

Mutational analysis of the specific priming signal essential for DNA replication of the broad host-range plasmid RSF1010

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To analyze the RSF1010-specific priming mechanism, a library of randomly mutagenized *ssiA* sequences was constructed by chemical synthesis using mixed nucleotide phosphoramidites. Synthetic *ssiA* sequences with the single base-substitutions were assayed for the SSI activity in *E. coli* JM109 expressing RepB' primase. It was demonstrated that the activity of *ssiA* was damaged markedly by single base-substitutions within the possible stem-loop structure and its 3'-flanking region. It is conceivable that these domains are critical in recognition and primer synthesis by RepB' primase.

DNA replication; Replication origin; Priming signal; Broad host-range plasmid RSF1010

1. INTRODUCTION

In the initiation process of DNA replication, priming enzymes are introduced onto each start site of DNA strand synthesis through various mechanisms depending on specific nucleotide sequences in the replication origins. Functional and structural features of the priming events are well-defined in prokaryotic replicons. The specific sequences on single strand DNA templates which direct entry of the primase molecules are called *ssi* (single strand DNA initiation) signals and play essential roles in the complementary strand synthesis of single strand phage DNA replication [1–3]. Many bacterial plasmids also contain one or two *ssi* signals at or near the replication origins [4–8]. These *ssi* signals are essential to maintain the wild-type copy numbers and should function as priming signals which lead to establishment of the replication forks during the initial process of plasmid DNA replication [9–12].

RSF1010 is a small (8684 bp) IncQ plasmid and has a remarkable property of replicating in a wide variety of Gram-negative bacteria and even in some Gram-

positive actinomycetes [13–16]. This prominent broad host-range property of RSF1010 is dependent on the specificity of its initiation mechanism of DNA replication. That is, it encodes all of the specific initiator protein, DNA helicase, and primase, namely RepC, RepA, and RepB' proteins, respectively. These plasmid-specified Rep proteins direct initiation of RSF1010 DNA replication without host replication functions such as *E. coli* DnaA, -B, -C, -G, and -T [17].

In RSF1010 DNA replication, the specific primase, RepB', is loaded on each template DNA strand depending on the specific nucleotide sequences, *ssiA* and *ssiB*, after the duplex unwinding step that is most probably directed by RepA and RepC proteins [15,18,19]. *ssiA* and *ssiB* are located on each complementary strand of the *oriV* region and have been cloned as 48-nt and 144-nt *Hae*III fragments, respectively [20]. Although *ssiA* and *ssiB* can be substituted functionally by heterologous *ssi* signals such as the primosome assembly sites or DnaG-dependent priming signals from other plasmids or phages, both of them are required for the normal plasmid replication in *E. coli* [19,21]. Deletion of either of the *ssi* signals causes abnormal replication products and RSF1010 cannot be maintained stably as a monomeric form. Thus the specific priming reaction dependent on RepB' primase and its target sequences, *ssiA* and *ssiB*, is essential for the plasmid DNA replication. Haring and Scherzinger [4] have reported that this priming reaction occurred in vitro in the absence of the ribonucleotide triphosphates. Such a unique priming system renders RSF1010 DNA replication independent of the vital priming functions of the host cells. However,

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Abbreviations: *ssi*, single strand DNA initiation; Inc, incompatibility; nt, nucleotide(s); *oriV*, origin of vegetative DNA replication; *oriC*, origin of complementary DNA strand synthesis; X-gal, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; IPTG, isopropyl- β -D-thiogalactopyranoside; [], designates plasmid-carrier state; pfu, plaque forming units

