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Primer RNA Synthesis by *E. coli* RNA Polymerase on the SSB-coated 229-nt *ssi* Signal of Lactococcal Plasmid pGKV21

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Plasmid pGKV21 contains a 229-nucleotide (nt) single-strand DNA initiation (*ssi*) signal. Using asymmetric PCR, we prepared a small single-stranded (ss) DNA fragment of the *ssi* signal and, using the 229-nt ssDNA fragment, determined the requirements of RNA polymerase for priming and DNA-protein interaction. The *ssi* fragment prepared was able to generate primer RNAs with almost the same efficiency as the M13Δ*lac*182/229 phage DNA. However, the *cssi* (complementary strand of the *ssi* signal) fragment could not synthesize primer RNAs. This result suggests that the 229-nt *ssi* signal functions in a strand specific manner. Gel retardation and DNase I footprinting demonstrated that the synthesized *ssi* fragment could interact with both *E. coli* RNA polymerase and SSB protein to synthesize primer RNA. In *Escherichia coli* [pWVAp], an addition of rifampicin resulted in an accumulation of ssDNA, indicating that the host-encoded RNA polymerase is involved in the conversion of ssDNA to double-stranded plasmid DNA.

Key words : Primer RNA synthesis, plasmid replication, single-strand initiation DNA (*ssi*) signal

Introduction

We have previously reported that plasmid pGKV21 contains a 229-nucleotide (nt) *ssi* signal located within the 109-nt upstream of the nick site of the putative plus origin [13]. This *ssi* signal was required for complementing the defective SSI function of an *ori*$_C$-defective M13 mutant phage (M13Δ*lac*182). Therefore we thought that in *E. coli*, this *ssi* signal may direct its lagging-strand synthesis as a minus origin of lactococcal plasmid pGKV21.

E. coli RNA polymerase is involved in synthesis of RNA primers on the 229-nt *ssi* signal of pGKV21 depending on the presence of *E. coli* SSB protein. It is well known that the M13 ssDNA phage for its complementary strand DNA synthesis takes advantage of one of simple priming system using *E. coli* RNA polymerase. In this system, RNA polymerase in association with SSB protein synthesizes 30-nt of primer RNA from a unique M13 origin sequence on the viral DNA strand [8]. This origin sequence, located between genes Ⅱ and Ⅳ, has a potential for generation of two hairpin loop structures. This system is rather different from the other priming systems in which primases act in concert with many other initiation proteins and then synthesize primer RNAs [1,2,22].

Phage G4 contains a specific origin of complementary-strand DNA synthesis (G4*ori*$_C$) that is required by primase to synthesize primer RNA [7,11,21]. *In vitro*, primase can synthesize 29-nt of primer RNA on either the G4 or the M13/G4*ori*$_C$ phage ssDNA template coated with *E. coli* SSB protein. [14,17].

Previous studies on the binding of *E. coli* SSB protein to the ssDNA template described that *E. coli* SSB protein existed in solution as a stable tetramer [23] and bound to ssDNA indifferent modes depending upon ionic conditions, salt concentrations, temperatures, pHs, and protein concentrations [3,4,16]. Griffith *et al*. [6,12] have reported that *E. coli* SSB protein also assembled on ssDNA as octamers that interacted with about 145-nt stretch of ssDNA, leaving about 30-nt of unbound ssDNA between the adjacent octameric SSB proteins.

In this study, to determine the priming ability of *E. coli* RNA polymerase on the 229-nt ssDNA fragment of lactococcal plasmid pGKV21, the *ssi* fragment by asymmetric PCR was artificially prepared. This prepared DNA fragment was used to examine not only primer RNA synthesis but also a DNA-protein interaction.

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Materials and Methods

Bacterial strains, phage and plasmids

E. coli TG1 was used as the host bacterium for M13 bacteriophages and plasmids used in this study. Plasmid pWVAp is a pGKV21 derivative carrying an *ssi* signal and the β-lactamase gene from pMW119 [13]. Plasmid pWVAp/Δ*ssi* is an *ssi* signal-deleted derivative from pWVAp. The filamentous phage vector M13Δ*lac*182 was constructed and a kind gift by Dr. N. Nomura.

