INITIATION MECHANISM OF DNA REPLICATION OF BROAD HOST-RANGE PLASMID RSF1010

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ABBREVIATIONS

A_{610}	absorbance at 610 nm
AGE	Agarose gel electrophoresis
Amp	Ampicilin
bp	base pair(s)
Cm	chloramphenicol
E. coli	*Escherichia coli*
Inc	incompatibility
IPTG	isopropyl-β-D-thiogaractopyranoside
kb	kilobase pair(s)
kDa	kilodalton
Km	kanamycin
LB	Luria Broth
LD_{50}	50 % lethal dose
MCS	multi clonig site
moi	multiplicity of infection
nt	nucleotide(s)
oriV	origin of vegetative DNA replication
ori_c	origin of complementary DNA strand synthesis
pfu	plaque-forming unit(s)
R	resistance
RF	replicative form
Sm	streptomycin
ssi	single-strand DNA initiation
SSI	phenotypic designation for *ssi*
Su	sulfonamide
Tc	Tetracyclin
Tn	transposon
X-gal	5-bromo-4-chioro-3-indolyl-β-D-garactopyranoside
[ ]	designates plasmid-carrier state

CONTENTS

INTRODUCTION

DNA replication is an essential phenomenon for cell division and most precisely regulated in its initiation process. Thus, the initiation of DNA replication is a key step for the regulation of cell division. Various biochemical and genetical studies on DNA replication have been done with phages and plasmids parastic on *E. coli* as model systems. However, the total mechanism by which DNA replication is controlled has not been elucidated yet.

Cis-acting elements that ensure replicon identity and are essential for the initiation of DNA replication are called replication origins, or *oris*. The specificity of replication depends on the structure of the replication origin which contains critical sequences for recognition by the *trans*-acting factors such as replicon-specific initiator proteins. In some replication origins such as *E. coli* chromosomal origin (*oriC*) and origin of bacteriophage λ, the molecular mechanisms of the initiation event have been elucidated with the aid of *in vitro* reconstituted systems composed of purified replication factors (Bramhill and Kornberg 1988; Schnös *et al.* 1988). It has been shown that melting and unwinding of the duplex DNA take place by initiator proteins and DNA helicases in the initiation stage of DNA replication dependent on such replication origins. The unwinding process is also required for the initiation of DNA replication of some eukaryotic replicons such as SV40 and yeast ARS elements (Dean *et al.* 1987; Umek and Kowalski 1990). It is plausible that unwinding of the duplex DNA within the replication origins should be one of the essential process in the replication of most replicons which replicate via Cairns-type intermediate molecules. However, relatively little is known about the mechanism by which priming enzymes are introduced onto each single-stranded DNA generated by the unwinding process and DNA strand synthesis is initiated with the concomitant establishment of the replication forks. Each

replicon should possess unique systems to recruit priming enzymes and replication machineries efficiently.

Specific nucleotide sequences on the template strand DNA which direct introduction of the priming enzymes and initiation of DNA synthesis have been isolated from various replicons and they are called single-strand DNA initiation (*ssi*) signals (Ray *et al.* 1981; Sakai and Godson 1985). In *E. coli*, three types of *ssi* signals have been shown to direct the complementary strand DNA synthesis of the small single strand DNA phages, such as M13, G4 and ϕX174 (Kornberg and Baker 1991). In the complementary strand synthesis of M13 and G4, a unique primer RNA is synthesized at a defined region within ori_C by *E. coli* RNA polymerase and DnaG primase, respectively (Bouché *et al.* 1978; Rowen and Kornberg 1978). The ori_C regions include specific nucleotide sequences with potential secondary structures which are recognized by the priming proteins (Geider *et al.* 1978; Hiasa *et al.* 1989; Sakai *et al.* 1987).

In contrast to the simple system of M13 or G4, the complementary strand synthesis of ϕX174 phage is more complicated. It requires a multiprotein complex, primosome, containing *E. coli* proteins, DnaB, DnaC, DnaT (i), PriB (n), PriA (n'), PriC (n") in addition to DnaG primase (Lee *et al.* 1990; Masai and Arai 1988; Nurse *et al.* 1990). The primosome assembles at a specific nucleotide sequence in ori_C of ϕX174, then it moves along single strand DNA template in 5' to 3' direction to initiate synthesis of multiple

Table 1. *ssi* signals in various plasmid replicons

mode of the priming	plasmid replicon	reference
i) DnaG-dependent (G4) type	R1 (pSY343)	Bahk *et al.* 1988
	R100	Nomura *et al.* 1991
	ColIb (pSM32)	Tanaka *et al.* 1991
	R6K	Nomura et al. 1991
ii) primosome-dependent (ϕX174) type	ColE1 (pBR322)	Nomura and Ray 1980; Marians *et al.* 1982; van der Ende *et al.* 1983a
	F	Nomura et al. 1991
	ColE2	Nomura *et al.* 1991
	p15A (pACYC184)	Bahk *et al.* 1987
iii) ABC-primosome-dependent type	R6K	Nomura *et al.* 1991
		Nomura *et al.* 1991
iv) plasmid-specific type	RSF1010	this thesis

Only those which are located in close proximity to the *oriV*s are listed.

primers (Arai *et al.* 1981; Schlomai and Kornberg 1980). Primosome, or at least some of its components should direct the initiation of lagging strand synthesis during replication of chromosomal DNA (Baker *et al.* 1987; LeBowitz and McMacken 1986).

