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Short Communication

Bacillus subtilis RNA polymerase incorporates digoxigenin-labeled nucleotide in vitro

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Transcription is the first key step of a gene expression. Many researchers have been trying to uncover the mechanisms of regulation of transcription, especially its initiation. RNAP is the key enzyme complex responsible for transcription in all kinds of species. In prokaryotes, the RNAP holoenzyme consists of sigma factor and the core enzyme that is composed of α, β, and β' (Ishihama, 1993). Sigma factor enables RNAP to bind to the specific promoter sequence of a gene and to initiate transcription. In most cases, the RNAP core enzyme is unique in each species, while sigma factors are diversified. Different sigma factors are activated in response to different conditions to change a gene expression to adapt to living conditions (Ishihama, 2000). Additionally another protein family called transcriptional regulators possessing DNA-binding motif of a helix–turn–helix signature also regulates the initiation of transcription in the manner of activation or repression (Huffman and Brennan, 2002; Ramos et al., 2005). Those transcription factors facilitate or inhibit the activity of RNAP mostly by modulating the affinity for promoter DNA. Together with these transcription factors, the adaptive responses are mediated.

During the course of bacterial genome-sequencing projects, vast numbers of the genes coding for sigma factors and transcriptional regulators have been found. The number of these transcription factors varies among bacterial species and their number seems to increase with the complexity of lifestyle. Larger genomes possess more transcription factors than smaller ones (Cases et al., 2003). Most of these transcription factors are unanalyzed and their functions remain unknown. One of the efficient ways to investigate these transcription factors is to reconstitute an in vitro transcription system with minimum components for RNA synthesis. In most cases, radioactively labeled nucleotides have been used for a portion of substrates so far with this technique. However, radioactive isotopes are sometimes unstable, harmful for the body, and hard to handle. Therefore this method is not suitable for comprehensive and time-consuming research. DIG, which is cardenolide in Digitalis plants and easily detected with a high affinity antibody, is one of the useful tools for the labeling of nucleotides (Höltke and Kessler, 1990). DIG can be incorporated into a chain of nucleic acids by the action of phage-related RNAP (e.g. T7 and SP6 RNAP) or thermo-tolerable DNA polymerase (e.g. *Taq* DNA polymerase). These polymerases are enzymes composed of a single polypeptide. There have been few reports that DIG could be used for the substrate of bacterial RNAP composed of multi subunits (Brutsche and Braun, 1997). They used commercial *Escherichia*

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Abbreviations: DIG, digoxigenin; RNAP, RNA polymerase.

coli RNAP core enzyme and purified *Bacillus subtilis* sigma factor. In this report, in order to apply DIG to a comprehensive study of unanalyzed transcription factors in vitro, we investigated a DIG-dependent in vitro transcription system constituted of purified *B. subtilis* RNAP and transcription factors.

It was reported that affinity purification with histidine-tag fused to the C-terminus of the β′ subunit of RNAP enables efficient and rapid isolation of the active RNAP holoenzyme (Fujita and Sadaie, 1998b). To avoid contamination of unnecessary sigma factors, sigma factors which expressed highly at the logarithmically growing phase except for SigA, which is essential for growth, were depleted from the host cell. The insertion mutations of *sigBΔ2* (Bacillus Genetic Stock Center), *sigHΔHB* (Asai et al., 1995), and *sigW*::cat (Asai et al., 2008) replaced by an appropriate antibiotic-resistant cassette by the plasmid described by Steinmetz and Richter (1994) were introduced by transformation (Anagnostopoulos and Spizizen, 1961; Asai et al., 2007) into the genome of strain 168rpoCHis (*trpC2 rpoC*ΩpMUTinHis) harboring a histidine-tagged β′ subunit of RNAP (Ishikawa et al., 2010) and the resultant strain, ASK2102 (*trpC2 rpoC*ΩpMUTinHis erm *sigBΔ2* cat *sigHΔHB* erm::neo *sigW*::cat::spc), was used for purification of RNAP.