Preparation of the synthesized *ssi* fragment

A replicative-form (RF) DNA of the recombinant phage carrying the 229-nt *ssi* signal of pGKV21 was cleaved with a restriction enzyme that has a single cleavage site at the 3′-end of the *ssi* signal (such as *Bam*HI in the polylinker). The linearized RF DNA was then electrophoresed on an 1% agarose gel in Tris-acetate-EDTA buffer. The linearized RF DNA band was excised from the gel, and DNA was electroeluted. The eluate was used for asymmetric PCR reaction to make the synthesized *ssi* fragment in the presence of an oligonucleotide primer. The PCR mixtures contained: 100 ng of template DNA, 10× *Taq* DNA polymerase, 0.25 mM of deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP) mixture, and 200 pmol of a primer. DNA sample was denatured to separate strands at 95°C for 20 sec. The denatured ssDNAs were annealed with a primer at 55°C for 50 sec and chain-elongated by *Taq* DNA polymerase at 68°C for 60 sec. They were then cycled 30 times. The PCR products were electrophoresed on an 1.5% agarose gel, and the expected *ssi* fragments were electroeluted. The eluted fragment (the synthesized *ssi* fragment) was used directly for primer RNA synthesis, a DNA-protein binding assay and DNase I digestion. M13 17-Base Probe Primer (5′-TCATGGT CATAGCTGTT-3′) was used as a primer for this PCR reaction (Fig. 1A).

The complementary-strand of the *ssi* signal (a *cssi* ssDNA fragment) was also made in the same way (Fig. 1B). The RF DNA of M13Δ*lac*182/229 was cleaved with a restriction enzyme that has a single cleavage site at the 5′-end of the *ssi* signal (such as *Pst*I in the polylinker). The asymmetric PCR reaction was carried out in the presence of a pUC/M13 Forward sequencing Primer (5′-GTAAAACGACGGCCAGT-3′).

In vitro primer RNA synthesis

Primer RNA synthesis was done by the method previously described [13].

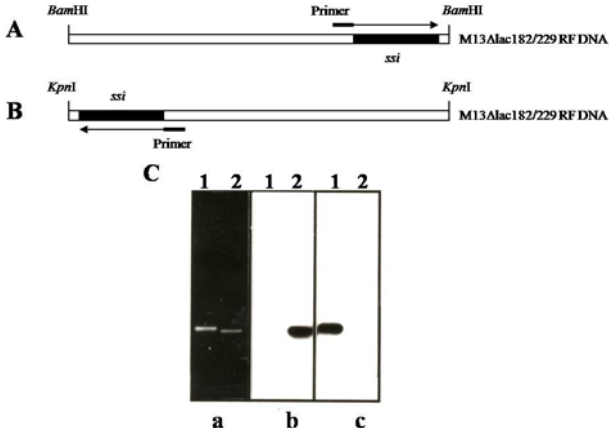

Fig. 1. Asymmetric PCR scheme for preparing the synthesized *ssi* fragment. (A) Preparation of the synthesized *ssi* fragments. (B) Preparation of the *cssi* fragments. (C) Detection of the synthesized *ssi* fragments. (a) DNA fragments on the agarose gel. The DNAs were transferred to the membrane and hybridized with (b) 5′-end-labeled *ssi* fragment and (c) 5′-end-labeled *cssi* fragment. Lane 1: the *ssi* DNA fragment, lane 2: the *cssi* DNA fragment.

Gel retardation

The standard reaction conditions for primer RNA synthesis were used for a DNA-protein binding analysis, except that no *E. coli* RNA polymerase was added. Various amounts of *E. coli* SSB protein were added to the synthesized *ssi* fragment, and the mixtures were incubated for 10 min at 30°C. One μl of loading buffer (50% glycerol, 0.4% bromophenol blue) was added to 10 μl of the binding reaction mixture. After mixing, the solution was immediately applied to a prewarmed (30 min at 100 V) 4% nondenatured polyacrylamide gel in 8×8 cm which is made up with a ratio of acrylamide to bisacrylamide of 38: 1 dissolved in Tris-borate-EDTA buffer.

