA in a purified in vitro transcription system. This is interesting in the light of previous findings that both *pcnB* transcription and PAP I levels are inversely



**Fig. 10** Activity of the pS2 promoter in vivo and its dependence on the *rpoS* gene function. Wild-type (WT) *E. coli* or ppGpp<sup>0</sup> (*relA spoT* double mutant), *rpoS* and ppGpp<sup>0</sup> *rpoS* (*relA spoT rpoS* triple mutant) strains were cultured in the LB medium at 37°C to exponential (exp) or stationary (st) phase of growth. Primer extension experiments were performed with primer PS2.rev as described in “Materials and methods”, and the products of the reactions were separated during polyacrylamide gel electrophoresis, with the products of the sequencing reaction (performed using the same primer) run at the same gel (lanes G, A, T and C)

proportional to bacterial growth rate (Jasiecki and Wegrzyn 2003). Since ppGpp levels increase moderately in slowly growing *E. coli* cells (see Potrykus and Cashel 2008, for a review), one might speculate that effects opposite to those described in this report could occur at the stationary phase of growth. However, it appears that physiological processes may be controlled differentially in slowly growing and non-growing bacteria. We suppose that physiological significance of such a regulation may reflect the control of energy resources in bacterial cells. In slowly growing cells, enhanced expression of certain genes might be required, and under poor nutritional conditions, nucleotides necessary for transcription of these genes could be obtained from rapidly degraded transcripts. Thus, an increased level of *pcnB* transcription (and expression), and resultant more efficient RNA polyadenylation and its more rapid degradation, might provide sufficient pool of nucleotides. However, when cell growth is totally inhibited, like at the stationary phase, production of an excess of PAP I, and subsequent intensive RNA polyadenylation, would be a waste of energy. Moreover, PAP I was proposed to be toxic for cells when occurring at significantly elevated levels

**Fig. 11** Effects of ppGpp and DksA on the pS1 (a) and pS2 (b) promoter activity. Wild-type (WT) *E. coli* or ppGpp<sup>0</sup> (*relA spoT* double mutant), DksA<sup>0</sup> (*dksA::kan* mutant) and ppGpp<sup>0</sup> DksA<sup>0</sup> (*relA spoT dksA* triple mutant) strains were cultured in the LB medium at 37°C to exponential (exp) or stationary (st) phase of growth. Primer extension experiments were performed as described in “Materials and methods”, with the use of PS1.rev primer (left panel) and PS2.rev primer (right panel), and the products of the reactions were separated during polyacrylamide gel electrophoresis, with the products of the sequencing reaction (performed using the same primers) run at the same gel (lanes G, A, T and C). Positions corresponding to pB, pS1 and pS2 transcription start sites are shown

