The role of the omega subunit of RNA polymerase in expression of the *relA* gene in *Escherichia coli*

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Received 9 August 2006; revised 17 October 2006; accepted 23 October 2006.
First published online December 2006.

DOI:10.1111/j.1574-6968.2006.00532.x

Editor: Robert Gunsalus

Keywords
stringent control; RNA polymerase; *rpoZ*; *relA*; ppGpp; promoter.

Abstract

The *rpoZ* gene for the omega subunit of *Escherichia coli* RNA polymerase constitutes single operon with the *spoT* gene, which is responsible for the maintenance of stringent response under nutrient starvation conditions. To identify the physiological role of the omega subunit, we compared the gene expression profile of wild-type *Escherichia coli* with that of an *rpoZ* deleted strain by microarray analysis using an *E. coli* DNA chip. Here we report on a set of genes which show changes in expression profile following the removal of *rpoZ*. We have seen that *relA*, which is responsible for the synthesis of the stringent factor ppGpp and many ribosomal proteins, exhibited noticeable changes in mRNA levels and were therefore further analyzed for their expression using a GFP/RFP two-fluorescent protein promoter assay vector. In the absence of *rpoZ*, the promoter for the *relA* gene was severely impaired, but the promoters from the ribosomal protein genes were not affected as much. Taking these results together we propose that the omega subunit is involved in regulation of the *relA* gene, but induction of the stringently controlled genes in the absence of *rpoZ* is, at least in part, attributable to a decrease in ppGpp level.

Introduction

Within any living organism, DNA-dependent RNA polymerase is one of the most important enzymes as it plays a central role in transferring the message embodied in the DNA to the synthesis of proteins. RNA polymerse is a multi-subunit enzyme with a fascinating structure–function relationship. Prokaryotic RNA polymerase is composed of a core enzyme (subunit composition $\alpha_2\beta\beta'\omega$) with the catalytic function of RNA polymerization and one of the σ subunits, which are involved in promoter recognition (Burgess, 1971; Gross *et al.*, 1992). The roles of the α, β and β' subunits in the diverse functions of bacterial RNA polymerase have been a subject of active investigation for some time (Ishihama, 1981) and much is now known, particularly of its crystallographic structure (Murakami & Darst, 2003). However, there is one more subunit, ω, which is ubiquitously present across bacterial species (Burgess, 1969), and which is often ignored as this is the only RNA polymerase subunit which can be deleted from a growing culture of *Escherichia coli* without compromising much of its enzyme function (Mathew & Chatterji, 2006). Subunit ω is encoded by the gene *rpoZ*, which is situated in the same operon as *spoT*, responsible for the maintenance of a stringent response in *E. coli* (Gentry & Burgess, 1993). The alarmone ppGpp is produced by the *relA* gene during carbon or amino acid starvation in *E. coli* and this process is known as a stringent response (Cashel *et al.*, 1996). Subunit ω mostly acts through the largest subunit, β', during RNA polymerase assembly and shows several important structural and functional characteristics (Mathew & Chatterji, 2006). There have been conflicting reports in the literature regarding the role of *rpoZ* in stringent responses in *E. coli* (Igarashi *et al.*, 1989; Gentry *et al.*, 1991). However, a recent study has conclusively proven that there is another protein, DksA, which, along with ω, plays a major role *in vitro* in eliciting the stringent response of *E. coli* (Vrentas *et al.*, 2005). In the present work, we have shown that *rpoZ* has a direct role in the expression of *relA in vivo*. Using microarray and promoter expression analysis we report that the deletion of *rpoZ* in *E. coli* abrogates the expression of *relA*.

Materials and methods

Bacterial strains and media

An *E. coli* strain (MG 1655) was constructed with a disruption in the *rpoZ* genes by P1 transduction (Miller, 1972) of

an earlier strain CF2790 (a gift from from D.R. Gentry, University of Wisconsin, WI), and by insertion of a chloramphenicol-resistant gene (*cat*). Wild-type MG1655 and its *rpoZ* disruptant were growth in Luria–Bertani (LB) broth until the stationary phase (0.65 A_{600} units) when stringent controls started to operate without the use of artificial treatments such as amino acid deprivation. An equal cell mass was subsequently used for the microarray analysis.