DNase I footprinting

The standard reaction condition for primer RNA synthesis was used for a DNA-protein binding analysis. The synthesized *ssi* fragments were ^{32}P-labeled at their 5′-end. A 20 μl reaction mixture contained approximately 0.1 pmol of the ^{32}P-labeled synthesized *ssi* fragment. The reaction mixtures were stored on ice for 5 min and then were incubated at 30°C for 5 min. Various amounts of DNase I were added. The incubation was continued for 1 min at 30°C. The reaction was stopped by an addition of 400 μl of "DNase I stop buffer" containing 0.3 M sodium acetate, 20 mM EDTA, and 100 μg/ml of *E. coli* tRNA. DNA was precipitated by an addition of 1ml of ethanol, washed once with 70% ethanol,

and dried under vacuum. The final pellet was resuspended with formamide dye and applied onto an 8% polyacrylamide gel containing 7M urea. Size markers were generated by Maxam-Gilbert sequencing reactions [18] on the same end-labeled fragment.

Results and Discussion

Primer RNA synthesis on the synthesized 229-nt *ssi* DNA fragment

To determine requirement of *E. coli* RNA polymerase for priming on the 229-nt ssDNA fragment, asymmetric PCR was performed to prepare the synthesized *ssi* DNA fragment. (see "preparation of the synthesized *ssi* fragment" under "Materials and methods"). To know whether the synthesized *ssi* fragments were prepared exactly as expected, the prepared *ssi* fragments were electrophoresed on an 1.5% agarose gel. The *ssi* and the *cssi* DNA bands stained with ethidium bromide were detected as shown in Fig. 1C panel a. The DNAs were transferred to a nitrocellulose filter, and hybridized with a 5′-end-labeled *ssi* fragment (Fig. 1C panel b) or a 5′-end-labeled *cssi* fragment (Fig. 1C panel c). As shown in Fig. 1C panel b, only the *cssi* fragment could hybridize with a 5′-end-labeled *ssi* fragment probe. The DNA band hybridized with a 5′-end-labeled *cssi* ssDNA fragment probe was also detected only in the synthesized *ssi* fragment (Fig. 1C panel c). These results indicated that the synthesized *ssi* fragments were prepared exactly as expected.

To know whether the synthesized 229-nt *ssi* fragment can serve as a substrate for *E. coli* RNA polymerase to generate primer RNA, its activity was compared with that of M13Δ*lac*182/229, a recombinant phage containing the same 229-nt sequence inserted in the M13Δ*lac*182 vector. As shown in Fig. 2, the synthesized 229-nt *ssi* fragment was able to synthesize RNA primers with the same efficiency as M13Δ*lac*182/229 phage ssDNA could. No primer RNA synthesis was observed with M13Δ*lac*182 phage DNA, which was used as a negative control. Also, the *cssi* fragment, complementary-strand DNA of the *ssi* signal, was also unable to synthesize RNA primers, suggesting that primer RNA synthesis proceeds in a strand specific manner.

Requirement of *E. coli* SSB protein for primer RNA synthesis

ssDNA of filamentous phage M13Δ*lac*182/229 requires both *E. coli* SSB protein and *E. coli* RNA polymerase for

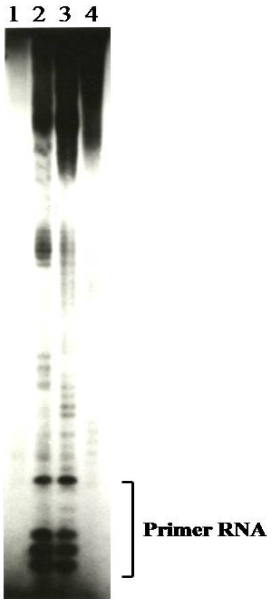

Fig. 2. Primer RNA generation of the synthesized *ssi* fragment template. Lane 1: M13Δ*lac*182, lane 2: M13Δ*lac*182/229, lane 3: the synthesized *ssi* fragment, lane 4: the synthesized *cssi* fragment.