ASK2102 cells were grown at 37°C and the cells were harvested by centrifugation when the cell density of the culture reached OD_{600} of 1. The preparation of the histidine-tagged RNA polymerase was performed according to the following procedures based on the manufacturer's instructions (Novagen) at 4°C as much as possible. The collected cells were washed with buffer D [20 mM Tris-HCl (pH 8.0), 15 mM imidazole, and 150 mM NaCl]; resuspended in buffer B [20 mM Tris-HCl (pH 8.0), 5 mM imidazole, 5% (v/v) glycerol, 150 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride]; and disrupted by sonication. The extracts were centrifuged for 3 min at 10,000×*g* (14,000 rpm). The supernatants were applied to 0.3 ml of His Bind Resin（Novagen）equilibrated with buffer B. The resin was washed with buffer E [20 mM Tris-HCl (pH 8.0), 50 mM imidazole, 5% (v/v) glycerol, and 500 mM NaCl] and buffer F [20 mM Tris-HCl (pH 8.0), 50 mM imidazole, 20% (v/v) glycerol, and 150 mM NaCl]. The proteins bound to the resin were eluted with buffer G [20 mM Tris-HCl (pH 8.0), 500 mM imidazole, 20% (v/v) glycerol, and 150 mM NaCl]. The eluted samples containing the RNAP complex designated extracted holoRNAP were supplemented with glycerol to the final concentration of 30%, and stored at −20°C till used. Extracted holoRNAP was further purified with phospho-cellulose according to the methods described by Qi and Hulett (1998) in order to remove sigma factors and to obtain the RNAP core enzyme designated purified coreRNAP. C-terminally histidinie-tagged SigA was purified from *E. coli* BL21(DE3)pLysS according to the methods described previously (Fujita and Sadaie, 1998a). Purified coreRNAP was incubated with histidinie-tagged SigA for 30 min at 4°C to reproduce holoenzyme of RNAP designated reconstituted holoRNAP.

In order to check whether our preparation of RNAP still possessed the activity of RNA synthesis, each fraction of RNAP was used for in vitro transcription assay. We used the promoter sequence of the *abrB* gene as a template, whose transcriptions is well known to be directed by RNAP containing SigA (Strauch et al., 1989). First, we performed an experiment of in vitro transcription using non-labeled substrate by the procedure based on the methods described previously (Fujita and Sadaie, 1998b). The DNA templates for in vitro transcription were amplified by PCR with the oligonucleotide primer pair of abrBF 5′-GAAGAATTCGA GTCTCTACGGAAATAGCG and abrBR 5′-GGAGGATC CAACGCAAACAACAAGCTGATCC for *abrB* from the *B. subtilis* 168 chromosome (248 bp). The PCR products were purified before use by excision and extraction from the gel after polyacrylamide gel electrophoresis (PAGE). Reconstituted holoRNAP or its components were mixed with the DNA template and incubated for 10 min at 4°C in transcription buffer [20 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM NaCl, 0.02 mM EDTA, 0.1 mM DTT, 10% glycerol, and 0.05% Tween 20]. The mixture was supplemented with substrate, 1 mM each of ATP, CTP, GTP and UTP, to start the reaction of transcription and incubated for 8 min at 37°C. The reaction was stopped by treatment with phenol/chloroform/isoamyl alcohol (25∶24∶1) and precipitated with ethanol. The resultant products were separated by 6% PAGE in denatured condition [20 mM MOPS (pH 7.0), 5 mM sodium acetate, 1 mM EDTA, and 7 M urea], blotted on a positively charged nylon membrane [Hybond-N (GE healthcare)] and detected by Northern hybridization using the DIG-labeled riboprobe specific for *abrB* (Fig. 1A) as noted previously (Asai et al., 2000).

In this method, template DNA besides newly synthe-

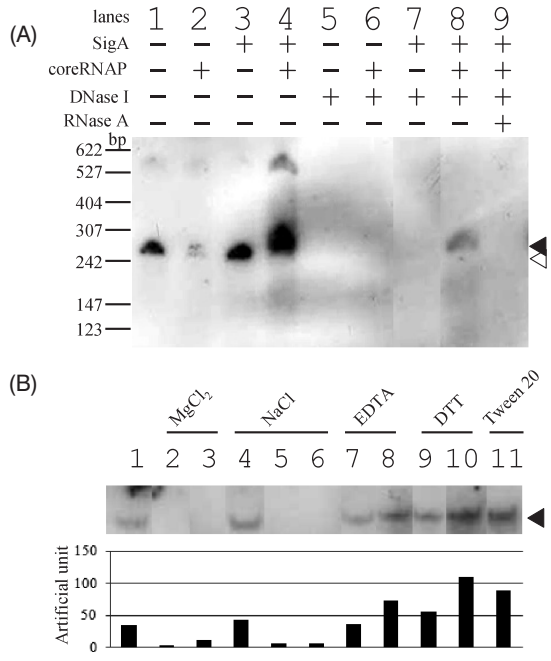

Fig. 1. In vitro transcription assay under the non-radioactive condition; detection of non-labeled transcripts (A) and DIG-labeled transcripts (B).