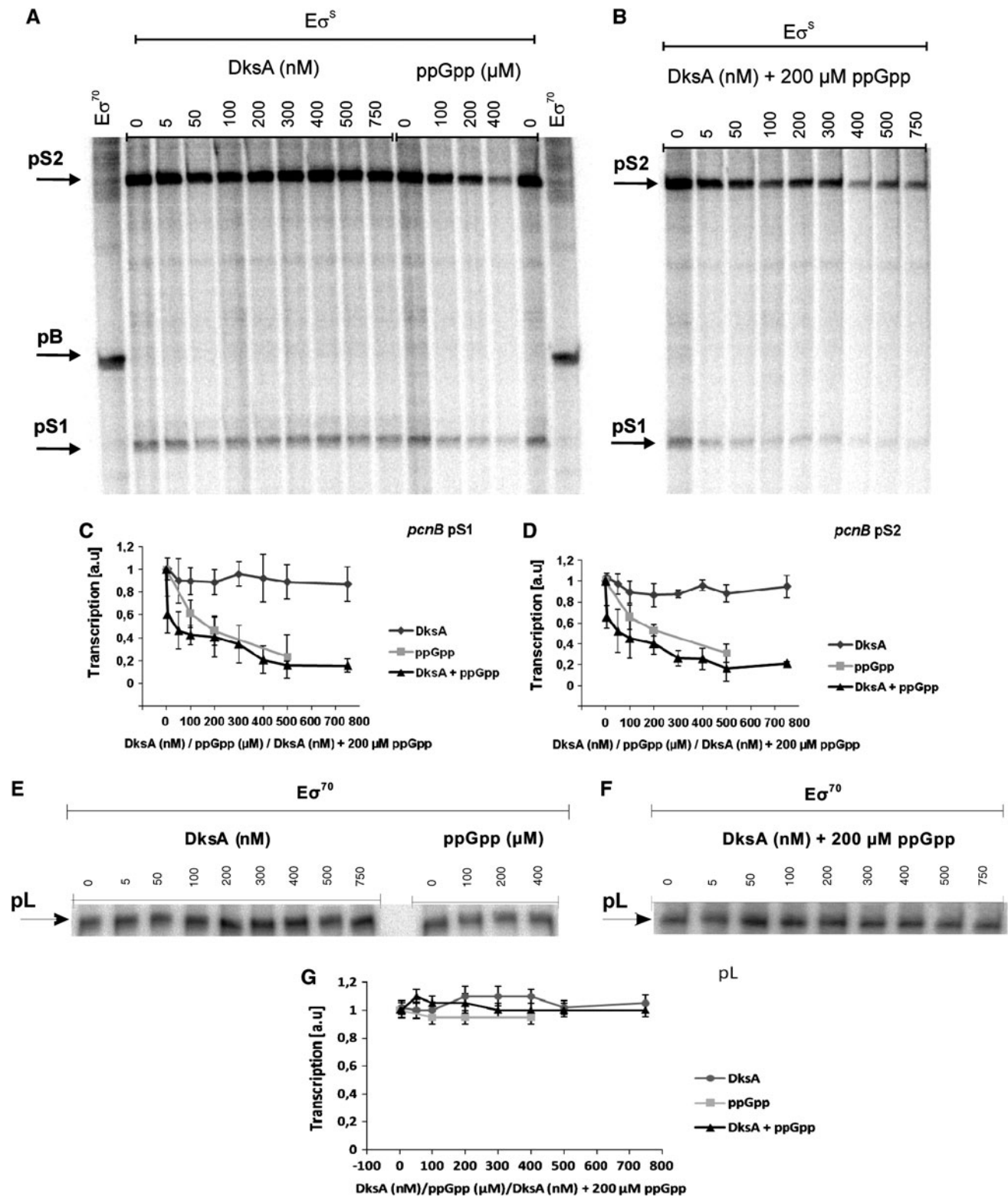


(Binns and Masters 2002); thus, an impaired transcription of *pcnB* might be a mechanism, which together with a specific regulation of translation from a non-canonical start codon (Binns and Masters 2002) could protect the cell from PAP I toxicity.

The localization of the functional *pcnB* gene promoter was controversial for several years. Early description of such a promoter, on the basis of nucleotide sequence analysis (Liu and Parkinson 1989), was subsequently criticized as a erroneous, due to demonstration that *pcnB* translation starts from the AUU codon located upstream of the promoter mentioned above, and identification of another functional promoter (Binns and Masters 2002). Perhaps surprisingly, we have found that the promoter described by Liu and Parkinson (1989), although located downstream of the translation start codon AUU, is also active in vitro, but requires  $E\sigma^S$  for maximal activity (contrary to pB, which is  $E\sigma^{70}$ -dependent). This promoter functions also in vivo, but only under specific conditions (inhibition of bacterial culture growth in the absence of ppGpp). As suggested by Liu and Parkinson (1989), there are putative Shine-Dalgarno and AUG sequences that could potentially promote translation of the shorter mRNA produced on the *pcnB* gene template. It is intriguing that there is a common picture described in the literature, showing that after electrophoretic separation of *E. coli* proteins and Western blotting, two protein bands react strongly with anti-PAP I antibodies (Jasiecki and Wegrzyn 2003; Mohanty et al. 2004;

Mohanty and Kushner 2006). Comparison of migrations of these two proteins during SDS-PAGE indicates that it is possible that there are two variants of PAP I, one whose translation starts from the AUU codon and the second, initiated at the AUG codon proposed by Liu and Parkinson (1989) (compare Fig. 1). The question whether such a putative N-terminally truncated PAP I is really produced in cells and might function in RNA polyadenylation remains to be answered, especially because one might argue that this is unlikely as pS1 requires specific conditions for its activity.

Although it is reasonable to assume that expression of the *pcnB* gene must be precisely regulated to avoid PAP I overproduction, one should ask about a physiological role for  $\sigma^S$ -dependent expression of *pcnB*. We speculate that there might be specific environmental conditions, under which the cells would benefit from enhanced synthesis of PAP I, resulting in liberation of nucleotides from rapidly degraded transcripts, even when cell's growth is halted. This might facilitate expression of specific genes required for bacterial survival, but if so, transcription of the *pcnB* gene from  $\sigma^S$ -dependent promoter(s) would be required. However, as demonstrated in this report, pS1 and pS2 promoters are active only in the absence of ppGpp. In fact, conditions that inhibit cell growth but do not provoke production of ppGpp can occur, indeed, and may be exemplified by sub-lethal concentrations of toxins or antibiotics. In this light, it is worth reminding that treatment of wild-type *E. coli* cells with chloramphenicol induces the relaxed



**Fig. 12** Effects of DksA and ppGpp on in vitro transcription from the pS1, pS2 (a–d) and  $\lambda p_L$  (e–g) promoters. Representative results are shown on autoradiograms (a, b, e and f). The presence and concentrations of particular factors in the reaction mixtures are indicated and positions of bands corresponding to certain transcripts are shown.

