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Construction of an Efficient *In Vitro* System for Analysis of Transcription from Sigma 54-Dependent *pspA* Promoter

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IbsC is a small toxin protein in *Escherichia coli*, whose expression is repressed by a *cis*-acting small noncoding RNA, SibC (renamed from RygC or QUAD1c).[1,2] Overexpression of IbsC or the absence of SibC transcription induces the expression of both *psp* operon (*pspABCDE*) and *pspG* gene encoding phage shock proteins,[1] whose expression were known to be induced by multiple environment stresses or agents such as filamentous phage infection, osmotic shock, continued incubation in stationary phase, heat shock, and ethanol treatment.[4,5] Although exogenous overexpression of IbsC leads to cell death, physiological roles of IbsC remain unknown especially because the strain without *ibsC* gene in the chromosome shows no growth defects.[1] Considering that only Psp proteins are induced by expression of IbsC, not by other similar small toxin proteins such as LdrD, ShoB, and TisB,[1] the role of IbsC might specifically be related to phage shock proteins.

The *psp* operon is transcribed from *pspA* promoter by RNA polymerase holoenzyme containing the alternative stress-responsive sigma factor 54 (σ^{54}).[5] Transcription from *pspA* promoter requires PspF as an activator protein that binds to the upstream region of the core promoter elements.[6,7] Furthermore, PspA inhibits the σ^{54}-dependent *pspA* transcription by interacting with PspF and exerting its negative effects on transcription activation by PspF.[8] Since the IbsC expression causes the induction of *pspA* transcription, it is attempting to see how the signal of IbsC gets transduced into the transcription activation. The mechanism involved in linking IbsC to *pspA* transcription is essential for understanding not only the physiological functions of IbsC, but also the regulation mechanism of transcription from σ^{54}-dependent promoters in response to environmental stresses. To understand the molecular mechanism of the *pspA* induction by IbsC, it is necessary to know which factors are involved in the activation of *pspA* transcription under conditions of IbsC expression. Although an *in vitro* transcription system for *pspA* promoter analysis was previously set up,[8] it is difficult to identify *in vitro* transcripts because their transcription termination sites are unclear. To overcome the drawback of the previous *in vitro* transcription system, in this study, we constructed plasmid pPR56 containing a transcription fusion of *pspA* promoter and *rnpB* terminator by replacing the *rnpB* promoter-containing DNA fragment of pLMd23-wt with the *pspA* promoter-containing fragment.[9,10] The *pspA* promoter-containing fragment spanning from −320 through +56 of *pspA*, which also includes two UAS sequences (UAS I and UAS II) as PspF-binding sites and the IHF binding site,[6] was subcloned into the *Bam*HI/*Eco*RI linearlized pLMd23-wt not having the *rnpB* promoter-containing DNA fragment, to generate the fusion plasmid pPR56 (Fig. 1). The *rnpB* terminator region in the fusion construct contained the *rnpB* sequence from +331 to +1286, which includes the three *rnpB* terminators T1, T2, and T3 leading to transcription termination at +413, +526, and +638, respectively. Therefore, this pPR56 construct was designed to generate *pspA-rnpB* fusion transcripts of 146 nt terminating at T1 (*pspAT1*), 259 nt at T2 (*pspAT2*), and 371 nt at T3 (*pspAT3*) if transcription starts at the transcription initiation site of *pspA*. Using supercoiled plasmid pPR56 DNA as a template, *in vitro* transcription was carried out by adding $E\sigma^{54}$ and PspF. The *pspAT1* transcript of 145 nt was produced as a major band although minor *pspAT2* and *pspAT3* products were also observed (Fig. 2). The increased abundance of three transcripts was observed with the incremental amount of PspF protein, while RNA I[11] of 108 nt (a transcript transcribed from its own σ^{70} specific promoter of the plasmid DNA) was not produced. In contrast, the same transcription reaction performed with $E\sigma^{70}$ did not produce the *pspAT* transcripts. Instead, this reaction generated RNA I. All our data confirmed that the *pspA* transcripts were produced from the σ^{54}-specific *pspA* promoter of pPR56. To

Figure 1. Schematic representation of pPR56 transcription fusion. The *pspA* promoter region (−320 to +56) was fused to the *rnpB* terminator region (+331 to +1286). Three transcripts are possible from this construct by transcription termination at +413 (T1), +526 (T2), and +638 (T3): *pspAT1* of 145 nt, *pspAT2* of 258 nt, and *pspAT3* of 371 nt. The arrow indicates the transcription start site of *pspA* (+1). P_{pspA}, *pspA* promoter; I and II, UAS I and II as PspF binding sites; IHF, IHF binding site; E, the 7-bp *Eco*RI linker as the fusion site.

