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Functional division and reconstruction of a plasmid replication origin: Molecular dissection of the *oriV* of the broad-host-range plasmid RSF1010

(single-stranded DNA initiation/primosome assembly site/DNA primase/*dnaG*/replication fork)

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ABSTRACT **Two single-stranded DNA initiation signals (designated *ssi*) present in the origin of vegetative DNA replication (*oriV*) of the broad-host-range plasmid RSF1010 are essential for the priming of replication of each complementary DNA strand of this plasmid in *Escherichia coli*. Each of the RSF1010 *ssi* signals, *ssiA* and *ssiB*, could be replaced by a primosome assembly site from plasmid pACY184 or from bacteriophage φX174. In these chimeric origins, replication of the strand complementary to that containing the primosome assembly site was no longer dependent on the RSF1010 primase, protein RepB', but required the *E. coli* primase, DnaG. If both *ssiA* and *ssiB* sites of RSF1010 were replaced by primosome assembly sites, protein RepB' was no longer essential for the replication at this origin, whereas proteins RepA and RepC of RSF1010 were still required. These results strongly suggest that the two *ssi* sites and the RepB' protein actually direct the priming of DNA synthesis in the replication of RSF1010, and the proteins RepA and RepC are involved in the prepriming events—i.e., the opening of the DNA duplex at *oriV*. It is evident that the origin of RSF1010 can be separated into three functional domains and reconstructed by replacing the *ssi* sites with heterologous elements.**

Cis-acting elements that ensure replicon identity and are essential for the initiation of vegetative DNA replication have been called replication origins (*oriV*). In some cases, with the aid of reconstituted systems composed of purified replication proteins, it has been shown *in vitro* that target sequences recognized by specific initiator proteins and helicases exist and that melting of duplex DNA takes place at *oriV* upon initiation (1, 2). On the other hand, relatively little is known about the mechanisms that introduce priming enzymes onto each template strand and initiate DNA strand synthesis with the concomitant establishment of the replication forks. There seem to be unique mechanisms responsible for specific initiation events on each strand of a replicating DNA molecule (3–6).

RSF1010 is an 8684-base-pair (bp) incompatibility group IncQ plasmid able to replicate in a wide range of Gram-negative host bacteria (7, 8). It is very similar to another IncQ plasmid R1162, particularly in the sequence and organization of its *oriV* (3, 9). Although not self-transmissible, it is efficiently mobilized if transfer functions are supplied by a coexisting plasmid, particularly an IncP group plasmid, into Gram-negative bacteria (10) or even into plant cells from *Agrobacterium* species (11).

The replication of RSF1010 proceeds either bi- or unidirectionally from a unique origin located at nucleotides 2347–2742 and is independent of the functions of the *E. coli* gene products of *dnaA*, *-B*, *-C*, *-G*, *-T*, and *rpoB* (12–16). Instead three plasmid-specified proteins, RepA, RepB', and RepC, function as specific DNA helicase, primase, and initiator protein, respectively, and are essential for RSF1010 replication (15, 16). The RepB' protein is a novel type of DNA primase by the following criteria. It does not require ribonucleotide triphosphates for the priming reaction (15), and it requires specific sites *ssiA* and *ssiB* (corresponding to *oriR* and *oriL* in ref. 8, respectively), located on each of the complementary strands of DNA within the *oriV* RSF1010, as the exclusive templates for initiation. When cloned into single-stranded phage vectors, these *ssi* sequences can direct the priming of DNA synthesis in the presence of RepB' protein *in vivo* (4) and *in vitro* (8, 15).

