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Functional difference between the two oppositely oriented priming signals essential for the initiation of the broad host-range plasmid RSF1010 DNA replication

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ABSTRACT

The broad host-range plasmid RSF1010 contains two oppositely oriented priming signals, *ssiA* and *ssiB*, for DNA synthesis dependent on the origin of vegetative DNA replication (*oriV*). If either *ssiA* or *ssiB* was deleted or inverted, the RSF1010 miniplasmids containing engineered *oriV*s were maintained at low copy numbers, replicated abnormally as dimers, and accumulated specific single strands in the *Escherichia coli* strain supplying the three RSF1010-encoded RepA, RepB′, and RepC proteins. Interestingly, an additional intracellular supply of the Sog primase (the *sog* gene product of plasmid ColIb-P9) reversed the replication deficiency of these miniplasmids with respect to all three aspects described above. These were also true for the RSF1010 miniplasmids in which either *ssiA* or *ssiB* was replaced by the primosome assembly site (PAS) or by the G4-type *ssi* signal (G site). Furthermore, comparative analysis of the functional contribution of the two oppositely oriented *ssi* signals to the DNA replication of RSF1010 showed that, irrespective of their types, *ssi* signals conducting the initiation of DNA chain elongation away from the iterons were functionally more important than ones in the inverted orientation. We consider that this functional difference reflects the inherent properties of the initiation mechanism of RSF1010 DNA replication.

INTRODUCTION

Almost all the replicons that replicate via θ-type intermediate molecules in *E.coli* have three sequential initiation processes (1): (i) initial interaction between *ori*, especially direct repeats (iterons), and *trans*-acting factors such as initiator proteins specific for its replicon; (ii) melting and unwinding of the duplex DNA in the *ori* region by the action of an initiator protein and DNA helicase in a replicon-specific way; and (iii) priming reaction by priming enzymes that are introduced onto each template strand, leading to initiation of DNA strand synthesis.

In *E.coli*, each replicon has a unique mechanism to introduce priming enzymes. In some replicons, priming enzymes are recruited in consequence of a series of protein–protein interactions (1). In *oriC*, the origin of chromosome DNA replication, priming enzymes are introduced by interaction with the initiator protein DnaA (2). On the other hand, in the proximity to *oriV*s, many plasmids have specific nucleotide sequences, single-stranded DNA initiation (*ssi*) signals, directing the specific priming of DNA chain elongation (3, 4, 5, 6). The *ssi* signals direct the loading of priming enzymes onto the single-stranded (ss) template at the specific nucleotide sequences. PAS is an *ssi* signal at which a multiprotein complex, the primosome, containing the *E.coli* proteins DnaB, DnaC, DnaT, PriB, PriA, and PriC in addition to DnaG primase, assembles (7). In several plasmid replicons, PAS is crucial in efficient lagging strand synthesis (8, 9). The G site is an *ssi* signal directing synthesis of a unique RNA primer at a defined site solely depending on the *E.coli* DnaG primase (10). The G site functions as an initiation site for the continuous leading strand synthesis in plasmid replication (4, 11).

Broad host-range plasmids of the incompatibility group Q (IncQ) in *E.coli*, represented by RSF1010 (12), R300B (13), and R1162 (14), are small, non-self-transmissible, multicopy replicons conferring resistance to streptomycin and sulfonamide. The most remarkable feature of plasmids of this group is their capability to replicate in a broad range of bacterial hosts, including most of the Gram-negative bacteria and at least some of the Gram-positive species (15, 16).

RSF1010 is an 8684-base pair (bp) plasmid and is very similar to another IncQ plasmid, R1162, particularly in the sequence and organization of its *oriV* (17). In *E.coli*, vegetative replication of RSF1010 absolutely depends on *oriV*, and at least three plasmid-encoded proteins, RepA (essentially identical to RepIA of R1162), RepB′ (RepII of R1162), and RepC (RepIB of R1162) (18, 19). RepC binds specifically to the three and a half iterons of 20-bp in the *oriV* region and functions as an initiator protein (19, 20). RepA has two enzymatic activities: an ss DNA-dependent ATPase activity and a DNA helicase activity (19). RepA acts as a helicase

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in unwinding of the duplex DNA with an apparent 5′ to 3′ polarity in a reaction requiring ATP hydrolysis. RepB′ has primase activity dependent on *ssiA* and *ssiB* as the only template sequences, directing the priming events for their complementary strand synthesis (3, 19, 21). By virtue of these three plasmid-specific proteins, the replication of RSF1010 is independent of the host DnaA, DnaB, DnaC, DnaG, and DnaT proteins and the RNA polymerase, while it is dependent on the DNA polymerase III and the DNA gyrase (19, 22, 23). These properties provide an adaptive advantage for survival in varied intracellular environments.