Many bacterial plasmids of different incompatibility groups also have been shown to possess *ssi* signals located in close proximity to the *oriV*s (Table 1). Although some of them are dispensable for the plasmid replication *in vivo*, most of these *ssi* signals have shown to be essential to maintain wild-type plasmid copy numbers (Masai and Arai 1989; van der Ende *et al.* 1983b; unpublished results). Furthermore, *in vitro* studies have shown that in plasmids such as pBR322 and R1, *ssi* signals are actually essential to initiate DNA replication (Masai and Arai 1988; Masai and Arai 1989; Minden and Marians 1985). Judging by the mode of function and the genomic location of *ssi* signals in various plasmids, it is conceivable that the *ssi* signals should contribute to the specificity of plasmid-directed initiation events and to establishment of the replication forks (Masai *et al.* 1990a; 1990b).

Plasmid RSF1010 is a small multicopy plasmid belonging to the incompatibility group Q , and has remarkable property of replicating in a wide variety of Gram-negative bacteria (Grinter and Barth 1976; Guerry *et al.* 1974; Scholz *et al.* 1989). Thus, this plasmid provides a good model for studying the molecular basis of broad host-range. In order to elucidate the common mechanism which is essential and conserved in the initial stage of DNA replication among many bacterial species, it is impotant to understand how RSF1010 DNA replication is initiated.

In this thesis, the author elucidates the unique initiation mechanism of RSF1010 DNA replication. The aim of this study is to clone and detect *ssi* signals in RSF1010 and to interpret their biological significances in the initiation of the plasmid DNA replication.

Chapter 1. Isolation and functional analysis of the two single-strand DNA initiation signals in *oriV* of plasmid RSF1010

Signals for single strand DNA initiation (*ssi* signals) are defined as nucleotide sequences on single stranded DNA templates required for priming DNA synthesis. Origins for complementary DNA strand synthesis of single strand DNA phages are typical *ssi* signals (Kaguni and Ray, 1979; Strathearn *et al.*, 1984; Sakai *et al.*, 1987). Plasmids are also demonstrated to carry one or more *ssi* signals on their DNA (Zipursky and Marians, 1980; Bahk *et al.*, 1988). Although their roles in the plasmid DNA replication have not yet been specified, it is most probable that structual and functional characteristics of *ssi* signals are quite diverse among plasmid species. Each *ssi* signal of a type of plasmid may carry its own structure, function and biological implications.

Plasmid RSF1010 (Guerry *et al.*, 1974) is a 8.7-kb multicopy plasmid confering resistance to Sm and Su. It belongs to the incompatibility group Q (Grinter and Barth, 1976) and has the remarkable property of replicating in a wide variety of Gram-negative bacteria. In *E. coli*, this plasmid requires the functions of at least three plasmid-specified proteins, RepA, RepB' (formerly designated as RepB by Scherzinger *et al.*, 1984) and RepC, for its replication. The RepC protein has been demonstrated to act as a positive replication factor binding specifically to the direct repeats in the origin of RSF1010 replication (*oriV*) (Haring *et al.*, 1985). RepA and RepB' make RSF1010 independent of the host genes *dnaB*, *dnaC*, *dnaG*, and *rpoB* (Scholz *et al.*, 1985).

RSF1010 is identical, or at least very similar, to another IncQ plasmid R1162. The known nucleotide sequences of the two plasmids are almost the same. For R1162, it has been demonstrated that the DNA chain elongation is initiated at two separate positions within the *oriV* region (Lin and Meyer, 1987).

The author shows here that two *ssi* signals are found in separate DNA

strands within the *oriV* region of RSF1010, which correspond to two initiation positions in R1162.

MATERIALS AND METHODS

(a) Bacterial strains, plasmids and phages

Filamentous phage vector M13Δ*lac*110, which contains single strand circular DNA molecule as its genome, was constructed and kindly gifted by Dr. N. Nomura (personal communication). It was constructed by introducing the truncated *lac* operon from M13mp11 into M13ΔE101 (Fig. 1). M13ΔE101 was derived from filamentous phage M13 by deleting most of the M13 ori_C (Kim *et al.*, 1981). Phage M13Δ*lac*110 forms small and turbid