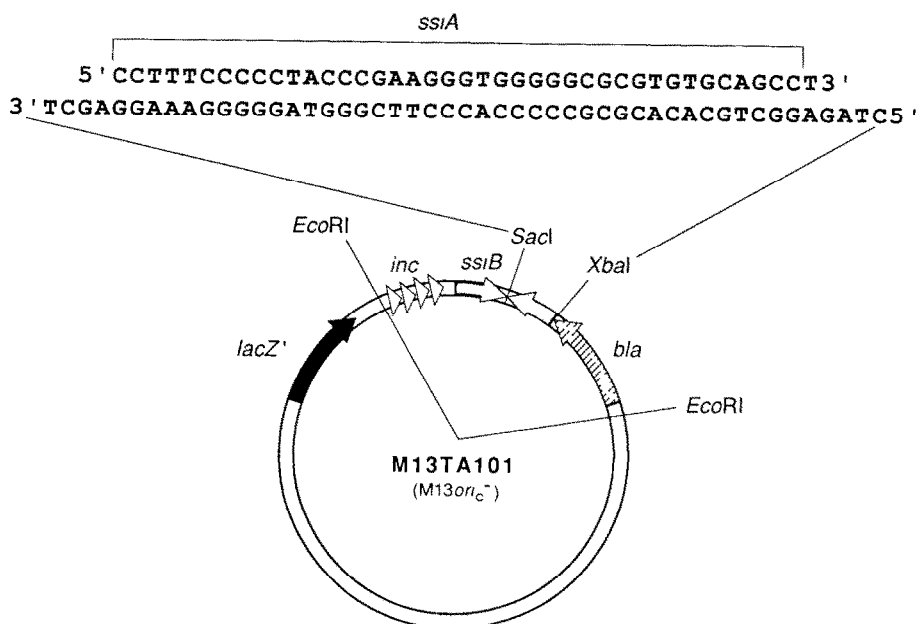


Fig. 1. Physical and genetic map of M13TA101. This recombinant phage contains the mini-RSF1010 plasmid pYH101VS [19] in the *ori_C*-defective filamentous phage M13ΔE101 [26] (see section 2). Upon infection to *E. coli* cells expressing RepB' primase, this phage makes plaques the size of which is dependent on the activity of the *ssiA* sequence it contains. *inc* indicates *mc* repeats; *bla*, the coding region of β -lactamase from pBR322. The synthesized oligonucleotides containing the conserved 40-nt sequence in *ssiA* is shown in the upper part of the diagram.

it is not clear how RepB' interacts with the *ssi* sequences and synthesizes primer molecules.

The nucleotide sequences of *ssiA* and *ssiB* involve 40-nt stretches homologous to each other [20]. This region contains a potential stem-loop structure and the DNA initiation start sites proposed in plasmid R1162 which is almost identical to RSF1010 [22]. The stem-loop structures have been found in all the *ssi* signals isolated from a variety of plasmids and phages investigated to date, and it is conceivable that such specific secondary structures play crucial roles in the recognition by the priming proteins [6,23,24]. To discover the importance of the conserved 40-nt sequence in *ssiA* and *ssiB* for the recognition and primer synthesis by the RepB' primase, mutational analysis of *ssiA* was undertaken.

2. MATERIALS AND METHODS

2.1. Bacterial strains, plasmids, and phages

E. coli JM109 [25] and JM109 harboring pMMB2(*repA*⁺, *repB*⁺, *repC*⁺), pMMB2Δ23(*repB*⁺) and pMMB2ΔSS(*rep*⁻) [18] were used as host bacteria. A recombinant bacteriophage, M13TA101 (Fig. 1), was constructed by introducing the EcoRI-digested mini-RSF1010 plasmid, pYH101VS [19], into the unique EcoRI site of M13ΔE101 [26] in the orientation such that *ssiA* was located on the viral (+) strand. This recombinant phage lacks most of the M13 *ori_C* and contains the pUC19-derived *lacZ'* gene. Thus, upon infection to JM109 cells, M13TA101 forms small blue plaques in the presence of X-Gal and IPTG. However, when RepB' primase is expressed by a helper plasmid such as pMMB2 or pMMB2Δ23, this phage forms large plaques depending on the activity of *ssiA*.

2.2. Chemical synthesis of mutagenized *ssiA* sequences

Chemical synthesis was done with an Applied Biosystems model 394

DNA/RNA synthesizer. Standard operating procedures were used except for the preparation of the mutagenic nucleotide phosphoramidite mixtures [27]. Before the synthesis, each of the four nucleotide phosphoramidites was contaminated with the other three. Upon this cross-contamination, they contained 1.25% of each of the three mutagenic species. Although an average of 1.5 substitutions should be incorporated per oligonucleotide, the mutations per clone in the library were expected to be about half of this, according to the results of Huchison III et al. [27].