In *E.coli*, the replication of RSF1010 proceeds via a θ-form intermediate structure either bi- or unidirectionally from a unique origin at nucleotides 2347−2742 (19, 22, 24). The same origin is also used in *Pseudomonas aeruginosa* (22). In the *oriV* of RSF1010 ($oriV_{RSF1010}$), three major features (25) essential for the plasmid replication are: (i) iterons, binding of RepC to which is essential for the origin function; (ii) two adjacent regions, the GC-rich and the AT-rich regions, flanking the iterons in the proximal and distal positions, respectively; and (iii) two convergently plasmid-specific DNA priming signals, *ssiA* and *ssiB*, which are on the *l*- and the *r*-strands, respectively, directing the priming of their complementary strand syntheses in a RepB′-dependent manner (19, 21, 26).

RepB′ independently directs the RSF1010-specific priming events on *ssiA* and *ssiB* after completion of the duplex unwinding step that is dependent on RepA and RepC (26). Our previous studies showed that other types of priming signals, PAS and G site from plasmids and phages, can functionally substitute for both *ssiA* and *ssiB* (26, 27). When both *ssiA* and *ssiB* are replaced by foreign ones, the function of the chimeric $oriV_{RSF1010}$ carrying the heterologous *ssi* signals is no longer dependent on RepB′, but is still dependent on RepA and RepC. However, both the two convergently located ssi signals are required for the normal plasmid replication in *E.coli* and deletion of either of the ssi signals makes the DNA replication abnormal, that is, the resulting plasmid is not maintained stably as a monomeric form (26, 28).

We have shown that, in *Pseudomonas aeruginosa*, only one RSF1010-specific *ssi* signal that primes the DNA chain elongation away from the iterons is sufficient for the replication of RSF1010 (29). In this study, we examined the functional differences between the two convergently located *ssi* signals in the replication of RSF1010 in *E.coli* by comparative analysis of replication properties of various mini-RSF1010 plasmid derivatives carrying heterologous *ssi* signals.

MATERIALS AND METHODS

Bacterial strains and plasmids

E.coli JM109 (30) was used as a host bacterium. A mini-RSF1010 plasmid pYH101VS (26) contains the coding region of β-lactamase from pBR322 and a 444-bp *oriV* segment from RSF1010 [nucleotides 2335−2778] consisting of *ssiA*, *ssiB*, the GC-rich region, the AT-rich region, and the iterons. Seven recombinant miniplasmids, pYH101RA, pYH101RB, pYH101DA, pYH101DB, pYH101DAB, pYH174A, and pYH174B, containing a deletion, inversion, or substitution of either of the ssi signals were derived from M13mp19/YH101VS (26). Three additional recombinant miniplasmids, pYHG4A, pYHG4B, and pYHDAG4B, carrying heterologous ssi signals, were derived from pHSG399/YH101VS (27). Generation of Ampicillin (Amp)-resistant transformants was a primary criterion for the functional $oriV_{RSF1010}$ (26, 27). The helper plasmids pMMB2 (ColD plasmid-based pKT101 carrying repA, repB′, and repC of RSF1010) and pMMB2D67 (ColD plasmid-based pKT101 carrying repA and repC of RSF1010) are as described (18, 21). The helper plasmid pLG214′ is an Amp-sensitive derivative of plasmid pLG214 (31) (a pBR325-based recombinant plasmid carrying the *sog* gene of ColIb-P9). In pLG214′, the *bla* gene is inactivated by removing the *PvuI-VspI* segment in the coding region. This was done by cleavage with *PvuI* and *VspI* followed by blunting and ligation.