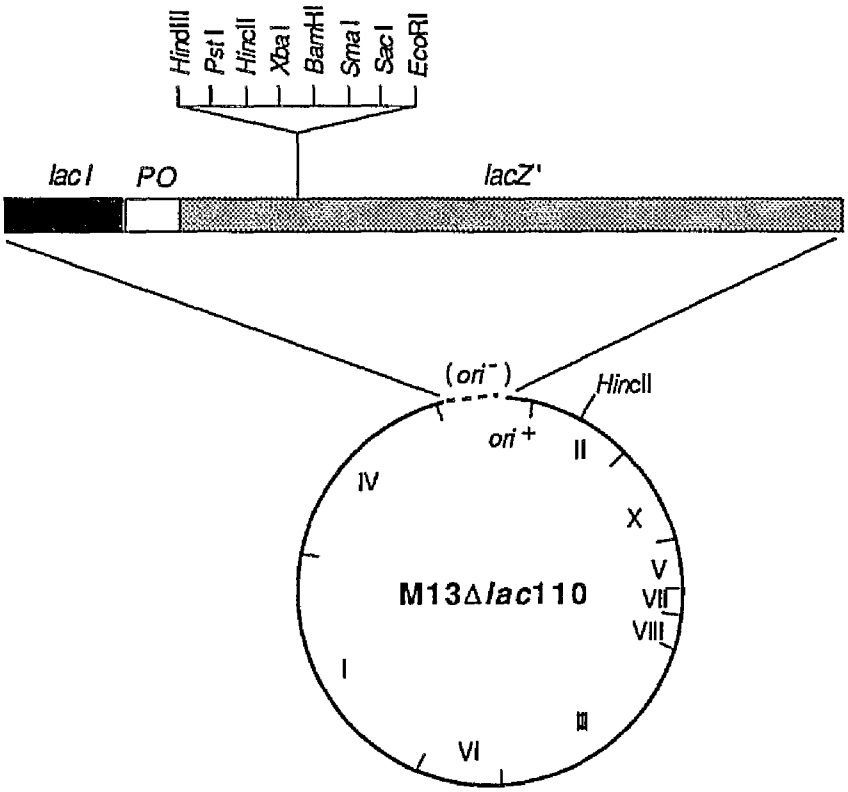


Fig. 1. Map of the M13Δ*lac*110. This filamentous phage vector has been constructed by Dr. N. Nomura as described in MATERIALS AND METHODS, section (a). Roman numerals indicate the positions of M13 genes. The position of the M13 viral strand replication origin is indicated by ori^+. Most of the complementary DNA strand origin is deleted as indicated by (ori^-). The deleted tract is replaced by the truncated *lacPO-lacZ* operon fragment from M13mp11 (Nomura, personal communication).

plaques. Upon insertion of an single strand DNA segment containing an *ssi* signal, the recombinant filamentous phage comes to form larger and clearer plaques. *E. coli* RL108 (F^+, Tc^R, *recA*56, *leu*, *met*, r_K^-, m_K^+), (Kim *et al.*, 1981) and RL108[RSF1010], which was constructed in our laboratory by introducing plasmid RSF1010 into strain RL108, were used as host bacteria for filamentous phage vector.

(b) Enzymes and reagents

Restriction endonucleases and DNA-modifying enzymes were purchased from Takara Shuzo, Toyobo, Bethesda Research Laboratories, Inc., Pharmacia and Boeringer Mannheim GmbH. [α-^{32}P]dCTP was purchased from Amersham International plc and ICN Radiochemicals.

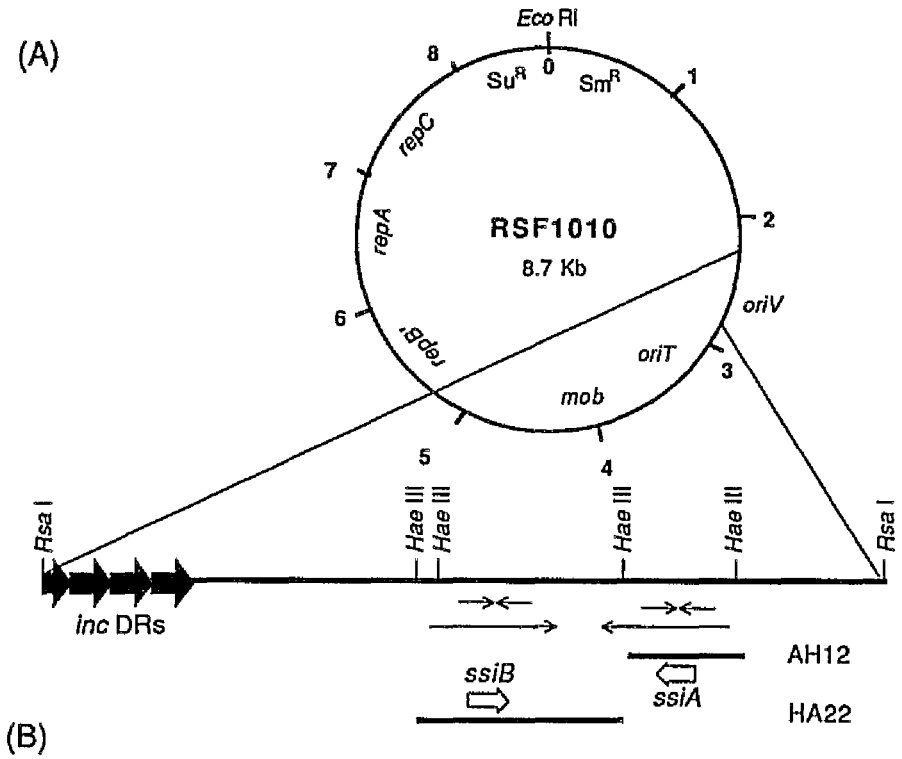
(c) Synthesis of replicative intermediates *in vivo*

The procedure was carried out essentially according to the method of Lambert *et al.* (1987). A 75 ml culture of RL108 or RL108 [RSF1010] grown in L broth medium at 37°C was pelleted when A_{610} of the culture reached to 0.6. The pelleted cells were resuspended in 3.1 ml fresh L broth medium and split into three aliquots. Each aliquot was infected with phage at an moi of 100, and incubated at 37°C. At each time indicated, an 0.25 ml of sample was removed, mixed with 0.4 ml of a saturated culture of HB101[pBR322], and DNA was isolated by the alkaline procedures of Birnboim and Doly (1979).