synthesis of primer RNA *in vitro*. To determine whether the *ssi* signal itself should be coated with *E. coli* SSB protein in this reaction, *in vitro* primer RNA synthesis was performed by using the synthesized *ssi* fragment as a template, *E. coli* RNA polymerase, and various concentrations of *E. coli* SSB protein (Fig. 3). In the absence of *E. coli* SSB protein the synthesized RNAs were random in length. However, when increased gradually by adding up to 8 μg of *E. coli* SSB protein, only the primer RNAs were more clearly observed. These results demonstrated that the synthesized *ssi* fragment was able to synthesize primer RNA at high SSB concentrations.

Using a standard condition of primer RNA synthesis, the interaction of *E. coli* SSB protein with the 229-nt *ssi* signal was monitored by a gel retardation assay. The ssDNA fragment was 5′-end-labeled with [γ-^{32}P]ATP. When *E. coli* SSB protein was added up to 8 μg of the ^{32}P-end-labeled *ssi* fragment, migration of the SSB-*ssi* signal complex was significantly retarded (Fig. 4). These results suggest that *E. coli* SSB protein was bound to the 229-nt *ssi* DNA fragment. It demonstrates that *E. coli* SSB protein is necessary for *E. coli* RNA polymerase to synthesize primer RNA.

DNase I footprinting analysis of RNA polymerase bound to the SSB-coated 229-nt *ssi* signal

To investigate the binding of RNA polymerase to the

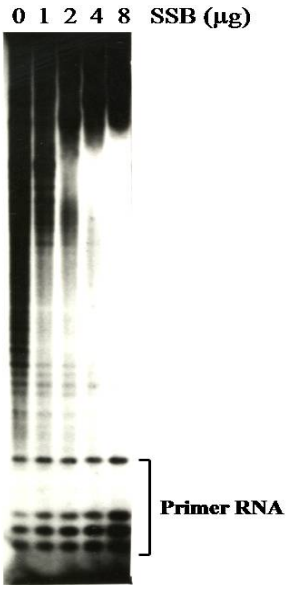

Fig. 3. Primer RNA synthesis on the synthesized *ssi* fragment with increasing amounts of *E. coli* SSB protein. The synthesized *ssi* fragment was used as a template by adding *E. coli* RNA polymerase and increasing amounts (0 to 8 μg) of *E. coli* SSB protein.

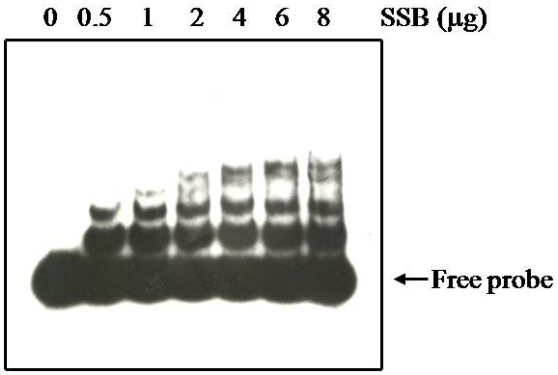

Fig. 4. Gel retardation of the synthesized *ssi* fragment by *E. coli* SSB protein. The synthesized *ssi* fragment was used as a template by adding *E. coli* RNA polymerase and increasing amounts (0 to 8 μg) of *E. coli* SSB protein.