DNA manipulation

Restriction endonucleases and DNA-modifying enzymes were purchased from Takara Shuzo Co., Ltd. and New England Biolabs, Inc. [γ-^{32}P]ATP was from Dupont NEN Research Products. Plasmid DNA was extracted by the alkaline denaturation procedure (32). Transformation was done by the method of Chung *et al.* (33). Concentrations of antibiotics in selective media were: Amp, 50 μg/ml; Chloramphenicol, 30 μg/ml; Kanamycin, 100 μg/ml; and Tetracycline, 10 μg/ml.

Measurement of the relative copy numbers of the miniplasmids with chimeric $oriV_{RSF1010}$

Relative copy numbers of the miniplasmid with chimeric $oriV_{RSF1010}$ were measured by estimation of the single-cell Amp resistance in the strain JM109 (34).

Accumulation of specific single strands during replication of miniplasmids with chimeric $oriV_{RSF1010}$

E.coli JM109 strains that harbor various helper plasmids (pMMB2, pMMB2Δ67, pMMB2 plus pLG214′, or pMMB2Δ67 plus pLG214′) and mini-RSF1010 plasmid derivatives containing heterologous ssi signals were grown at 37°C overnight. Plasmid DNA was then extracted and electrophoresed on a 1% agarose gel. DNA bands were transferred onto a nylon membrane (GeneScreenPlus) without alkaline denaturation. Southern hybridization was done with strand-specific probes. Oligonucleotides, 5′-TGTTGGAAAAATCCATCCATGATTATCT-AAGAATAATCCACTA-3′ (the nucleotide sequence from 2456 to 2498 in the RSF1010 genome (25), designated AT-*l*), and 5′-TAGTGGATTATTCTTAGATAATCATGGATGGATT-TTTCCAACA-3′ (complementary to AT-*l* and designated AT-*r*), were synthesized in a DNA synthesizer (Applied Biosystems 394 DNA/RNA synthesizer) and 5′-end labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. The ^{32}P-labeled oligonucleotides, AT-*l* and AT-*r*, were used as strand-specific probes to detect the *r*- and the *l*-strands, respectively.

RESULTS

Replication properties of mini-RSF1010 plasmids containing a deletion or inversion of the RSF1010-specific *ssi* signals

Miniplasmid pYH101VS and its five derivatives containing deletions or inversions of the RSF1010-specific *ssi* signals, *ssiA* and *ssiB* (Fig. 1), were used to transform the strain JM109 harboring pMMB2 as a helper plasmid (JM109[pMMB2]). This plasmid supplies RepA, RepB′, and RepC, essential for the replication dependent on $oriV_{RSF1010}$. Miniplasmid derivatives lacking *ssiA* or *ssiB* (pYH101DA or pYH101DB respectively),