Microarray analysis

The microarray analysis was performed essentially as described previously (Yamamoto & Ishihama, 2005). In brief, total RNA was isolated from fresh cells using a Qiagen RNA kit (Chatsworth, CA) or by phenol/chloroform extraction. To remove the genomic DNA, the samples were treated with RNase-free DNase I (Takara) followed by phenol/chloroform extraction and precipitation with ethanol. Fluorescence labeled cDNA was prepared in a 40 μL reaction mixture containing standard AMV-RT-XL buffer (Takara), 0.5 mM each of dATP, dGTP and dCTP, 0.2 mM dTTP, 10 μg of random primers (Takara), 0.4 nmol of Cy3-dUTP or Cy5-dUTP, and 20 μg RNA. The reaction mixture was heated for 5 min at 65 °C and cooled to room temperature. After the addition of 50 units of reverse transcriptase (AMV-RT-XL, Takara), a cDNA synthesis was carried out at 42 °C for 1 h and, after the addition of a further 50 units of AMV-RT-XL, was continued for an additional 1 h. Synthesized cDNA was purified using Centri-Sep spin columns pre-equilibrated with 0.1 M NaCl. Subsequently, an Intelligene™ *E. coli* CHIP (Takara, Japan, Version 1.0) was used for the microarray analysis.

Promoter assay

Quantitative measurement of the promoter activity *in vivo* was performed as described previously (Makinoshima *et al.*, 2002; Shimada *et al.*, 2004). In brief, test promoter sequences upstream from the respective translation initiation codon, up to about 500 base pairs, were PCR-amplified and inserted into pGRP, a two-fluorescent-protein (TFP) vector, in which the RFP (red fluorescent protein) gene was under the control of a reference promoter *lac*UV5 and the GFP (green fluorescent protein) gene was under the control of a test promoter. The promoter assay vectors thus constructed were transformed into their respective host strains. For measurement of the fluorescent intensity of RFP or GFP expressed in transformed *E. coli*, cells were grown in LB medium for various periods, harvested by centrifugation, and resuspended in PBS to give approximately the same cell density at 0.6 A_{600}. For the measurement of bulk fluorescence, 0.3 mL aliquots of the cell suspension were applied onto 0.4 × 96 flat-bottom wells, and their fluorescence was measured with a FL600 Bio-Tek microplate reader (Bio-Tek Instruments, USA). The net fluorescence value was measured after subtraction of a background fluorescence determined from *E. coli* cultures with pGRP vector but without the promoter insertions.

Results and discussion

Influence of the *rpoZ* deletion on mRNA patterns

The level of mRNA in growing *E. coli* fluctuates for a group of genes, as analyzed by repeated microarray assays (Ozoline *et al.*, manuscript in preparation). After removing the fluctuating *E. coli* genes from the microarray data analysis, we identified a set of genes that showed significant variations following *rpoZ* deletion. A more than twofold increase in mRNA level was observed for more than 20 genes (Table 1), including some ribosomal protein genes such as *rplE* (L5), *rplN* (L14), *rplS* (L19), *rpsB* (S2), *rpsL* (S12), *rpsM* (S13) and *rpsU* (S21). Other genes organized into the same operons with these ribosomal protein genes were also induced, albeit at lower levels (less than twofold). The genes for the RNA polymerase core enzymes, *rpoA* (α), *rpoB* (β) and *rpoC* (β′), were induced at high levels, in agreement with the finding that *rpoA* is co-transcribed with *rpsM*, *rpsK*, *rpsD* and *rplQ*, whereas *rpoB* and *rpoC* are also organized in the ribosomal protein L10–L12 (*rplJL*) operon, although there is a minor promoter in front of *rpoB* (Ishihama, 1981).

In the *rpoZ* deletion mutant, a decrease in mRNA level was observed for a small number of genes. Interestingly, a large reduction was detected for the *relA* gene encoding ppGpp synthase I. The stringent factor ppGpp binds to the RNA polymerase β subunit (Chatterji *et al.*, 1998) and represses the transcription of stringently controlled genes (Paul *et al.*, 2004). RelA is induced during either the stationary phase or under nutrient starvation conditions (Cashel *et al.*, 1996; Chatterji & Ojha, 2001). The finding of a decreased expression of *relA* in the *rpoZ* mutant raises the possibility that transcription induction of the stringently controlled genes in the *rpoZ* mutant is, at least in part, due to the decrease in ppGpp levels. Indeed, we carried out such experiments and saw that about a 50% reduction in ppGpp synthesis takes place following the removal of *rpoZ* (D. Chatterji, unpublished data).