In this report we show that both *ssiA* and *ssiB* of RSF1010 are essential for the replication of this plasmid and constitute unique functional elements of its *oriV*. Each of the *ssi* sites of RSF1010 may be substituted *in vivo* by the primosome assembly site of bacteriophage φX174 or of plasmid pACYC-184 (also known as the n' site)—the *ssi* signals that introduce the primosome onto the template DNA strand. Such substitution renders the replication of the affected strand dependent on the function of the DnaG primase.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The strains were derivatives of *E. coli* K-12, JM109 (17), and BW86 [*dnaG3 leu thyA deoB rpsL* ColI^r Δ(*chlA-uvrB*)] (18). Plasmid RSF1010, helper plasmid pMMB2 (ColD-based recombinant plasmid carrying *repA*, *repB'*, and *repC* of RSF1010), and deletion derivatives pMMB2Δ5, pMMB2Δ67, pMMB2ΔAE, and pMMB2ΔSS have been described (4, 16). For construction of RSF1010 miniplasmids, we first constructed a recombinant bacteriophage, M13mp19/YH101, which contains the coding region of β-lactamase from pBR322 and a 444-bp *oriV* segment from RSF1010 [nucleotides 2335–2778 (8)] consisting of *ssiA*, *ssiB*, and the *inc* repeats. A 1574-bp fragment containing the *oriV* and *bla* can be excised as an *Eco*RI fragment from the replicative form (RF) DNA of M13mp19/YH101 (Fig. 1). The mini-RSF1010 plasmid, pYH101, was obtained by self-ligation of this *Eco*RI fragment. An *Eco*RV site was introduced between the *inc* and *ssi* domains of this plasmid, at nucleotide 2568, as a G·C → T·A transversion, by oligonucleotide-directed mutagenesis, and a *Sac* I linker was introduced into the *Eco*47III site between *ssiA* and *ssiB* to produce M13mp19/YH101VS. Twelve recombinant plasmids con-

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Abbreviations: Amp, ampicillin; Amp^R, Amp resistance; RF, replicative form.

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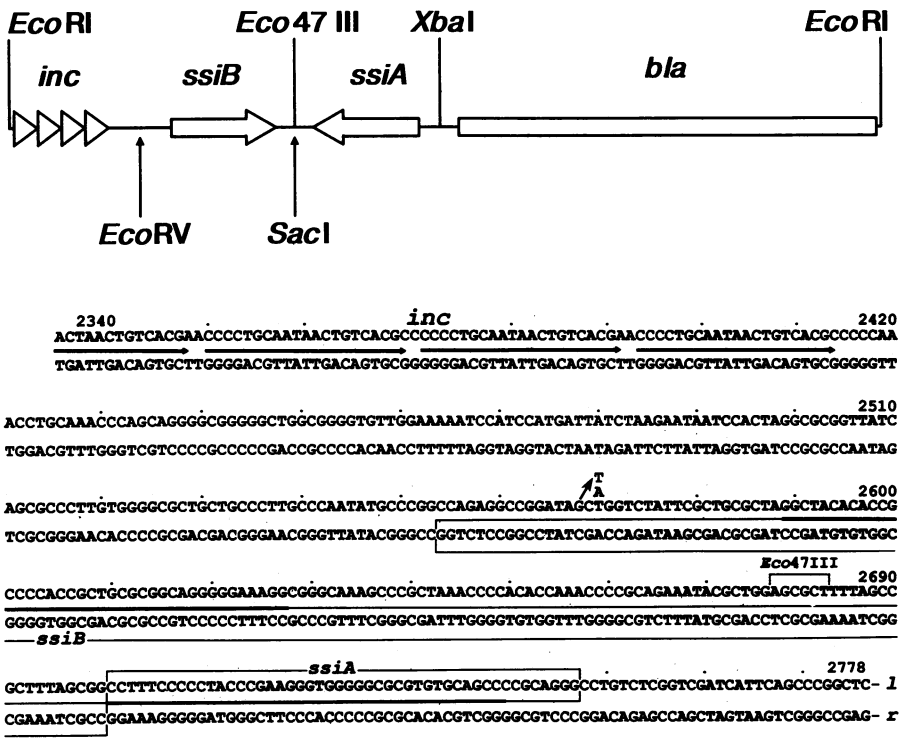