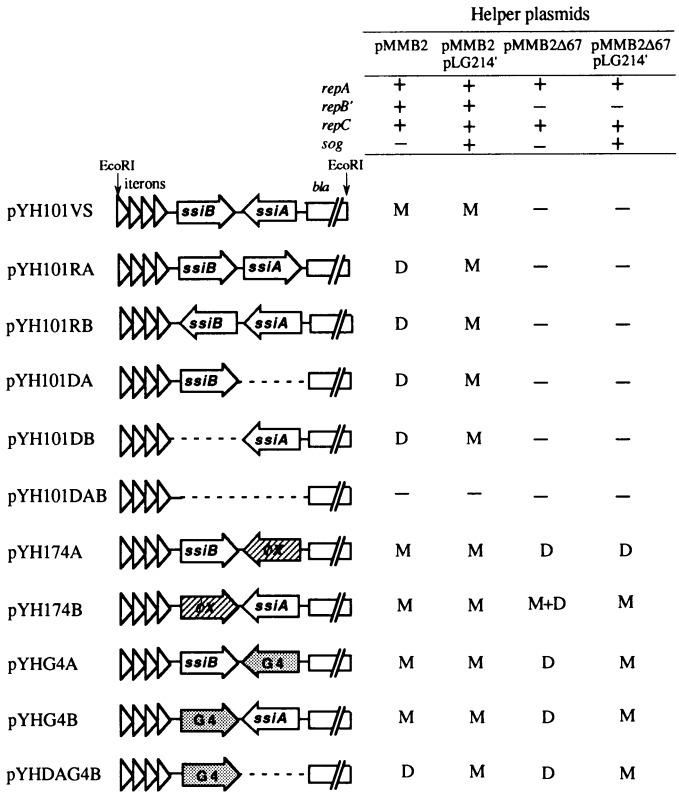

Figure 1. Chimeric mini-RSF1010 plasmids and their replication properties in *E.coli* in the presence or the absence of the helper plasmids supplying the RSF1010-specific Rep proteins and ColIb-P9-derived Sog. Schematic representations of the miniplasmids derivatives were drawn as linearized upon cleavage at the unique *Eco*RI site. Arrows indicate the locations and the priming orientations of the *ssi* signals: arrows marked with '*ssiA*' or '*ssiB*', the original RSF1010-specific *ssi* signals; arrows marked with 'ϕX', PAS from bacteriophage ϕX174; arrows marked with 'G4', G site from bacteriophage G4. Open triangles, iterons; broken lines, the deleted regions; the open boxes, the coding region of the β-lactamase from pBR322. The multimeric forms of replication products of the miniplasmid derivatives in the strains JM109[pMMB2], JM109[pMMB2, pLG214'], JM109[pMMB2Δ67], and JM109[pMMB2Δ67, pLG214'] are indicated: M, monomers; D, dimers; –, inability to produce transformants.

and ones containing one of the *ssi* signals in the reverse orientation (pYH101RA and pYH101RB) gave Amp (50 μg/ml)-resistant transformants, while a miniplasmid derivative lacking both *ssiA* and *ssiB* (pYH101DAB) gave no transformants (Fig. 1). However, the miniplasmids pYH101DA, pYH101DB, pYH101RA, and pYH101RB, which gave Amp-resistant transformants, replicated mostly as dimers in JM109[pMMB2] cells (Fig. 1 and 2).

Furthermore, we examined the replication properties of the miniplasmid pYH101VS and its five derivatives by transformation of JM109 harboring the helper plasmids pMMB2 plus pLG214' (JM109[pMMB2, pLG214']). Plasmid pLG214' carries the *sog* (suppression of *dnaG*) gene derived from plasmid ColIb-P9, which is responsible for suppressing the *dnaG* mutation of *E.coli* (35). It is known that the Sog primase (the *sog* gene product) has a low template specificity and can prime discontinuous DNA synthesis on ss DNA templates (31). Our preliminary results indicated that ColIb-P9 or pLG214' carrying the *sog* gene can completely restore the growth of the filamentous phage vector M13Δ*lac*183 to the normal level; it usually grows poorly and

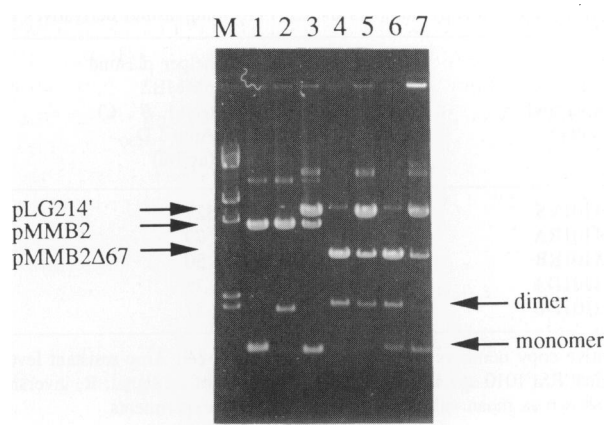

Figure 2. Molecular species of replication products of the miniplasmid derivatives. The plasmid DNA extracted from each transformant was resolved by agarose (0.7%) gel electrophoresis. Lane 1, pYH101VS in JM109[pMMB2]; lane 2, pYH101DA in JM109[pMMB2]; lane 3, pYH101DA in JM109[pMMB2, pLG214']; lane 4, pYH174A in JM109[pMMB2Δ67]; lane 5, pYH174A in JM109[pMMB2Δ67, pLG214']; lane 6, pYH174B in JM109[pMMB2Δ67]; lane 7, pYH174B in JM109[pMMB2Δ67, pLG214']; M, size marker: λ DNA digested with *Hin*dIII. Positions corresponding to the helper plasmids, pMMB2, pMMB2Δ67, and pLG214', and monomers and dimers of the miniplasmid derivatives are indicated by arrows.