RESULTS

(a) Cloning of *ssi* signals of RSF1010

DNA of RSF1010 was cleaved separately with restriction endnuclease *Alu*I, *Eco*RI* or *Hae*III, followed by insertion into M13Δ*lac*110. Primary detection of SSI activity was done by plaque morphology procedures using M13Δ*lac*110 recombinant phage and *E. coli* RL108 host. But no larger plaques than those of M13Δ*lac*110 itself could be found. Then the author repeated the procedures using *E. coli* RL108[RSF1010] as a host bacterium instead of RL108. A number of M13Δ*lac*110 recombinant phages which formed larger and clearer plaques were obtained. The result indicated that the SSI activities on the cloned ss DNA segments compensated for the defect in



(B)

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5'ACTAACTGTCACGAACCCCTGCAATAACTGTCACGCCCCCCTGCAATAACTGTCACGAACCCCTGCAATAACTGTCACGCCCCCAA
3'TGATTGACAGTGCTTGGGGACGTTATTGACAGTGCGGGGGGACGTTATTGACAGTGCTTGGGGACGTTATTGACAGTGCGGGGGTT

ACCTGCAAACCCAGCAGGGGCGGGGGCTGGCGGGGTGTTGGAAAAATCCATCCATGATTATCTAAGAATAATCCACTAGGCGCGGTTATC
TGGACGTTTGGGTCGTCCCCGCCCCCGACCGCCCCACAACCTTTTTAGGTAGGTACTAATAGATTCTTATTAGGTGATCCGCGCCAATAG

AGCGCCCTTGTGGGGCGCTGCTGCCCTTGCCCAATATGCCCGGCCAGAGGCCGGATAGCTGGTCTATTCGCTGCGCTAGGCTACACACCG
TCGCGGGAACACCCCGCGACGACGGGAACGGGTTATTCGGGCCGGTCTCCGGCCTATCGACCAGATAAGCGACGCGATCCGATGTGTGGC

CCCCACCGCTGCGCGGCAGGGGGAAAGGCGGGCAAAGCCCGCTAAACCCCACACCAAACCCCGCAGAAATACGCTGGAGCGCTTTTAGCC
GGGGTGGCGACGCGCCGTCCCCCTTTCCGCCCGTTTCGGGCGATTTGGGGTGTGGTTTGGGGCGTCTTTATGCGACCTCGCGAAAATCGG
    HA22                    AH12
GCTTTAGCGGCCTTTCCCCCTACCCGAAGGGTGGGGGCGCGTGTGCAGCCCCGCAGGGCGTGTCTCGGTCGATCATTCAGCCCGGCTCAT
CGAAATCGCCGGAAAGGGGGATGGGCTTCCCACCCCCTCGCACACGTCGGGGCGTCCCGGACAGAGCCAGCTAGTAAGTCGGGCCGAGTA

CCTTCTGGCGTGGCGGCAGACCGAACAAGGCGCGGTCGTGGTCGCGTTCAAGGT
GGAAGACCGCACCGCCGTCTGGCTTGTTCCGCGCCAGCACCAGCGCAAGTTCCA
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Fig. 2. Location and sequence of the *oriV* region of RSF1010. (A) Maps of RSF1010 and of an *Rsa*I fragment containing the *oriV* region. Genetic map is based on results of Haring *et al.* (1985) and Derbyshire *et al.* (1987). Very thick solid arrows indicate three and a half direct repeats responsible for incompatibility. Thin facing arrows indicate inverted repeats. And thick open arrows indicate the directions of DNA chain elongation initiated from the two *ssi* signals. Regions *repA-C* encode functions required for replication; *mob* encodes mobilization functions; *oriT*, origin of conjugal DNA transfer.

(B) Nucleotide sequence of the *Rsa*I fragment illustrated above. The locations of segments AH12 and HA22, which are cloned on M13Δ*lac*110, are indicated by boxes. Direct repeats responsible for incompatibility are indicated by arrows, and correspond to 3 1/2 very thick arrows in way A.

ori_C of M13Δ*lac*110 caused by the deletion. DNAs isolated from some of the clones were cleaved separately with restriction endonuclease *Hae*III, *Rsa*I or *Sac*I, followed by insertion into M13Δ*lac*110. Through the subcloning, the author found out two *ssi* signals located in two single strand DNA segments, AH12 and HA22, which were 48 nt and 144 nt in size, respectively. When the nucleotide sequences of AH12 and HA22 were compared with those of the RSF1010 *oriV* region which was determined in our laboratry, these two single strand DNA segments were proved to be located side by side on separate DNA strands within the *oriV* region (Fig. 2). The nucleotide sequences of these two segments were identical to those of R1162 (Meyer *et al.*, 1985) except for 3 nt.