FIG. 1. (*Upper*) Physical map of the miniplasmid pYH101 linearized at the unique *Eco*RI site. Open arrows represent *ssi* sites on the *l* and *r* strand, respectively; the direction of the arrows indicates the functional orientation of the *ssi* signals. ▷, *inc* repeats; □, β-lactamase gene from pBR322. The figure is not drawn to scale. (*Lower*) Nucleotide sequence of RSF1010 *oriV* region present in pYH101. Sequences of *ssiA* and *ssiB* are boxed-in. Thick lines represent regions of high homology between *ssiA* and *ssiB*. Arrows indicate the *inc* repeats. The G·C base pair converted to T·A by site-directed mutagenesis and the *Eco*47III site used to insert the *Sac* I linker to construct pYH101VS are also indicated.

taining deletion, inversion, or substitution of either of the *ssi* signals were derived from M13mp19/YH101VS (Fig. 2). The primosome assembly site of plasmid pACYC184 was cloned previously as a 119-bp fragment into the *Sma* I site of M13Δ*lac184*, an ori_c-defective derivative of M13 phage containing *lacZ'* of M13mp18 (5). The primosome assembly site of bacteriophage φX174 was cloned as 103-nucleotide *Acc* II fragment L containing ori_c into the *Sma* I site of M13Δ*lac183*, identical to M13Δ*lac184* (19). The DNA fragments containing these primosome assembly sites were excised from the RF DNA of appropriate bacteriophages with *Sac* I, *Xba* I, or *Hinc*II. Deletions or replacements of *ssiA* and/or *ssiB* were obtained by cleavage of RF DNA of M13mp19/YH101VS at *Sac* I, *Eco*RV, or *Xba* I sites followed by ligation. Miniplasmid derivatives containing the *ssi* signals in reverse orientation were constructed by replacement of the DNA fragment specific for each signal with the corresponding fragment derived from M13Δ*lac110/ssiA* or -*ssiB* (3).

Plasmid pTK2 was constructed by insertion of the 4296-bp *Sau*3AI–*Eco*RI fragment of RSF1010 (base pairs 4384–8680) into the *Bam*HI–*Eco*RI site of the plasmid pHSG399, a chloramphenicol-resistance derivative of pUC19 (20). In this plasmid the expression of *repA*, *repB'*, and *repC* is under the control of the *lac* promoter. It provided the Rep functions for the replication of RSF1010 miniplasmids.

DNA Manipulation. Restriction endonucleases and DNA-modifying enzymes were from Takara Shuzo (Kyoto). [α-^{32}P]dCTP was from Amersham. Plasmid DNA was extracted by the alkaline denaturation procedure (21). Oligonucleotide-directed mutagenesis was done with a kit from Amersham. Base substitutions and deletions as well as substitutions of *ssi* signals were confirmed by restriction analysis and nucleotide sequence determination (22). Transformation was done by the method of Chung *et al.* (23). Concentrations of antibiotics in selective media were: ampicillin (Amp), 50 μg/ml; chloramphenicol, 30 μg/ml, and kanamycin, 100 μg/ml.

RESULTS

Replication Activities of Recombinant RSF1010 Origins. Miniplasmid pYH101 and its derivatives, presented in Fig. 2, were transferred by transformation into the strain JM109 harboring the plasmid pMMB2 which supplied the Rep functions of RSF1010. Miniplasmid derivatives lacking *ssiA* and/or *ssiB* (pYH101DA, pYH101DB, and pYH101DAB) or containing one of the *ssi* signals in the reverse orientation (pYH101RA and pYH101RB) could not replicate (Fig. 2). These findings indicate that both of the two *ssi* signals in the proper orientation are essential for the replication of RSF1010 *oriV*. This is consistent with the results obtained *in vitro* with RSF1010 (8) and with R1162 plasmid (24).

If primosome assembly sites from φX174 or pACYC184 were substituted for *ssiA* or *ssiB* (pYH174A, pYH174B, and pYH184A), replication of the recombinant miniplasmids was restored. Moreover, miniplasmid pYH174A184B, in which both *ssi* signals were replaced by primosome assembly sites, could replicate in the presence of Rep proteins of RSF1010. However, recombinant plasmids pYH174ADB, pYH174ARB, and pYH174ARA could not replicate, indicating that each of the DNA strands requires a priming signal, either an *ssi* site or a primosome assembly site, and that one primosome could not induce the formation of a replisome.