SSB-coated 229-nt *ssi* signal, a DNase I footprinting experiment was performed. ^{32}P-end-labeled DNA was bound to *E. coli* RNA polymerase and *E. coli* SSB protein, and then digested with DNase I . As shown in Fig. 5, additions of both *E. coli* RNA polymerase and *E. coli* SSB protein prohibited DNase I cleavages and induced new cleavage sites on the 229-nt *ssi* signal. The cleavages in the right stem of stem-loop I were protected from DNase I digestion, and strong DNase I hypersensitive sites were appeared in the right stem and the 3′-spacer region of stem-loop II. These results suggest that conformational changes were occurred

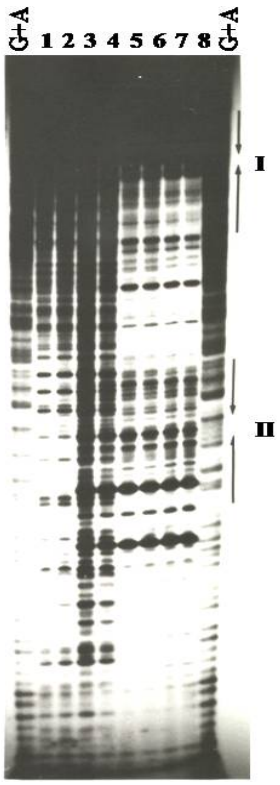

Fig. 5. DNase I footprinting by binding of both *E. coli* RNA polymerase and SSB proteins to the synthesized *ssi* fragment. Lane 1 and 2: the *ssi* DNA fragment alone, lane 3 to 8: the *ssi* DNA fragment with *E. coli* SSB protein and RNA polymerase. Concentrations of DNase I were used as follows; lane 1 and 4: 1 unit, lane 2 and 5: 0.5 unit, lane3: 3 unit, lane 6: 0.1 unit, lane 7: 0.05 unit, lane 8: 0.01 unit. Stem-loops I and II are indicated by two sets of arrows to the right. G+C is a size marker.

in the 229-nt ssDNA fragment containing stem-loops I and II by binding of *E. coli* RNA polymerase and SSB protein.

Dependence of *E. coli* RNA polymerase in the initiation of DNA synthesis on the 229-nt *ssi* signal

The RNA primers used in the conversion of ssDNA to RF DNA of the ssDNA phages are synthesized by either DNA primase (in ΦX174 and G4) or RNA polymerase (in M13). As described previously using *in vitro* system [13], *E. coli* RNA polymerase directly recognizes the 229-nt *ssi* signal and synthesizes primer RNAs depending on the presence of *E. coli* SSB protein.

To determine requirement of *E. coli* RNA polymerase for primer RNA synthesis *in vivo*, cells harboring either plasmid pWVAp (*ssi*+) or pWVAp/Δ*ssi* (*ssi*-) were grown in the presence or absence of rifampicin, to which only RNA polymerase is sensitive.

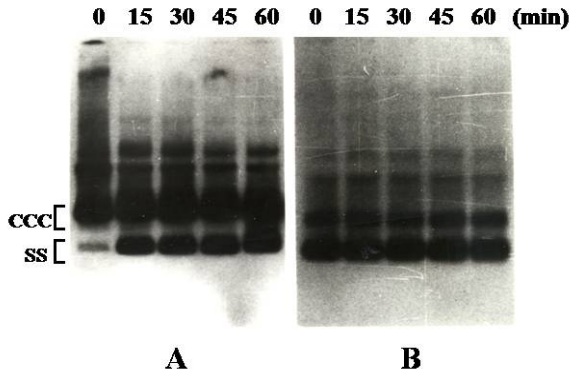

Fig. 6. Effect of rifampicin on ssDNA accumulation of (A) plasmid pWVAp or (B) pWVAp/Δ*ssi*. Rifampicin was added to cells to a final concentration of 100 μg/ml, and aliquots of cells were removed at the times indicated above. The autoradiograms were taken from denatured gels prior to transfer to a nitrocellulose filter. Relative positions of covalently closed circular (ccc) and ssDNA (ss) are indicated on the left.