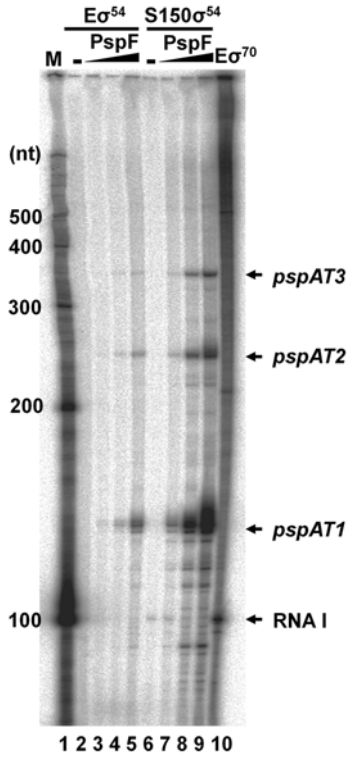

Figure 2. Analysis of *in vitro* transcription from the *pspA* promoter. Plasmid pPR56 DNA was used as a template for *in vitro* transcription either $E\sigma^{54}$ (lanes 2-5) or S150 supplemented with σ^{54} (lanes 6-9). *In vitro* transcription reactions were carried out in the presence of PspF protein. The amounts of PspF added in the transcription reactions were 0 (lanes 2 and 6), 4 (lanes 3 and 7), 40 (lanes 4 and 8), and 400 nM (lanes 5 and 9). The resulting RNA products were analyzed in a polyacrylamide sequencing gel. Three transcripts (*pspAT1* of 145 nt, *pspAT2* of 258 nt, and *pspAT3* of 371 nt) are indicated. The same *in vitro* transcription was carried out with $E\sigma^{70}$ (lane 10) and the $E\sigma^{70}$-derived product RNA I is indicated. M, size marker (lane 1).

search factors involved in the *pspA* induction by IbsC, *in vitro* transcriptional analysis with extracts from cells grown under the IbsC expression condition is necessary. Therefore, we prepared an S150 fraction from *E. coli* lysates as an *in vivo*-mimic transcription machinery for the *pspA* induction by IbsC. When σ^{54} and PspF were added into the S150 fraction, *pspAT* transcripts were produced and their production was increased with the increasing amount of PspF, suggesting that the S150 fraction can be used as an *in vivo* mimicry for analysis of *pspA* induction by IbsC.

In conclusion, our results show that the pRS56 fusion was appropriately constructed for efficient analysis of *in vitro* transcription from the σ^{54}-specific *pspA* promoter. The *in vitro* transcription system including the utilization of S100 we set up in this study can be used to analyze factors involved in transcription induction of *pspA* by IbsC.

Experimental Section

Construction of a Template DNA for Analysis of Transcription from pspA Promoter. A fusion plasmid containing both *pspA* promoter- and *rnpB* terminator-regions was constructed from plasmid pLMd23-wt, which was a derivative of pGEM3 (Promega) carrying the *rnpB* transcription unit.[10] The *Bam*HI-*Eco*RI fragment of pLMd23-wt was replaced with a *pspA* promoter-containing DNA fragment. The promoter-containing DNA fragment was obtained by PCR with a primer pair of BHIpspA-320 (5'-CGC GGA TCC GCA GTT AAG GGA AAT AAA CG-3') and ERIpspAre+56 (5'-CCG GAA TTC AGAA AAA ATA CCC ATA ATG TTG-3'). The PCR products were then digested with *Bam*HI and *Eco*RI, and cloned into the *Bam*HI-*Eco*RI site of pLMd23-wt to generate the fusion plasmid pPR56, carrying the promoter regions from −320 to +56 of *pspA*. For the plasmid construction and propagation, *E. coli* JM109 was used as the bacterial host strain.[12]