The two *ssi* signals, designated *ssiA* and *ssiB*, which were included in AH12 and HA22, respectively, were consequently located on the separate DNA strands (Fig. 2). Directions of possible DNA chain elongations under the control of the two *ssi* signals were opposite to each other, in such a way that the two DNA chain elongation apparatuses passed each other in the region between the two *ssi* signals.

(b) Functional activity of the *ssi* signals estimated by propagation of M13Δ*lac*110 recombinant phage

Growth of M13Δ*lac*110/AH12 and M13Δ*lac*110/HA22 phages in *E. coli* RL108 and RL108[RSF1010] was examined (Fig. 3). Both recombinant phages grew more efficiently than M13Δ*lac*110 in *E. coli* RL108[RSF1010]. On the contrary, their propagation in *E. coli* RL108 was as efficient as that of M13Δ*lac*110. These findings showed that coexistence with plasmid RSF1010 in the host cell was required for the SSI function. Since AH12 was more efficient than HA22, it is conceivable that the activity of *ssiA* was higher than that of *ssiB*.

(c) Analysis of RF DNA synthesis *in vivo* of M13Δ*lac*110 recombinant phage

To monitor more directly the effect of the RSF1010-specific SSI activities, synthesis of replicative form I (RFI) DNA was monitored after infection of M13Δ*lac*110 recombinant phage to *E. coli* RL108[RSF1010] and RL108. This experimental approach was chosen because the *ori* for complementary

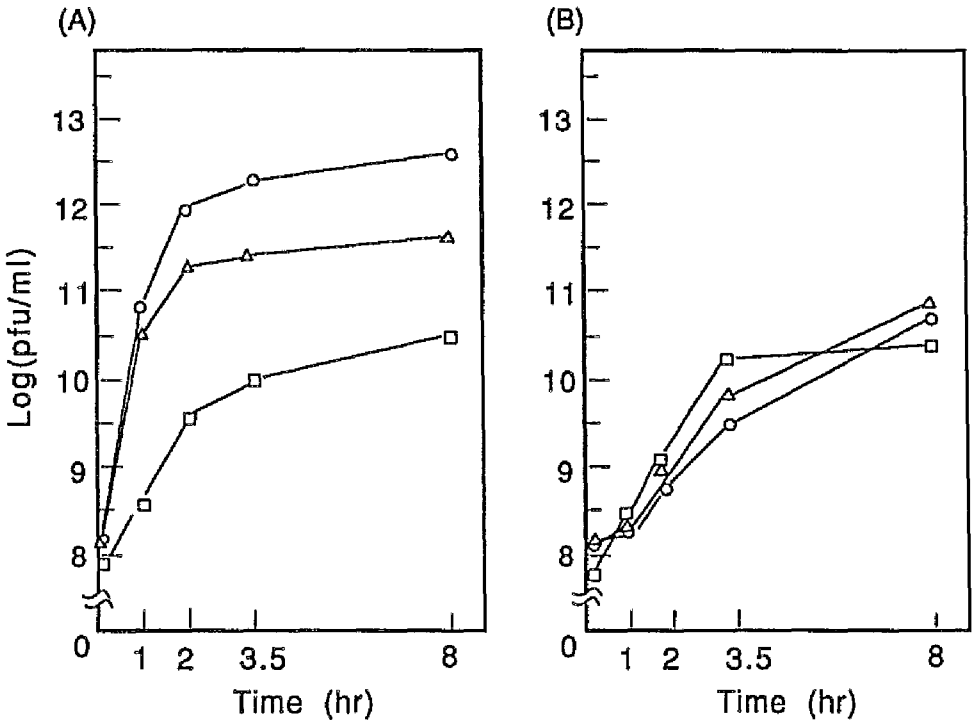

Fig. 3. Propagation of M13Δ*lac*110 recombinant phages. Host bacteria are *E. coli* RL108[RSF1010] in (A), and RL108 in (B). Bacterial growth and phage infection were carried out at 37°C in LB medium (pH 7.2) containing, per liter, 10 g of bactotryptone, 8 g of NaCl and 5 g of yeast extract, to which were added 5 ml of 20% glucose, 1 ml of 1 M $CaCl_2$ and 10 ml of 0.2% thymine after sterilization by autoclaving. Growth of phages M13Δ*lac*110; squares, M13Δ*lac*110/AH12; circles, and M13Δ*lac*110/HA22; triangles was examined.

strand synthesis was utilized both in the conversion of incoming viral ss DNA to the double-stranded RFI, and in the RF DNA replication (Hourcade and Dresler, 1978; Staudenbauer *et al.*, 1978). Therefore, initial accumulation of RFI DNA should be a measure of the activity of a cloned *ssi* signal *in vivo*. Accumulation of the RFI DNA was monitored in the course of infection with M13Δ*lac*110 recombinant phage (Fig. 4). The extent of RFI DNA accumulation was positively consistent with that of propagation of the corresponding M13Δ*lac*110 recombinant phage. These results indicated that both of *ssiA* and *ssiB* possessed RSF1010-specific SSI activities, and that the activity of *ssiA* was higher than that of *ssiB*.