3. RESULTS

3.1. Construction of the *ssiA* mutant library

To construct a mutant library of *ssiA*, two oligonucleotide strands which were complementary to each other were synthesized (Fig. 1). One was 49 nt long with 5' *XbaI* and 3' *SacI* cohesive ends. The other strand was 41 nt long and included the conserved 40-nt sequence in *ssiA*. Both strands except for the nucleotides in each of the cohesive ends were synthesized by using the same doped mixtures of monomers (see section 2). The annealed strands were inserted into M13TA101 in place of the wild-type *ssiA* sequence, followed by transfection into JM109 by the method of Chung et al. [28]. The plates, containing about 750 plaques, were washed by overlaying with 4 ml of LB medium for 5 min. The medium was removed and centrifuged to give the *ssiA* mutant library used in the subsequent analysis.

The library was diluted and infected to JM109[pMMB2] which harbored the helper plasmid pMMB2 encoding RepB' primase. In this strain, the activity of mutagenized *ssiA* can be roughly estimated based on the plaque morphology of each recombinant

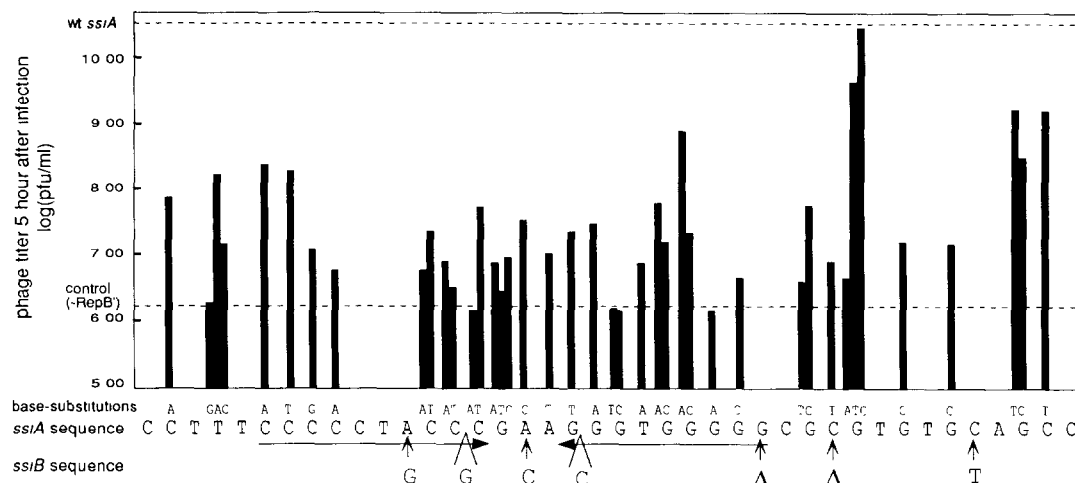


Fig. 2. Single base-substitution mutants and their SSI activities. The wild-type *ssiA* sequence and each base-replacement found in the single base-substitution mutants are indicated. Facing arrows indicate the inverted repeat sequences present in *ssiA*, and difference of the nucleotide sequences between *ssiA* and *ssiB* are also indicated as follows; →, replacement; ^, insertion; Δ, deletion. Each single base-substitution mutant phage was infected to the *E. coli* cells expressing RepB' primase at the titer of 10^5 pfu/ml. The phage titer 5 hour after infection was measured and log(pfu/ml) are depicted in the histogram. Dashed lines indicate positive and negative control levels corresponding phage titer of M13TA101 infected to *E. coli* JM109[pMMB2Δ23] and JM109[pMMB2ΔSS], respectively. Since the activity of *ssiA* completely depends on RepB' primase [4,18], phage titer of each mutant in the absence of RepB' primase should be the same level of the indicated negative control level.

clone. Most of the small plaques and some of the large ones were picked and the sequences of the inserted oligonucleotides were analyzed with an Applied Biosystems model 373A DNA sequencer. Of 114 mutant clones obtained, 41 clones contained single base-substitutions, 33 clones were double mutants, and the others were more than double mutants or contained some unexpected deletions or insertions in the *ssiA* sequence. Only the recombinant phages with single base-substitutions are shown in Fig. 2.