***In vitro* Transcription by *E.coli* Polymerase.** Plasmid pPR56 DNA was used as a template for *in vitro* transcription. *In vitro* transcription reaction was carried out basically as described previously,[2] with minor modification. Proteins σ^{54} and PspF were purified using the corresponding clones from the ASKA library,[13] as previously described.[2] $E\sigma^{54}$ was prepared either by combining the core enzyme and σ^{54} in a ratio of 1:4 or by supplementing σ^{54} to S150 fraction (containing RNA polymerase core). For preparing the S150 fraction, S30 fraction from *E. coli* strain MG1655 prepared as described previously[14] and the S30 fraction was further subjected to ultracentrifugation for 2 h at 4 °C at 150,000 × *g*. The S150 fraction was used as a crude RNA polymerase. Briefly, $E\sigma^{54}$ (the core enzyme of 90 nM) or S150 fraction (about 0.3 μg/μL protein) supplemented with σ^{54} of 360 nM was incubated at 37 °C for 5 min in the reaction buffer (40 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 10 mM DTT, 150 mM KCl, 0.05% Triton X-100) with 6 nM of template DNA and 40 mM rATP. The reaction was started by adding rNTP mixtures (500 μM of GTP, UTP, and 25 μM of CTP including 10 μCi of [α-^{32}P]CTP) in the presence of varying amounts of PspF protein (0 to 400 nM). After 25 min, the reaction was terminated by the addition of the same volume of phenol:chloroform (5:1) mixture and the products were ethanol-precipitated. *In vitro* transcription reaction with $E\sigma^{70}$ (Epicentre) was also conducted as described above except for using 1 unit of $E\sigma^{70}$. The products were analyzed on a 5% polyacrylamide sequencing gel containing 7 M urea and quantitated by BAS1500 (Fuji).

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References

1. Fozo, E. M.; Kawano, M.; Fontaine, F.; Kaya, Y.; Mendieta, K. S.;

Jones, K. L.; Ocampo, A.; Rudd, K. E.; Storz, G. *Mol. Microbiol.* **2008**, *70*, 1076.
2. Han, K.; Kim, K. S.; Bak, G.; Park, H.; Lee, Y. *Nucleic Acids Res.* **2010**, *38*, 5851.
3. Darwin, A. *J. Mol. Microbiol.* **2005**, *57*, 621.
4. Kobayashi, R.; Suzuki, T.; Yoshida, M. *Mol. Microbiol.* **2007**, *66*, 100.
5. Weiner, L.; Brissette, J. L.; Model, P. *Genes Dev.* **1991**, *5*, 1912.
6. Jovanovic, G.; Model, P. *Mol. Microbiol*. **1997**, *25*, 473.
7. Weiner, L.; Brissette, J. L.; Ramani, N.; Model, P. *Nucleic Acids Res.* **1995**, *23*, 2030.
8. Dworkin, J.; Jovanovic, G.; Model, P. *J. Bacteriol.* **2000**, *182*, 311.
9. Ko, J.; Lee, S. J.; Cho, B.; Lee, Y. *FEBS Lett*. **2006**, *580*, 539.
10. Kim, S.; Kim, H.; Park, I.; Lee, Y. *J. Biol. Chem.* **1996**, *271*, 19330.
11. Jung, Y. H.; Lee, Y. *Mol. Biol. Rep.* **1996**, *22*, 195.
12. Yanisch-Perron, C., Vieira, J.; Messing, J. *Gene* **1985**, *33*, 103.
13. Kitagawa, M.; Ara, T.; Arifuzzaman, M.; Ioka-Nakamichi, T.; Inamoto, E.; Toyonaga, H.; Mori, H. *DNA Res.* **2005**, *12*, 291.
14. Roberts, H. D.; Altman, S.; Smith, J. D. *J. Biol. Chem*. **1972**, *247*, 5243.