Requirements of Rep Functions for the Replication of Miniplasmids with Substitutions of *ssi* Signals. DNAs of the plasmid pYH101 and of its derivatives containing substitutions of the *ssi* signals were transformed into the strain JM109 harboring pMMB2 or one of its deletion derivatives as helper plasmids, and the number of transformants was scored. The replication of all miniplasmids required RepA and RepC proteins (Table 1). They also required the RepB' primase

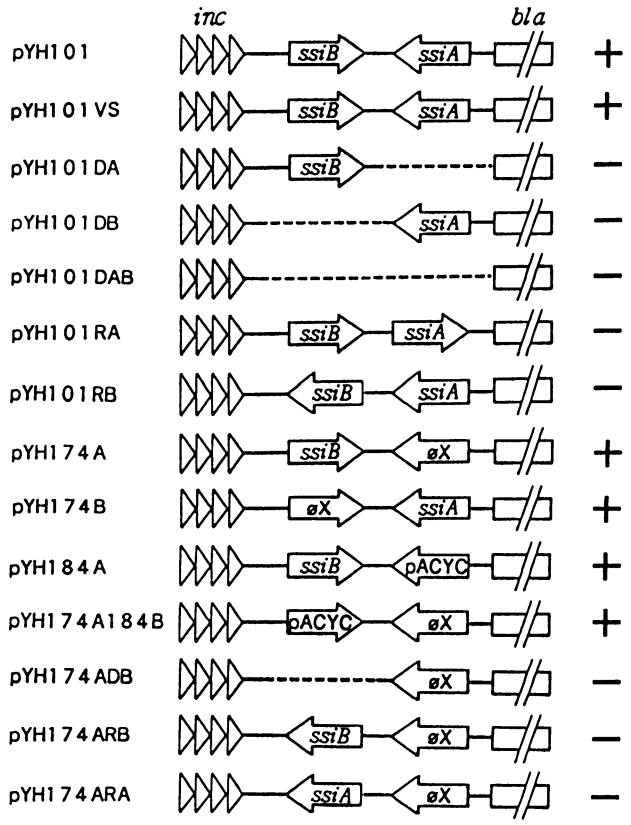

FIG. 2. Chimeric origins constructed from the plasmid pYH101VS and their ability to replicate in *E. coli* in the presence of RSF1010 Rep proteins. Broken lines indicate the areas of deletion. T4 DNA ligase was used to self-ligate 1.875 ng of linear DNA of each miniplasmid prepared by *Eco*RI cleavage of the recombinant M13mp19 RF DNA containing the corresponding miniplasmid segment. The ligated mixture was used to transform strain JM109 harboring the pMMB2 helper plasmid. Amp^R colonies were selected and screened for plasmid content. The presence (+) or absence (−) of stable transformants is indicated for each class of miniplasmid derivatives.

except for the miniplasmid pYH174A184B in which both *ssi* sites were substituted by primosome assembly sites. These results indicate that the RepB′ protein-dependent priming reaction may be separated from processes that are considered to occur earlier than the priming in the RSF1010 replication and are dependent on the RepA helicase and RepC initiator protein.

Copy Number of Miniplasmids with Substitutions of *ssi* Signals. Relative copy numbers of miniplasmids with different substitutions of *ssi* signals were determined by estimation of the single-cell Amp resistance (Amp^R) in the strain JM109 in the presence of coresident helper plasmid pMMB2 or pMMB2Δ67. Miniplasmids pYH174B and pYH184A were maintained at the same copy number as pYH101VS, whereas pYH174A and the plasmid with both *ssi* signals replaced had

Table 2. Relative copy number of *ssi*-replaced miniplasmid derivatives

Miniplasmid	*ssi* site A	*ssi* site B	Helper plasmid	Amp LD_{50}, μg/ml	Relative copy number, %
pYH101	+	+	pMMB2	1122.0	100.0
pYH101VS	+	+	pMMB2	812.8	72.4
pYH174A	øX	+	pMMB2	426.6	38.0
pYH174B	+	øX	pMMB2	831.8	74.1
pYH184A	pACYC	+	pMMB2	841.4	75.0
pYH174A 184B	øX	pACYC	pMMB2	309.0	27.5
	øX	pACYC	pMMB2 Δ67	302.0	26.9