DISCUSSION

Specific stem-loop structures were demonstrated to be actually plausible in the *ori* regions of some replicons (Tomizawa and Itoh, 1981; Lacatena and Cesareni, 1981; Ueda *et al.*, 1985). In the case of *ssiA* and *ssiB*, both of the signals involve stretches of 40-nt homologous to each other. This region contains a 9-nt inverted repeat, and a sequence homologous to a stretch of 7-nt, GAAGCGG, which is known as the conserved part of the n' protein recognition sites (van der Ende *et al.*, 1983a). Potential secondary structures of the 40-nt stretches are shown in Fig. 5. The 40-nt stretch conserved in both *ssiA* and *ssiB* seems to play the major role in the SSI function.

The findings presented here indicate that functions of *ssiA* and *ssiB* are independent of each other. They are located within the *oriV* of RSF1010 in the opposite priming direction. Since these signals require some factors supplied in *trans* by RSF1010 for their normal functions, they are RSF1010-

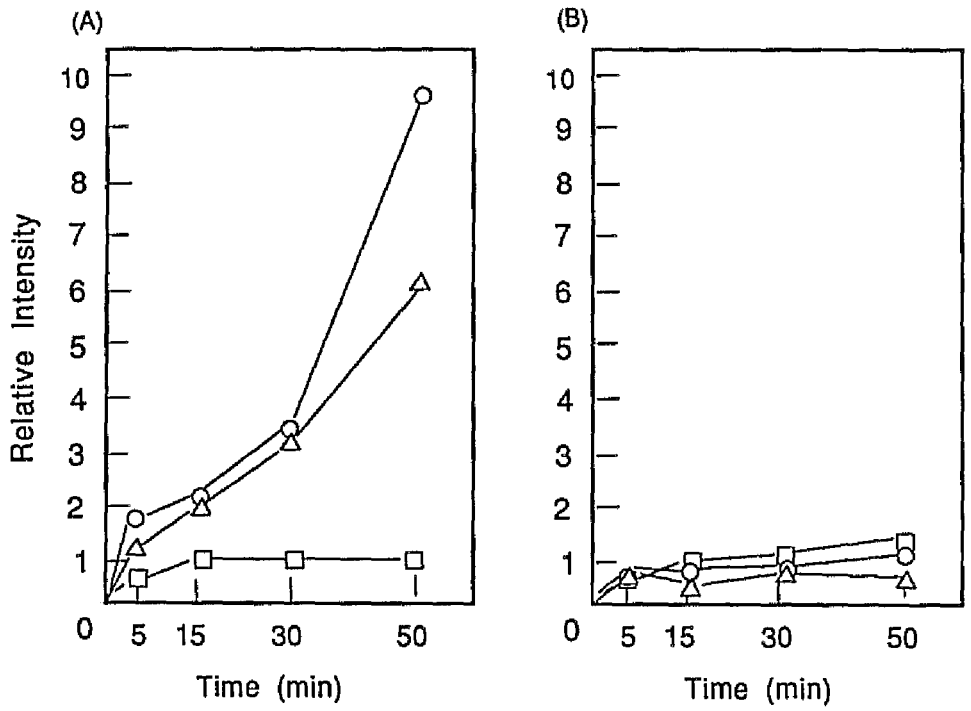

Fig. 4. Analysis of RFI DNA synthesis. Host bacteria are *E. coli* RL108[RSF1010] in (A) and RL108 in (B). DNA samples were run on 1% agarose gels, then Southern hybridization was performed using multiprimed M13 RF [^{32}P]DNA as a probe. Diagrams quantitate relative intensity of densitometric scannig of RFI in each lane of the autoradiograms. Differences in DNA recovery were corrected, as based on the relative recovery rate of pBR322 DNA, which is estimated by densitometric sccanning of a photographic negatives of the agarose gels. Symbols used are: squares, M13Δ*lac*110; circles, M13Δ*lac*110/AH12; triangles, M13Δ*lac*110/HA22 (see also Fig. 3).

specific. Judging from high homology in DNA structure between RSF1010 and R1162, replication systems of these two plasmids should be quite similar. The nucleotide sequences and relative location of *ssiA* and *ssiB* correspond to those of the two initiation start positions in R1162, which have been demonstrated to be the actual *oriV* of R1162 by Lin and Meyer (1987) (Fig. 5). Therefore, *ssiA* and *ssiB*, in cooperation with each other, seem to function as the *oriV* of RSF1010 under a certain RSF1010-specific control.