A, purified coreRNAP (0.3 pmol), SigA (25 pmol), and template DNA (0.4 pmol) were subjected to the assay. Closed and open arrowheads indicate position of transcripts and template, respectively. RNA probe was prepared as described previously (Asai et al., 2000). The bands were visualized with anti-DIG antibody according to the manufacturer's instructions (Roche Diagnostics). The sample was treated with 2.5 U of DNase I, RNase-free (Roche Diagnostics) and with 50 μg/ml RNase A (Wako Pure Chemical Industries) for 10 min at 37°C. B, Extracted holoRNAP (0.6 pmol), SigA (25 pmol), and template DNA (0.4 pmol) were subjected to the assay. Arrowhead indicates position of transcripts. Lanes are indicated as follows: 1, primary used buffer [20 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM NaCl, 0.02 mM EDTA, 0.1 mM DTT, and 10% glycerol]; and different condition from primary buffer are shown: 2, 0 mM $MgCl_2$; 3, 100 mM $MgCl_2$; 4, 0 mM NaCl; 5, 100 mM NaCl; 6, 350 mM NaCl; 7, 0 mM EDTA; 8, 0.2 mM EDTA; 9, 0 mM DTT; 10, 1 mM DTT; 11, 0.05% (v/v) Tween 20. The intensities of signals on the image measured by NIH image software (http://rsbweb.nih.gov/nih-image/) are shown below with a bar graph.

sized RNA was hybridized with the riboprobe and was detected (Fig. 1A, lane 1 and 5). Therefore in order to distinguish them, the products were subjected to treatment with DNase I and RNase A. Neither purified coreRNAP nor SigA alone synthesized DNase I-resistant products (Fig. 1A, lane 2, 3 and 6, 7). On the other hand, reconstituted holoRNAP synthesized DNase I-resistant and RNase A-sensitive product (Fig. 1A, lane 4, 8, and 9). These results suggested that purified coreRNAP was free from SigA and formation of reconstituted holoRNAP was successful. However, this method has a hybridization step and is a little troublesome. Next we tried to introduce DIG-labeled substrate into RNA and to detect the products directly. The extracted holoRNAP and the promoter DNA of *abrB* were incubated in the presence of 1 mM ATP, CTP, GTP, 0.65 mM UTP and 0.35 mM DIG-11-UTP (Roche Diagnostics) in the buffer [20 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM NaCl, 0.02 mM EDTA, 0.1 mM DTT, and 10% glycerol]. The product was visualized without a hybridization step and was detected (Fig. 1B, lane 1). We investigated whether the buffer condition was appropriate for incorporation of DIG-labeled substrate as shown in Fig. 1B. The increased concentration of $MgCl_2$ (lane 2 and 3) and NaCl (lane 4 to 6) abolished the band. On the other hand, increased concentration of EDTA (lane 7 and 8) and DTT (lane 9 and 10), and especially the addition of Tween 20 (lane 11) improved markedly the intensity of the band as compared to lane 1. Therefore we added 0.05% (v/v) Tween 20 to the buffer for in vitro transcription.

Next we applied transcriptional regulators to in vitro transcription using DIG. *B. subtilis* can utilize glucomannan, whose main component is β-(1→4)-linked D-mannose and D-glucose, by the action of proteins encoded by the *gmu* operon that is constituted of eight genes (*gmuBACDREFG*) (Sadaie et al., 2008). It was suggested by the genetic analysis that the transcription of the *gmu* operon is regulated by GmuR repressor, the fifth gene of the operon (Fig. 2A), and SigA; however, biochemical analysis was not performed.

To express the C-terminally histidine-tagged GmuR in *B. subtilis* and *E. coli*, the *gmuR* region, which had been amplified by PCR using the primer pair of gmuRF 5′-GTCGTCGACGATATCAAACAGCTGCCGG and gmuRHisR 5′-GCAGCATGCGCGCCGTATGTTTA CAGAAG from chromosomal DNA of *B. subtilis*, followed by digestion with *Sal*I and *Sph*I, was cloned in *E. coli* C600 into vector pDGHisC, which was a derivative of plasmid pDG148 (a shuttle vector between *E. coli* and *B. subtilis*) (Stragier et al., 1988) and the resultant plasmid was designated pGMURHis. The histidine-tagged GmuR protein was purified from *E. coli* BL21(DE3)pLysS harboring pGMURHis accordingly to the methods described previously (Fujita and Sadaie, 1998a). In order to investigate whether the histidine-tagged GmuR specifically bound to the promoter