forms small and turbid plaques because the origin of complementary DNA strand synthesis is deleted (unpublished result). Interestingly, in this JM109[pMMB2, pLG214']) strain, most of the miniplasmid derivatives could replicate normally as monomers (Fig. 1 and 2). This indicates that the intracellular supply of Sog makes it possible for the miniplasmid derivatives lacking either of the two oppositely oriented priming signals to replicate normally as monomers. Perhaps this is caused by virtue of its lower specificity for the priming function as described above. However, a miniplasmid derivative lacking both the *ssi* signals (pYH101DAB) gave no transformants even with Sog present (Fig. 1).

Replication properties of chimeric mini-RSF1010 plasmids containing heterologous *ssi* signals

We analyzed the replication properties of miniplasmid derivatives containing ssi substitutions in a strain supplying no RepB'. Under these conditions, the RSF1010-specific ssi signals, ssiA and ssiB, are not functional, while only PAS from phage ϕX174 and the G site from phage G4, which depend on the *E.coli* priming factors, are functional. An additional five miniplasmid derivatives containing a PAS or G site instead of ssiA or ssiB (Fig. 1), pYH174A, pYH174B, pYHG4A, pYHG4B, and pYHDAG4B, were used to transform the strain JM109 harboring pMMB2Δ67 as a helper plasmid (JM109[pMMB2Δ67]). This helper plasmid supplies RepA and RepC, but no RepB'. Most of the miniplasmid derivatives replicated largely as dimers, although all the miniplasmid derivatives could give Amp-resistant transformants. Interestingly, a miniplasmid derivative, pYH174B, which contained PAS instead of ssiB, replicated not only as dimers but also as monomers in JM109[pMMB2Δ67] (Fig. 1 and 2). This finding is remarkable in that this is the first case in which the priming of DNA chain elongation of only one of the two complementary strands can support the RSF1010 miniplasmid replication as monomers in *E.coli*.

Table 1. Relative copy numbers of RSF1010 miniplasmid derivatives containing a deletion or inversion of RSF1010-specific *ssi* signals

Miniplasmid derivatives	*ssi* site A	B	helper plasmid pMMB2 (*repA, B', C*) Amp LD_{50} (μg/ml)	Relative copy number (%)	pMMB2+pLG214' (*repA, B', C, sog*) Amp LD_{50} (μg/ml)	Relative copy number (%)
pYH101VS	+	+	527	100	570	100
pYH101RA	R	+	79	15	335	59
pYH101RB	+	R	50	9	313	55
pYH101DA	−	+	75	14	308	54
pYH101DB	+	−	37	7	262	46

Relative copy numbers were estimated by single-cell Amp-resistant levels. The *ssi* signals contained in the miniplasmid derivatives are indicated as follows: +, original RSF1010-specific *ssi* signal; −, deletion of *ssi* signal; R, inversion of *ssi* signal. Amp LD_{50} was measured by the method of Nordström *et al.* (34). Results are shown as mean values of three independent experiments.

Table 2. Relative copy number of RSF1010 miniplasmid derivatives containing heterologous *ssi* signals

Miniplasmid derivatives	*ssi* site A	B	helper plasmid pMMB2Δ67 (*repA, C*) Amp LD_{50} (μg/ml)	Relative copy number (%)	pMMB2Δ67+pLG214' (*repA, C, sog*) Amp LD_{50} (μg/ml)	Relative copy number (%)
pYH101VS	+	+	527	100	570	100
pYH174A	*φX*	+	57	11	69	13
pYH174B	+	*φX*	109	21	251	48
pYHG4A	G4	+	55	10	240	46
pYHG4B	+	G4	104	20	530	93
pYHDAG4B	−	G4	101	19	583	102

Relative copy numbers were estimated by single-cell Amp-resistant levels. The *ssi* signals contained in the miniplasmid derivatives are indicated as follows: +, original RSF1010-specific *ssi* signal; −, deletion of *ssi* signal; φX, PAS from phage φX174; G4, G site from phage G4. Results are shown as mean values of three independent experiments.