This is the first case in which two *ssi* signals, or elements involving the *ssi* signals, combined head to head seem to function as an *ori* for vegetative DNA replication of a plasmid. Thus far, no similar situations with respect to *ssi*

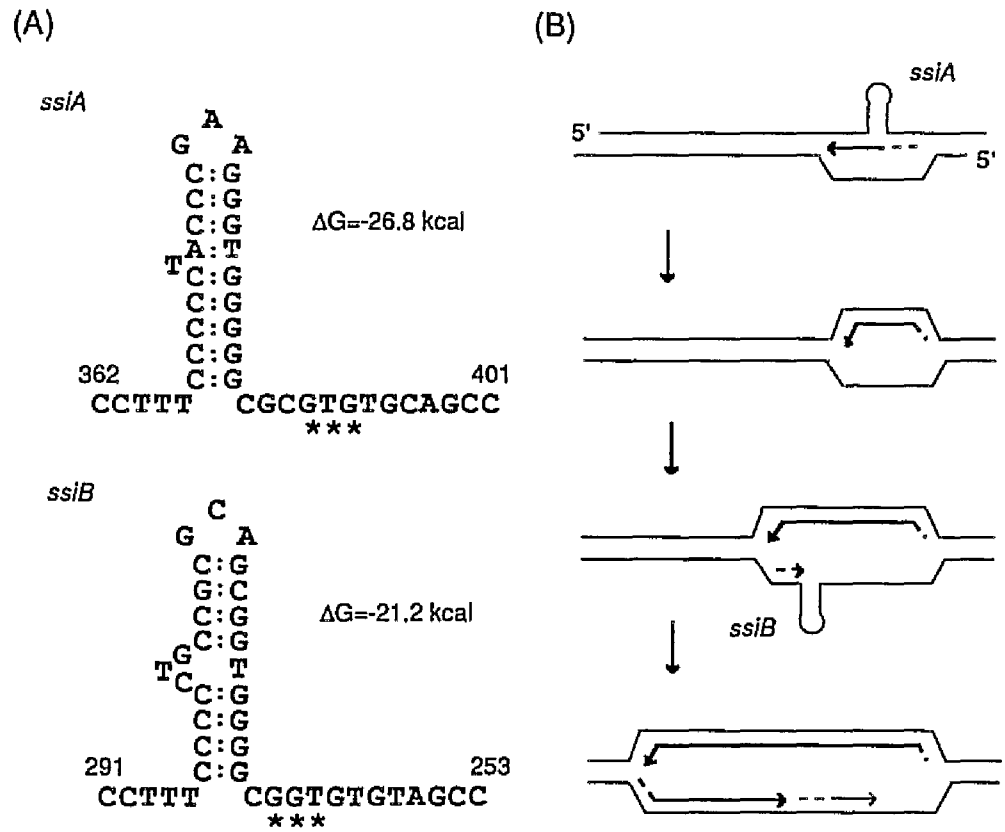

Fig. 5. Possible structure and function of the *ssi* signals. (A) Potential secondary structures of the 40 bp region of homology between *ssiA* and *ssiB*. Free-energy values for each stem-loop structures were calculated by the method of Tinoco *et al.* (1973). Asterisks indicate possible initiation positions in R1162, as proposed by Lin and Meyer (1987).

(B) Possible early events (progression is shown by downward arrows) in vegetative DNA replication of RSF1010, assuming that initial priming occurs exclusively at *ssiA*. Thick arrows represent frequent DNA chain elongation, while thin arrows represent less frequent ones.

signals have been found in the minimal essential region of *E. coli oriC*, and in the *ori* of ColE1 plasmid and its analogues. The function of *oriV* of RSF1010 may, therefore, be a unique one. The Rep proteins encoded by this plasmid have RSF1010-specific functions analogous to those of the *E. coli* gene products of *dnaB*, *dnaC* and *dnaG* (Scholz *et al.*, 1985). Some of the Rep proteins may play significant roles in functioning of *ssiA* and *ssiB*. It is very likely that these RSF1010-specific priming functions render the RSF1010 replication independent of the vital DNA priming functions of the host cell, and that this provides the basis of broad host-range properties of RSF1010.

Replication of RSF1010 has been demonstrated to proceed bidirectionally or unidirectionally by electron microscopic studies (de Graff *et al.*, 1978). In the case of unidirectional replication, the DNA chain elongation seemes to start exclusively from *ssiA*. It is conceivable that the difference in functional activities between ssiA and *ssiB* provides an initial event leading to determining which mode of DNA replication to be taken. To describe the mechanism in detail, functional relationship between the *ssi* signals and some other elements for initiating DNA replication, including the direct repeats responsible for the incompatibility, remains to be revealed.

SUMMARY

Two single strand DNA initiation (*ssi*) signals are found in the *oriV* region of broad host-range plasmid RSF1010, using a plaque assay system with a mutant M13 phage which lacks most part of the ori_C. These two signals, designated *ssiA* and *ssiB*, have RSF1010-specific properties, because they require one or more RSF1010-specific factors provided in *trans*. The functional activity of *ssiA* is higher than that of *ssiB*. The two signals are located on separate DNA strands, so that the DNA chain elongations initiated from them in the opposite directions may pass each other. It is conceivable that the signals, *ssiA* and *ssiB*, direct DNA priming functions at the initiation stage in vegetative DNA replication of RSF1010.