3.2. Estimation of the activity of the mutant *ssiA* with a single base substitution

To analyze effects of the single base substitutions on the activity of *ssiA*, propagation of the mutant recombinant phages was tested. Each mutant phage was used to infect *E. coli* JM109[pMMB2Δ23] (*repB*⁺) followed by measurement of the phage titer 5 hour after infection. As expected, propagation of each mutant phage with a single base substitution was less efficient than that of M13TA101 phage which contained the wild-type *ssiA* sequence. It is clear that the conserved 40-nt sequence in *ssiA* is critical in recognition and primer synthesis by RepB' protein. Furthermore, the propagation of some of the single base-substitution mutants in JM109[pMMB2Δ23] were even as poor as that of M13TA101 phage in JM109[pMMB2ΔSS] in which RepB' primase is not expressed, indicating that the RepB'-dependent activity of *ssiA* was completely abolished by the single base substitutions they contained.

Most of the base substitutions causing great phenotypic defectiveness of *ssiA* are localized near or in the inverted repeat sequence present in the conserved 40-nt sequence in *ssiA*. Moreover, at a defined nucleotide

position in the inverted repeat sequence, a transversion always causes more severe damage to the *ssiA* activity than a transition, with the exception of C at position 14 (Fig. 2). These results strongly suggested that changes in the secondary structure of this region had critical influence on the *ssiA* activity.

4. DISCUSSION

It has been reported that specific stem-loop structures exist in the primosome assembly sites isolated from bacteriophage ϕX174 or plasmids such as ColE1, ColE2, F, and R100 [6]. And mutational analysis of the primosome assembly sites from pBR322 showed that the stem-loop structures should be essential for the initial recognition by the PriA (n') protein [23,24]. The ABC-primosome dependent *ssi* signal from R6K also contains one stem-loop structure, and *E. coli* DnaA protein recognizes and binds in vitro to the dnaA box located in the stem region [5]. In addition, the DnaG-dependent *ssi* signals from phage G4 or the plasmids R1 and ColIb contains the stem-loop structures, too [7,8]. Particularly, the *ori*_c of phage G4 contains three stem-loop structures and the primer start site is located at the 3'-flanking region of one of the stem-loops [29,30]. It seems that the specific stem-loop structure is one of the essential requirements for the *ssi* signals to be recognized by the priming proteins.

The nucleotides of which substitutions resulted in the remarkable inactivation of *ssiA* were summarized in Fig. 3. All but one of them are located in a region containing the specific stem-loop structure and its 3'-flanking domain. These results indicated that nucleotide residues in this region were strictly required for the *ssiA*

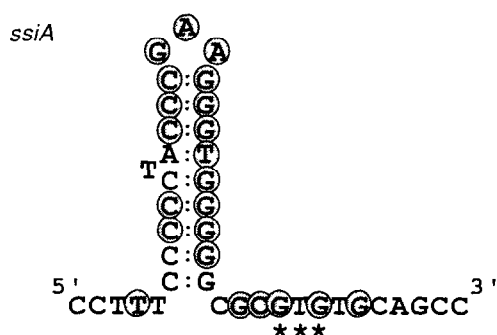


Fig. 3. Potential stem-loop structure and essential nucleotide residues in *ssiA*. The nucleotide residues of which substitutions were demonstrated to cause more than 1000-fold reduction of the *ssiA* activity from the wild-type level (Fig. 2) are indicated by shaded circles. The DNA start sites proposed in plasmid R1162 are indicated by asterisks.

activity. In plasmid R1162, it was demonstrated that DNA strand synthesis was initiated within the triple nucleotide residues in the 3'-flanking domain (Fig. 3) [22]. It is conceivable that the stem-loop structure and its 3'-flanking domain are crucial in the RSF1010-specific priming reaction depending on the *ssi* signals and RepB' primase. Probably, the stem-loop structure in each of the *ssi* signals is needed for specific and productive recognition by the RepB' primase, and the primer synthesis takes place in its 3'-flanking region.

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