When Sog was intracellularly supplied, most of the miniplasmid derivatives, except for pYH174A in which *ssiA* was replaced by PAS, could replicate normally as monomers as described above (Fig. 1 and 2). This result indicates that Sog can restore replication ability of the miniplasmid derivatives carrying substitutions, deletions, or inversions of the RSF1010-specific *ssi* signals.

Copy numbers of the miniplasmid derivatives containing heterologous *ssi* signals in the absence or the presence of Sog

We estimated the relative copy numbers of the miniplasmid derivatives containing heterologous *ssi* signals by measuring the single-cell Amp resistance in the host strains JM109[pMMB2], JM109[pMMB2, pLG214'], JM109[pMMB2Δ67], and JM109[pMMB2Δ67, pLG214']. Relative copy numbers of the miniplasmid derivatives pYH101RA, pYH101RB, pYH101DA, and pYH101DB, were about 7 to 15 per cent of that of pYH101VS in the strain JM109[pMMB2] (*repA*$^+$, *repB'*$^+$, *repC*$^+$) (Table 1). In the strain JM109[pMMB2, pLG214'] (*repA*$^+$, *repB'*$^+$, *repC*$^+$, *sog*$^+$), however, the relative copy numbers of these miniplasmid derivatives were 46 to 59 per cent of that of pYH101VS (Table 1). The restoration of the relative copy numbers was completely in agreement with the recovery of the monomeric forms among the population of replication products described above. That is, these results indicate that an intracellular supply of Sog can restore the replication deficiency of miniplasmid derivatives in the relative copy number as well as in the multimeric formation of replication products. Similar

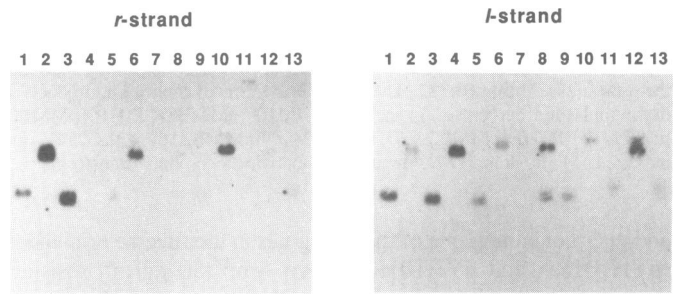

Figure 3. Accumulation of the specific single strands during replication of the miniplasmid containing chimeric $oriV_{RSF1010}$ in JM109 strains harboring various helper plasmids. Southern hybridization was done with strand-specific probes (see *Materials and Methods*). The ^{32}P-labeled oligonucleotides, AT-*l* and AT-*r*, were used as strand-specific probes to detect the *r*- and the *l*-strand, respectively. Lane 1, pYH101VS in JM109[pMMB2]; lane 2, pYH101DB in JM109[pMMB2]; lane 3, pYH101DB in JM109[pMMB2, pLG214']; lane 4, pYH101DA in JM109[pMMB2]; lane 5, pYH101DA in JM109[pMMB2, pLG214']; lane 6, pYH174A in JM109[pMMB2Δ67]; lane 7, pYH174A in JM109[pMMB2Δ67, pLG214']; lane 8, pYH174B in JM109[pMMB2Δ67]; lane 9, pYH174B in JM109[pMMB2Δ67, pLG214']; lane 10, pYHG4A in JM109[pMMB2Δ67]; lane 11, pYHG4A in JM109[pMMB2Δ67, pLG214']; lane 12, pYHG4B in JM109[pMMB2Δ67]; lane 13, pYHG4B in JM109[pMMB2Δ67, pLG214'].

Sog-dependent restoration of copy numbers was seen in the strain JM109[pMMB2Δ67, pLG214'] for the miniplasmid derivatives carrying *ssi* replaced by a PAS or G site, with the sole exception of pYH174A (Table 2).