Summaries of the results (mean values from three experiments with error bars indicating SD) are shown at the diagrams (c, d and g). The presence of following factors in the reaction mixtures is shown: DksA (diamonds), ppGpp (squares), DksA and ppGpp (triangles)

response (Baracchini and Bremer 1988), which is defined as a lack of ppGpp production under conditions of protein synthesis inhibition.

Results presented in this report may also shed some light on the mechanism(s) of the transcription regulation under conditions of the stringent control. In the generally accepted models of ppGpp/DksA-mediated regulation of transcription,  $\sigma^{70}$ -dependent promoters are either inhibited (if they form unstable open complexes) or stimulated (if they form stable open complexes), and transcription from promoters requiring one of alternative  $\sigma$  factors can be enhanced according to the passive model, due to releasing RNA polymerase core from  $\sigma^{70}$ -dependent promoters and facilitating the use of other  $\sigma$  factors (for a review, see Srivatsan and Wang 2008). In fact, there are examples of  $\sigma^N$ - and  $\sigma^E$ -dependent promoters stimulated under condition of the stringent response in vivo (see, e.g., Bernardo et al. 2006; Costanzo and Ades 2006; Szalewska-Palasz et al. 2007a; Costanzo et al. 2008). Stimulation of  $\sigma^N$ -dependent promoters by ppGpp/DksA observed in vivo could not be detected in in vitro transcription systems with only one type of the RNA polymerase holoenzyme,  $E\sigma^N$ ; however, the in vitro competition experiments could support the passive model for transcription regulation by ppGpp and DksA (Laurie et al. 2003; Szalewska-Palasz et al. 2007a).

There are also many examples of positive regulation of transcription from  $\sigma^S$ -dependent promoters under conditions of the stringent response and/or stationary phase of growth. Efficient expression of genes coding for integration host factor (Aviv et al. 1994), transketolase A (Harinarayanan et al. 2008), OpgG and OpgH (Costa et al. 2009), cyclopropane fatty acid synthase (Eichel et al. 1999), and glutaredoxin 2 (Potamitou et al. 2002) requires both ppGpp and  $E\sigma^S$  in vivo. In fact, expression of the *rpoS* gene (which is under control of a  $\sigma^{70}$ -dependent promoter), coding for  $\sigma^S$ , is stimulated by ppGpp and DksA (Gentry et al. 1993; Lange et al. 1995; Brown et al. 2002; Bougdour and Gottesman 2007). Thus, one might assume that most of the above-mentioned effects are indirect, and result from an increase in the level of this alternative  $\sigma$  factor. However, among the promoters requiring ppGpp and  $E\sigma^S$ , there are some which are apparently regulated by ppGpp independently from  $E\sigma^S$ . Moreover, it was suggested that  $\sigma^S$ -dependent promoters may require ppGpp for induction even in the presence of high levels of this  $\sigma$  factor (Kvint et al. 2000). Therefore, one might propose that apart from the passive model of the transcription stimulation by ppGpp/DksA under stress conditions, the active model may also operate through direct activation of promoters dependent on alternative  $\sigma$  factors (or, more precisely, at least on  $\sigma^S$ ) by these effectors of the stringent response. The weak point of such a hypothesis would be a fact that only activation of transcription of  $\sigma^S$ -dependent promoters by ppGpp/DksA

was reported to date. Clearly, a lack of the negative regulation would not be expected in the active model.

To our knowledge, the results presented in this report demonstrate for the first time an experimental evidence for direct inhibition of transcription from  $\sigma^S$ -dependent promoters by ppGpp/DksA. Therefore, they support the proposal that apart from the passive model of ppGpp/DksA-mediated regulation of transcription initiated by RNA polymerase bearing alternative  $\sigma$  factors, the active model may also be operating, at least in the case of  $E\sigma^S$ .

In summary, results reported in this article indicate that regulation of transcription of the *pcnB* gene in *E. coli* is a complex process, controlled by various factors, including different  $\sigma$  subunits of RNA polymerase ( $\sigma^{70}$  and  $\sigma^S$ ) and the stringent response factors (ppGpp and DksA). It appears that this complex regulation of transcription, together with the control of the *pcnB* expression at the translation initiation stage, described previously (Binns and Masters 2002), ensures production of PAP I in precisely desired amounts, depending on bacterial growth conditions. Moreover, we demonstrated for the first time that activities of  $\sigma^S$ -dependent promoters may be inhibited by ppGpp and DksA. This discovery supports the hypothesis that regulation of transcription from promoters requiring alternative  $\sigma$  factors by these effectors of the stringent response might occur according to both passive and active models. Such a regulation may have a physiological importance for survival of bacteria under specific environmental conditions.

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