Involvement of the ω protein in stringent control

Our next task was to further validate the microarray data and to reveal the role of the ω protein in stringent control. In order to identify the influence of *rpoZ* deletion on gene expression, we used an *in vivo* promoter assay system with an *E. coli* promoter collection constructed in TFP (GFP/RFP

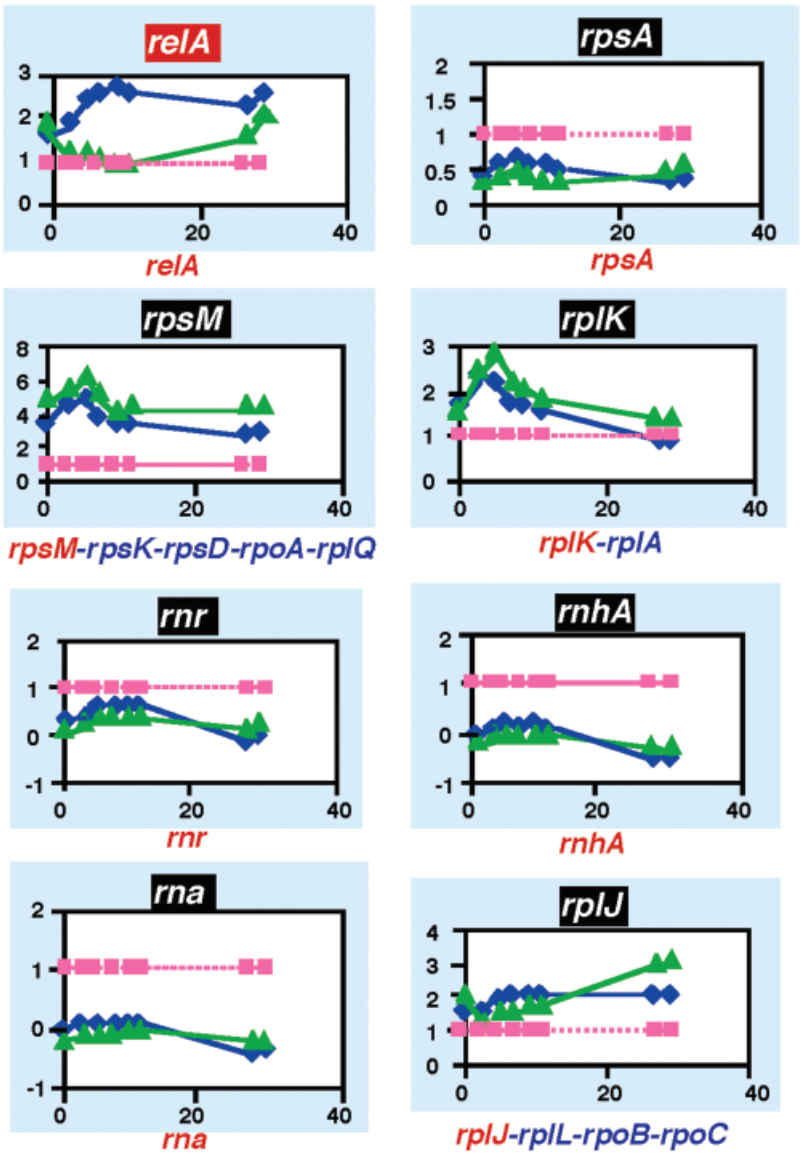

Fig. 1. Growth-dependent variation of the promoter activity. Test promoters were inserted into two fluorescent-protein vector pGRP (see Materials and methods) and each of the resultant promoter assay plasmids was transformed into both the wild-type MG1655 and its *rpoZ* deleted mutant CF2790. An overnight culture of each transformant grown in LB was transferred into fresh LB, and the culture was incubated at 37 °C with shaking. The promoter activity was measured at 0, 3, 5, 7, 9, 11, 27 and 29 h (*x*-axis). The activity of the test promoter relative to the reference promoter *lacUV5* was determined by measuring the GFP/RFP fluorescence ratio (*y*-axis). Only some typical promoters, marked in bold in Table 1, are shown here. Triangle symbol with green line = mutant; diamond symbol with blue line = wild type. Square symbol with purple line = *lac*UV5-RFP/*lac*UV5-RFP ratio. The names of the test promoters are indicated within each figure, while the respective transcription units are indicated below each figure.