On the other hand, we compared the functional contribution of the two oppositely oriented *ssi* signals to the DNA replication of RSF1010. In the strains JM109[pMMB2] and JM109[pMMB2, pLG214'], relative copy numbers of the miniplasmid derivatives, pYH101RA and pYH101DA in which *ssiA* was inverted and deleted, respectively, were higher than those of the miniplasmid derivatives, pYH101RB and pYH101DB in which *ssiB* was inverted and deleted, respectively (Table 1). Furthermore, in the strains JM109[pMMB2Δ67] and JM109-[pMMB2Δ67, pLG214'], relative copy numbers of the miniplasmid derivatives, pYH174B, pYHG4B, and pYHDAG4B in which *ssiB* was substituted for a PAS or G site, were much higher than those of the miniplasmid derivatives, pYH174A and pYHG4A in which *ssiA* was substituted (Table 2). Only the replaced PAS and G sites were functional in the initiation of the leading strand syntheses in the host strains JM109[pMMB2Δ67] and JM109[pMMB2Δ67, pLG214'] because they supply no RepB'. Therefore, these results indicate that irrespective of types of *ssi* signals, *ssi* signals conducting the initiation of DNA chain elongation away from the iterons are functionally more important than ones conducting the initiation in the opposite orientation. We presume that the difference in the functional importance between the two oppositely oriented *ssi* signals reflects the inherent properties of DNA initiation mechanisms dependent on $oriV_{RSF1010}$.

Accumulation of specific single strands during replication of miniplasmids with chimeric $oriV_{RSF1010}$

Our previous studies have suggested that the synthesis of each complementary strand in RSF1010 replication is independent and proceeds by a strand-displacement mechanism (26, 27). If the priming of DNA chain elongation of only one of the two complementary strands is directed with a chimeric $oriV_{RSF1010}$, the displaced strand should accumulate in the cells as a single strand without efficient conversion into a double strand. We tested this in the various host strains harboring miniplasmid derivatives carrying heterologous *ssi* signals. The ss replication products were resolved by agarose gel electrophoresis and detected by hybridization to strand-specific oligonucleotide probes.

The mini-RSF1010 plasmid pYH101VS that carries the wild type $oriV_{RSF1010}$ produced a small amount of ss DNA in JM109[pMMB2] (26) (Fig. 3). The miniplasmids pYH101DB in JM109[pMMB2], pYH174A in JM109[pMMB2Δ67], and pYHG4A in JM109[pMMB2Δ67] accumulated the *r*-strands and the miniplasmids pYH101DA in JM109[pMMB2], pYH174B in JM109[pMMB2Δ67], and pYHG4B in JM109[pMMB2Δ67] accumulated the *l*-strands (Fig. 3). In most miniplasmids, the ss DNAs were accumulated mainly at the position corresponding to dimeric forms of replication products. Exceptionally, the miniplasmid pYH174B in JM109[pMMB2Δ67] accumulated the *l*-strands of monomeric forms in addition to dimeric forms. These patterns of the multimeric form of the ss DNA accumulation were consistent with those of replication products described above (Fig. 2 and 3). These results also demonstrate that RSF1010-specific *ssi* signals, PAS, and G site in chimeric $oriV_{RSF1010}$s actually contribute to the priming of the DNA chain elongation dependent on the respective *ssi* signal-specific mechanism.

Furthermore, the intracellular supply of Sog led to attenuation of the ss DNA accumulation and/or shift of the electrophoretically resolved bands of the accumulated ss DNA from the position corresponding to the dimeric form to that of the monomeric form (Fig. 3). This fact strongly suggests that the low specificity of Sog actually makes it possible to initiate the complementary DNA chain elongation on a template strand lacking functional *ssi* signals, leading to concomitant restoration of the monomeric form production and of the replication ability of the miniplasmids.

DISCUSSION

The broad host-range plasmid RSF1010 has a unique plasmid-specific priming system. In replication of RSF1010, the plasmid-encoded RepB' primase is specifically introduced onto the two oppositely oriented *ssi* signals (*ssiA* and *ssiB*) to direct priming, leading to DNA chain elongation (19, 21). Besides this, RSF1010 has the remarkable characteristic that $oriV_{RSF1010}$ is composed of distinct functional domains. That is, the RepB'-dependent priming at the *ssiA* and *ssiB* site is independent of the prepriming events of origin recognition and opening of the duplex that involve RepC and RepA (26, 27). Thus, RSF1010 provides a good model for elucidating the general mechanism in the initial events of DNA replication used by many bacterial replicons.