Relative copy numbers were estimated by single-cell Amp^R levels. +, Original RSF1010-specific *ssi* signal; øX, the primosome assembly site from øX174; pACYC, the primosome assembly site from pACYC184. Amp LD_{50} was determined by the method of Nordström *et al.* (25). Results are mean values of two determinations with independent clones.

copy numbers reduced by a factor of 2 (Table 2). It seems that replication efficiency of the recombinant *oriV*s is influenced by the combination of *ssi* signal and the primosome assembly site. Of particular interest, however, was the fact that the copy number of plasmid pYH174A184B, with both *ssi* signals replaced by primosome assembly sites, was independent of the presence of a functional *repB′* gene. Obviously a different primase, most likely the product of *dnaG* was involved in its initiation. These facts taken together with the results shown in Table 1 indicate that *ssi* signals are the sites of RepB′ primase action in the initiation event of RSF1010 replication.

Effect of DnaG Function on the Replication of Miniplasmids with Substitution of *ssi* Signals. If the priming of DNA strand elongation in the recombinant *oriV*, in which an *ssi* signal of RSF1010 is substituted by a primosome assembly site, is indeed directed by the primosome, then the replication of that strand should be dependent on DnaG primase. In a *dnaG* conditional mutant harboring such a recombinant miniplasmid, the accumulation of the strand primed at the RSF1010 *ssi* signal should occur at nonpermissive conditions. To test this, DNAs of pYH101VS, pYH174A, pYH174B, and pYH174A184B were transformed into the strain BW86 (a *dnaG3* mutant, specifying temperature-sensitive primase) carrying pTK2 as a helper plasmid supplying the Rep functions of RSF1010. After incubation at permissive and nonpermissive temperatures, the single-stranded replication products were resolved by agarose gel electrophoresis and detected by hybridization to specific single-stranded probes.

Very little of single-stranded DNA was detected at either permissive or nonpermissive temperature in the cells containing pYH101VS (Fig. 3, lane 1). Cells carrying pYH174A or pYH174B exhibited the accumulation of those strands that replicated under the direction of the RSF1010 *ssi* signal (Fig. 3, lanes 2 and 3). The accumulation was detectable at the permissive temperature but became prominent upon incubation at 41°C. These results indicate that primosome assembly sites of φX174, introduced in place of the RSF1010-specific

Table 1. Requirements of Rep functions for the replication of miniplasmid derivatives

Helper plasmid	Rep function A	Rep function B	Rep function C	Miniplasmid transformants into JM109, no. per μg of DNA: pYH101	pYH101VS	pYH174A	pYH174B	pYH184A	pYH174A184B
pMMB2	+	+	+	3.9×10^5	4.4×10^5	3.1×10^5	2.9×10^5	2.2×10^5	3.1×10^5
pMMB2Δ5	−	−	+	<276	<276	<276	<276	<276	<276
pMMB2Δ67	+	−	+	<276	<276	<276	<276	<276	2.5×10^5
pMMB2ΔAE	−	+	−	<276	<276	<276	<276	<276	<276
pMMB2ΔSS	−	−	−	<276	<276	<276	<276	<276	<276

Preparation of miniplasmid pYH101 and derivative DNAs containing substitutions of *ssi* signals was described in the legend to Fig. 2. Results are mean values of two determinations.