Accumulation of ssDNAs as the plasmid replication intermediates in cells upon addition of rifampicin indicates that RNA polymerase is involved in synthesizing the primer. Fig. 6 shows that plasmid ssDNAs are accumulated in cells harboring pWVAp upon addition of rifampicin. In cells harboring pWVAp/Δ*ssi*, plasmid ssDNA is present before drug addition and its level does not increase albeit the addition of rifampicin. In the present *in vivo* study, the conversion of ssDNA to dsDNA of plasmid pGKV21 was found to be almost totally inhibited in the presence of rifampicin, suggesting that rifampicin-sensitive RNA polymerase is involved in the complementary-strand DNA synthesis.

Seegers *et al*. [19] have reported that in *L. lactis*, functional pWV01 minus origin is located on a 294-bp DNA fragment and consists of two inverted repeats, IR Ⅰ and IR Ⅱ.

Lagging strand DNA synthesis could occur via only IR Ⅰ, but it was sub-optimal. For full activity of the minus origin in *L. lactis*, the combination of IR Ⅰ and IR Ⅱ was required in a rifampicin-sensitive process. The plasmid ssDNA was gradually converted to dsDNA in the course of the experiment, apparently through an RNA polymerase-independent pathway. The IRI-mediated pathway renders the ssDNA conversion resistant to rifampicin because a conversion of ssDNA via IR Ⅰ in *L. lactis* was not affected by an addition of rifampicin [15,19].

In the present report with *E. coli* (Fig. 6), not with *L. lactis*, the plasmid ssDNA was gradually increased in the course of the experiment. Such an observation concerned with rifampicin sensitivity on the ssDNA to RF DNA conversion suggests that the 229-nt *ssi* signal during replication may be regulated by a slightly different mechanism from the case in *L. lactis*.

SSB proteins are required for DNA replication, repair, and recombination [5]. These proteins are present in high concentrations *in vivo* and bind nonspecifically to ssDNAs [5].

In vitro, primase can synthesize primer RNAs on the phage G4 ssDNA template only when this template is coated with *E. coli* SSB protein [14,18,27]. The nuclease protection experiment of Sims and Benz [21] using ϕK*ori*$_C$ demonstrated that primase protected various regions of ϕK*ori*$_C$ stem-loop structure. This protection, however, required *E. coli* SSB protein. Bacteriophage N4 virion RNA polymerase requires both supercoiled template and *E. coli* SSB protein for transcription of dsDNA promoter; the other SSB proteins cannot be replaced. The DNA determinants for virion RNA polymerase binding at the promoter region comprise a small template-strand hairpin [9,10]. Results of primer RNA synthesis, gel retardation, and DNase I footprinting showed that *E. coli* SSB protein does interact directly with the synthesized 229-nt DNA fragment, and that *E. coli* RNA polymerase also participates in primer RNA synthesis.

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초록 : Lactococcal plasmid pGKV21의 SSB-coated 229-nt *ssi* signal 상에서 *E. coli* RNA polymerase에 의한 시발체 RNA 합성

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플라스미드 pGKV21에는 229-nt single-strand DNA initiation (ssi) signal이 존재한다. Asymmetric PCR 기법으로 합성된 229-nt ssDNA 단편을 이용하여 실제로 RNA polymerase에 의한 priming ability와 protein interaction을 확인하였다. *in vitro* primer RNA 합성 실험 결과, 229-nt ssDNA 단편은 filamentous M13 phage의 주형 DNA에서와 비슷한 효율로 시발체 RNA를 합성하였으며, 이 반응은 strand-specific하게 이루어졌다. DNase I footprinting과 gel retardation 실험 결과, RNA polymerase와 SSB 단백질은 229-nt ssDNA 단편에 stable interaction을 하며, 시발체 RNA를 합성하였다. 또한, *in vivo* 조건 하에서 RNA polymerase의 저해제인 rifampicin을 처리하여 세포 내에 ssDNA 중간체가 집적되는 정도를 비교하여 본 결과, 플라스미드 pGKV21은 rifampicin-sensitive RNA polymerase가 상보가닥 합성에 관여 함을 보여 주었다.