We found that mini-RSF1010 plasmids defective in either of the two oppositely oriented *ssi* signals replicated as dimers in the JM109 strain (*recA*) harboring the helper plasmid pMMB2 or pMMB2D67, while ones defective in both the *ssi* signals could never replicate. Previously, Zhou and Meyer have shown that mini-R1162 plasmids with a single *ssi* signal were found as dimers and higher multimers in a *recA* strain and in an equilibrium between monomeric and dimeric forms in a $recA^+$ strain, and that their dimers had a head-to-tail plasmid composition (28). Now, we have no clear explanation for the mechanism of dimer formation, but it is unlikely that dimers are formed simply in the process of constructions of miniplasmid derivatives, because when the monomers of pYHG4B in JM109[pMMB2] were used to transform JM109[pMMB2Δ67], the resulting replication products were dimers (data not shown). On the contrary, when the dimer products of pYHG4B in JM109[pMMB2Δ67] were used to transform JM109[pMMB2], the resulting replication products remained as dimers (data not shown). Furthermore, our results indicate that appearance of dimers among the replication products of the RSF1010 miniplasmid derivatives reflects the decline in their replication abilities. It is conceivable that dimers are formed by some unknown mechanisms to survive in the cell, because dimer formation would result in an enhanced gene dosage of *bla*, and increase the chance for initiation of DNA synthesis on the displaced single strand, which lacks a specific priming signal.

The two oppositely oriented *ssi* signals in $oriV_{RSF1010}$ essential for the normal vegetative replication of RSF1010 are functionally independent of each other and can be replaced functionally by other types of priming signals (PAS and G site) (26, 27). That is, irrespective of the type of priming signals, two convergently oriented priming events for the DNA chain elongation of the leading strands are essential for the initiation of RSF1010 replication. In this study, however, we suggest some functional differences depending on the orientation of each priming signal. These experiments indicate that, for PAS, G site, and RSF1010-specific *ssi* signals, priming the DNA chain elongation away from the iterons has more functional significance in RSF1010 replication than priming in the opposite orientation. That is, the DNA chain elongation away from the iterons functionally contributes more to the RSF1010 replication than that toward the iterons. Probably, this substantially reflects the inherent properties of the initiation mechanisms of RSF1010

replication described as follows. Activation of *ssi* signals requires transient melting of the duplex DNA to make the priming enzymes accessible to the *ssi* signals. Subsequent to the initial destabilization of the duplex by RepC binding to the iterons, the duplex is unwound by the RSF1010-encoded RepA helicase, not by the host DnaB helicase. RepA is likely to be primarily introduced into the AT-rich region adjacent to the iterons through protein-DNA or protein-protein interaction in a plasmid-specific manner, and then translated in the 5′ to 3′ direction along both the strands. However, it has been postulated that RepA extends unwinding of the contiguous duplex region predominantly in one direction away from the iterons to activate *ssiA* and *ssiB* for priming (23, 36). The GC-rich regions between the iterons and the AT-rich region, and/or nucleoprotein complex involving the iterons and RepC, might initially prevent another RepA molecule on the *r*-strand from unwinding toward the iterons. A replication property of the miniplasmid derivative pYH174B is partially explicable according to this concept. The miniplasmid pYH174B contains PAS instead of *ssiB* on the *r*-strand, on which the PAS-dependent primosome can be formed. This primosome would move along the *r*-strand template in the 5′ to 3′ direction to initiate synthesis of multiple primers for the DNA chain elongation away from the iterons. This 5′ to 3′ helicase action of the primosome might help in destabilizing the duplex barriers such as the GC-rich region and/or nucleoprotein complex so that some replication forks can pass through them.

Similar models have been proposed by Scherzinger *et al.* (23) and Zhou *et al.* (28). Especially, Zhou *et al.* (36) supported this model by the experiment of *in vitro* probing the activation of plasmid R1162 origin with Tus, the *E.coli* anti-helicase protein. However, electron microscopy analysis of the replication intermediates has shown that the replication of RSF1010 proceeds either bi- or unidirectionally. It is not resolved yet what mechanism and which critical stage in the initiation processes are responsible for making a decision as to whether the replication of RSF1010 proceeds bi- or unidirectionally.

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