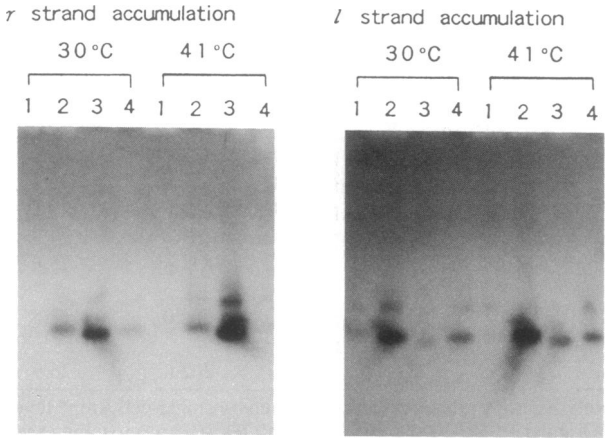

FIG. 3. Accumulation of specific single strands upon replication of miniplasmids with chimeric $oriV_{RSF1010}$ in a *dnaG3* mutant. *E. coli* BW86, harboring pTK2 helper plasmid and miniplasmid derivatives with *ssi* replacements, were grown at 30°C to an OD_{610} = 0.5. Isopropyl β-D-theogalactopyranoside was then added (0.2 mM), and the culture was divided in two portions. One was incubated at 30°C and the other at 41°C for 1.5 hr. Plasmid DNA was then extracted and subjected to electrophoresis on 1% agarose gels. DNA bands were transferred to nitrocellulose filters without alkaline denaturation. Southern hybridization was performed with strand-specific probes. ^{32}P-labeled (−)-strands f1R199/YH101DAB*r* and f1R199/YH101DAB*l*, which contain each of the DNA strands of pYH101DAB were used to detect the *r* and *l* strand, respectively. Lanes: 1, pYH101VS; 2, pYH174A; 3, pYH174B; 4, pYH174A184B.

ssi signals, actually directed the priming of the individual DNA strands in a DnaG-dependent process. It is possible that the synthesis of each complementary strand was independent of each other and was preceded by a strand-displacement mechanism. It should be noted that accumulation of a small amount of the *l* strand has occurred also in the case of the plasmid pYH174A184B at 41°C. Although this result was reproducible, the reason for this is at present unknown. It is possible that the DnaG3 primase is inactive at 41°C for the *ssi* signal of φX174 but weakly active for the *ssi* signal of pACYC184. The fact that the sequences of these two signals are significantly different lends some support to this argument. Moreover, miniplasmids with the wild-type RSF1010 origin seem to possess a system ensuring a balanced replication of both DNA strands since no biased strand synthesis is observed with pYH101VS (Fig. 3). This mechanism may be affected by replacement of *ssi* signals. It is possible, therefore, that the plasmid pYH174A184B cannot synthesize both complementary strands in a concerted manner. Since a strand displacement mechanism was implicated in the replication of RSF1010 (15), accumulation of one of the strands in a chimeric origin may be possible.

DISCUSSION

Many bacterial plasmids of different incompatibility groups have been shown to possess *ssi* signals located in close proximity of the *oriV*s. These include ColE1, pBR322, pACYC-177, and pACYC184 derived from p15A; CloDF13, F, R6K, R1, and pSY343 derived from R1, R100; and ColE2 (5, 6, 26–34). Plasmid pSM32 (a mini-ColIb plasmid) also has an *ssi* signal (unpublished). The priming mechanisms at the *ssi* signals in these plasmids include primosome-dependent priming (φX174 type), DnaG-dependent priming (G4 type), and ABC-primosome-dependent priming (36). Although the primosome assembly sites of pBR322 are dispensable for the vegetative replication of its DNA *in vivo* (32), at least one of them is required for synthesis of the lagging strand *in vitro* (33). Also, in a replication system reconstituted from purified components, initiation of the lagging strand of pBR322 is totally dependent on the primosome proteins (35). In the replication of plasmid R1, the leading-strand synthesis is primed by DnaG primase alone at the *ssi* site located downstream of *oriR* (34). Judging by the mode of function and the location of *ssi* sites in different plasmids, it seems that these signals contribute to the specificity of plasmid-directed initiation events and to the establishment of the replication forks.