two-fluorescent protein) vector (Makinoshima *et al.*, 2002; Shimada *et al.*, 2004). The expression of both GFP and RFP were scored to take account of copy number variation. Here we measured the promoter activity of the genes, which showed a significant difference in expression levels following *rpoZ* deletion (Table 1). Figure 1 shows some representative cases. Each experiment was repeated several times and the results were consistent. The empty vector, pGRP, alone without any promoter insertion or with *lac*UV5 promoter, which shows no dependence on the growth phase of *E. coli* or the *rpoZ* gene, were taken as controls. The complete statistical averaging protocol for signal detection has been discussed elsewhere (Makinoshima *et al.*, 2002; Shimada *et al.*, 2004).

Table 1. Genes showing variations in mRNA level following *rpoZ* deletion

Genes	Number of experiments	Positive or negative effects
nusA	3	+
rpsP	3	+
rpsS	3	+
rpoE	3	−
rpsH	3	−
rplE	3	+ +
rplS	3	+ +
cysH	3	−
ratE	3	−
cspE	3	+ +
asr	3	−
cspA	3	+ +
rplD	3	−
rpsD	3	−
rpoS	3	+ +
trmD	3	−
rpoB	3	+
rpoC	3	+
dps	3	+
groE	3	+
rplB	3	−
rpsC	3	−
rpsJ	3	−
rpsU	3	+ +
rna	3	−
rnhA	3	−
rnr	3	−
relA	3	− −
rpsM	3	+ +
rplJ	3	+
rpsL	3	+ +
rplN	3	+ +
rpoA	3	+
rplK	3	+
rpsB	3	+ +
rpsS	3	+
rpsA	3	−

The genes in bold type are shown in Fig. 1.

It can be seen from Fig. 1 that following *rpoZ* deletion, transcription of the *relA* gene is markedly reduced during the exponential growth phase (up to the 7 h time point). Even during the onset of the stationary phase at 9 h, *relA* expression was limited. This is in good agreement with the microarray data (Table 1). These observations raise the possibility that the ω subunit of RNA polymerase is directly involved in *relA* gene induction. At present, it remains to be seen whether the ω subunit is directly involved in transcription activation of the *relA* promoter or whether it provides the surface for interaction with a DNA-bound transcription factor, as proposed by Dove & Hoschild (1998).

The microarray data indicated that many of the genes for the ribosomal proteins and RNA polymerase core enzymes are positively regulated after ω deletion (Table 1). The promoter assay data, however, did not show a marked activation of promoters for these genes in the exponential growth phase of the *rpoZ* deletion mutant (Fig. 1), supporting the prediction that the increase in mRNA level for these genes was attributable, at least in part, to a decrease in ppGpp level because of the decreased expression of *relA* in the *rpoZ* deletion mutant. The *rpoZ* mutant strain recovered its *relA* promoter-directed GFP expression after 1 day (Fig. 1). This induction of *relA* promoter was observed for both the wild-type and *rpoZ* mutant, implying that an as-yet unidentified mechanism is involved in the regulation of *relA* expression in the stationary phase.

A recent report from the Gourse group has demonstrated that the transcription factor DksA rescues the ppGpp-unresponsiveness of a mutant RNA polymerase lacking ω (Vrentas *et al.*, 2005). This direct involvement of the ω subunit and stringent regulation as shown *in vitro* agrees with our previous *in vitro* work (Igarashi *et al.*, 1989). In addition, the work presented here indicates the involvement of the ω subunit in the control of ppGpp levels *in vivo*. It should also be noted that the gene for another protein, *SpoT*, which carries a predominantly ppGpp degradation activity, is organized in the same operon with *rpoZ* in *E. coli*. SpoT is thought to be involved in the maintenance of ppGpp levels by predominantly degrading the alamone molecule. However, it also has a minor synthetic activity, as does *relA* (Chatterji & Ojha, 2001). Taken together with the reduction in ppGpp levels following *rpoZ* deletion, we believe that the rpoZ is primarily regulating the expression of *relA*.

Acknowledgements

This work was supported by a grant through the Indo-Japan scientific exchange program sponsored by Japan Society for the Promotion of Science (JSPS), Grants-in-Aid from Ministry of Education, Culture, Science and Technology of Japan (to AI), and Department of Science and Technology (DST), Government of India (to D.C.). The authors thank K. Yamamoto for the microarray analysis and Renjith Mathew for a review of the manuscript.

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