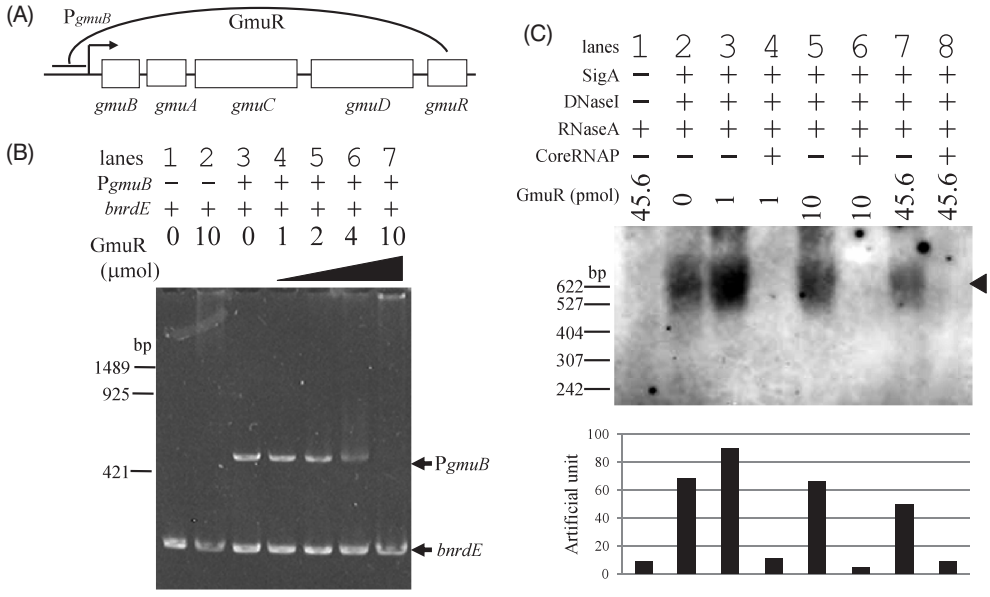

Fig. 2. Application of transcriptional regulator to in vitro transcription assay using DIG-labeled substrate.

A, Schematic model of negative regulation of the *gmuB* operon by GmuR transcriptional regulator. B, Gel mobility shift assay of GmuR protein. The promoter region of the *gmuB* operon (502 bp) and the internal region of *bnrdE* (229 bp) were used. In this case, the internal *bnrdE* region, which GmuR would apparently not bind with, was used as a negative control in the experiment. The mixture of both probes (10 ng of each) and GmuR protein were incubated at 25°C for 30 min in the buffer [10 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 1 mM DTT] and separated in 5% PAGE, followed by ethidium bromide staining. C, The effect of GmuR on *gmuB* transcription analyzed by in vitro transcription assay using DIG-labeled substrate. Purified coreRNAP (0.4 pmol), SigA (8 pmol), and template DNA (0.4 pmol) were subjected to the assay. Arrowhead indicates position of transcripts. Nuclease treatment was performed in a similar way as shown in Fig. 1A. The intensities of signals on the image measured by NIH image software (http://rsbweb.nih.gov/nih-image/) are shown below with a bar graph.

region of *gmuB* operon, we first performed a gel mobility shift assay (Fig. 2B). The promoter region of the *gmuB* operon was amplified by PCR using the primer pair of gmuMF 5′-GAAGAATTCAGGATTTTAC GGTTCCTGG and pHVR 5′-AGTGTATCAACAAGCTG G from chromosomal DNA of strain BSU71 (*trpC2 gmuB*ΩpMUTin2) (Sadaie et al., 2008) (502 bp). The DNA fragment of *gmuB* promoter disappeared when GmuR was added to the reaction cocktail, whereas the DNA fragment of *bnrdE* that was amplified by PCR using the primer pair of bnrdEinF 5′-AAGAAGCTTCTTTT AACTGAGAATGGATATG and bnrdEinR 5′-GGAGGAT CCACTCAGTTGCCCTAATTTC from chromosomal DNA of *B. subtilis* (229 bp) did not. The *bnrdE* gene that resides in prophage SPβ encodes the putative ribonucleotide reductase subunits alpha. These results suggested that GmuR protein bound specifically to DNA of the promoter of the *gmuB* operon, not to internal region of *bnrdE* (Fig. 2B).

Finally, the GmuR protein and the *gmuB* promoter DNA were subjected to the latter in vitro transcription analysis using DIG-labeled substrate (Fig. 2C). The DNase I-resistant product was observed when reconstituted holoRNAP was present in the reaction cocktail (Fig. 2C, lane 1 and 2). The amount of this product, which was DNase I-resistant and RNase A-sensitive, was slightly increased in the presence of 1 pmol GmuR protein (Fig. 2C, lane 2 and 3). It was previously suggested that the GmuR repressor protein seems to be required for full induction of the *gmuB* operon by inducer cellobiose since induction of the operon expression by cellobiose in wild type *gmuR* strain was stronger than in the *gmuR* mutant (Sadaie et al., 2008). It was probable that a low amount of GmuR protein stimulated *gmuB* in vitro transcription. On the other hand, the amount of the product was gradually decreased when

the amount of GmuR was increased much more (Fig. 2C, lane 3 to 8). Together with these results, it was possible that GmuR bound to the promoter of the *gmuB* operon and inhibited its initiation of transcription in in vitro transcription using DIG-labeled substrate. We propose that this safe non-radioactive system is useful in comprehensive analysis of an inexhaustible number of transcriptional regulators of unknown function.

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