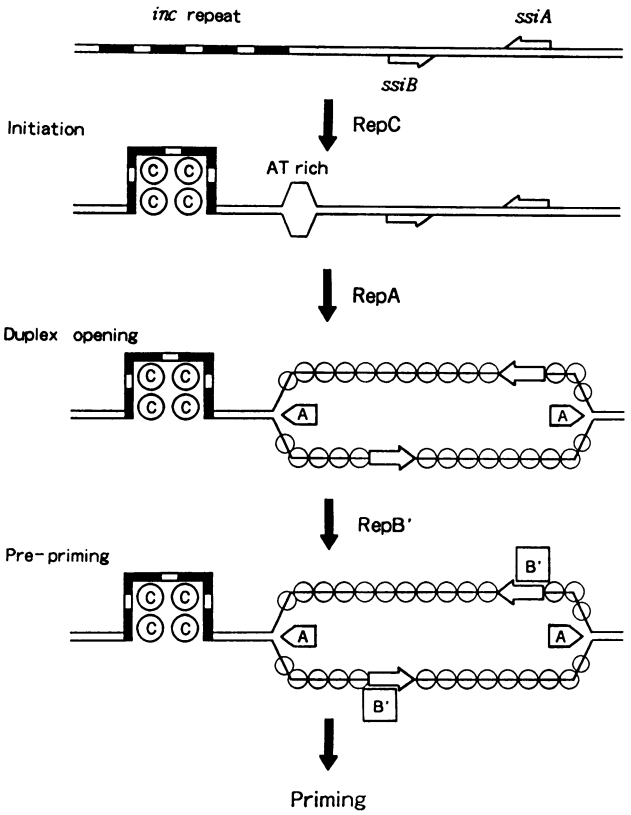

FIG. 4. Schematic representation of the proposed model for the initiation of DNA replication in RSF1010. The presumed chronology of events is as follows: (*i*) RepC protein binds to the *inc* repeats; specific loop structures proposed by Haring and Scherzinger (15) are not shown. (*ii*) RepA helicase binds, presumably, to the A+T-rich region and unwinds the duplex to expose the *ssi* sites. (*iii*) Single-stranded regions are stabilized by the single-stranded DNA binding protein (○), and RepB' primase forms the priming complexes with the *ssi* sites.

We have shown that the *ssi* sites of RSF1010 constitute a discrete class of initiation signals that differs from the other types of initiation signals described to date. Both *ssiA* and *ssiB* sites are essential for the function of $oriV_{RSF1010}$ *in vivo*, they are required as priming signals on each strand of the plasmid, and they are recognized by the plasmid-specific primase, RepB', and not by host priming protein DnaG. On the basis of the results presented in this paper, we can recognize and locate three distinct cis-acting functional domains in the $oriV_{RSF1010}$ region, the *inc* repeats, *ssiA*, and *ssiB*. Furthermore, we have shown that heterologous initiation sites, such as the primosome assembly site of the phage φX174, may be substituted for the RSF1010 *ssi* signals. These substitutions result in origins still functional *in vivo*, but with altered specificity in respect to the recognition by the priming enzyme. Initiation at such chimeric origins still depends on the RSF1010 initiator protein, RepC, and the RSF1010-encoded helicase, RepA. However, the priming at the φX174 or PACYC184 primosome assembly site is now dependent on the host primase, DnaG.

Domains of the RSF1010 *oriV* with their specific functions and the chronology of events taking place during initiation could be envisaged as in the scheme presented in Fig. 4. Since *ssiA* and *ssiB* are active on single-stranded DNA (3, 8), we suggest that the prepriming events of initiation at the $oriV_{RSF1010}$ involve origin recognition by the RepC protein and DNA duplex unwinding by the RepA helicase. Functionality of the chimeric origins of RSF1010 constructed in this study suggests that priming at the *ssiA* or *ssiB* site is independent of the prepriming events of origin recognition and opening of the DNA duplex that involve RepC and RepA proteins. The requirement of these proteins for the function of all chimeric *oriV*s, including those where both priming sites were substituted by primosome assembly sites (Table 2), is consistent with this model. This consistency also indicates that the events occurring in an *in vitro* replication system, reconstructed from purified components specific for RSF1010 initiation (15, 16), closely mimic the initiation events taking place *in vivo*. Propagation of the RSF1010 replication fork is considered to involve the RepA helicase (13), and the replication of this plasmid possibly proceeds by a strand-displacement mechanism. In this respect, it may be of interest to establish whether G4-type *ssi* signals that do not introduce a primosome onto the DNA strand can substitute for the *ssiA* and *ssiB* in the RSF1010 *oriV*.

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