Chapter 2. Identification of the *trans*-acting factor required for the priming reaction dependent on *ssiA* and *ssiB*

The author has shown in the preceding chapter that RSF1010 contains two plasmid-specific *ssi* signals, *ssiA* and *ssiB*, on separate DNA strands within the *oriV* region, and that these two signals cloned in the ori_C-defective M13 mutant phage required, for their functional activities, one or more factors provided in *trans* by RSF1010. The locations of *ssiA* and *ssiB* are analogous to those of the two DNA initiation positions in R1162, which have been demonstrated to be the actual *oriV* of R1162 by Lin and Meyer (1987). Therefore, *ssiA* and *ssiB*, in cooperation with each other, seem to function as the *oriV* of RSF1010 under a certain RSF1010-specific control.

In this chapter, the author shows that *repB'* is required in *trans* for the functional activities of *ssiA* and *ssiB*.

MATERIALS AND METHODS

The filamentous phage vector M13Δ*lac*110 and its recombinants, M13Δ*lac*110/AH12 and M13Δ*lac*110/HA22 which carry *ssiA* and *ssiB* respectively, have been described in Chapter 1. Plasmid pMMB2 and its derivatives, pMMB2Δ5, pMMB2Δ23 and pMMB2Δ67 have been described by Scherzinger *et al.* (1984). The recombinant plasmid pMMB2 carries *repA*, *repB'* and *repC*, genes of RSF1010 essential for its replication. Factors provided by these genes are essential for the establishment of the miniplasmid, pMMB12, containing the origin of RSF1010 replication (Scherzinger *et al.*, 1984). Two other derivatives of pMMB2, pMMB2ΔAE and pMMB2ΔSS, were constructed in this work by deleting the *Acc*I-*Eco*RI and *Sac*I-*Sac*I region, respectively. pMMB2ΔSS carried no DNA segment originating from the essential genes of RSF1010, and was used as a negative control. The physical and genetic map of pMMB2 and deletion map of its derivatives are

shown in Fig. 1.

E. coli JM109 (*recA*1, *endA*1, *gyrA*96, *thi*, *hsdR*17, *supE*44, *relA*1, λ⁻, Δ(*lac-proAB*), [F', *traD*36, *proAB*⁺, *lacI*ᵠZΔM15]) (Yanisch-Perron *et al.*, 1985) and JM109 harboring the pMMB2 series were used as host bacteria for M13Δ*lac*110 recombinant phages.

RESULTS

(a) propagation of M13Δ*lac*110 recombinant phages in the presence of pMMB2 derivatives

To identify the *trans*-acting factors for *ssiA* and *ssiB*, the propagation of phages M13Δ*lac*110/AH12 and M13Δ*lac*110/HA22 in JM109 harboring pMMB2 and a series of its derivatives was tested (Fig. 2). Growth of the two M13Δ*lac*110 recombinant phages was efficient in the presence of pMMB2, pMMB2Δ23 and pMMB2ΔAE, but poor in the presence of pMMB2Δ5, pMMB2Δ67 and pMMB2ΔSS, and the recombinant phage propagation in the presence of pMMB2Δ23 or pMMB2ΔAE was as efficient as that in the presence of pMMB2. These findings indicated that M13Δ*lac*110/AH12 and

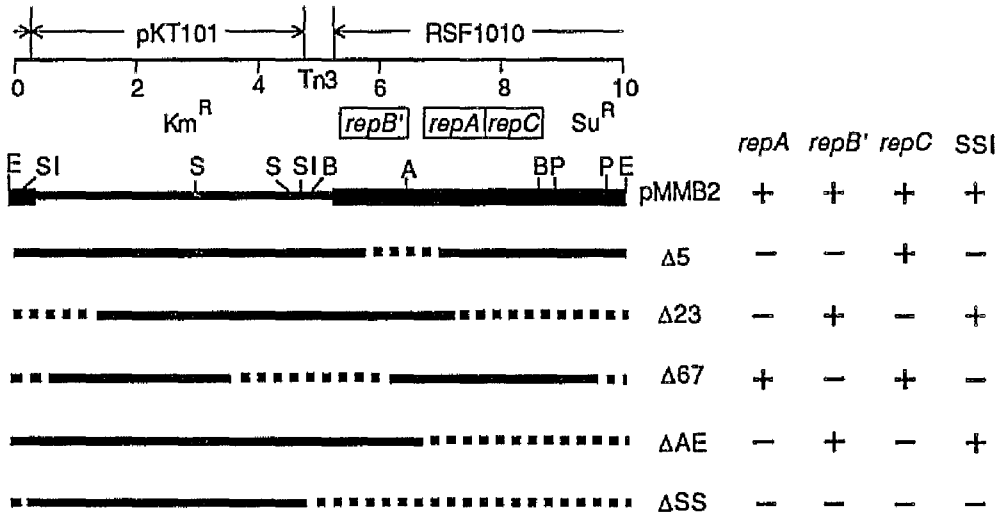

Fig. 1. Physical and genetic maps of pMMB2 and its deletion derivatives, and their effects on propagation of the M13Δ*lac*110 recombinant phages. Distances are given in kb from the unique *Eco*RI site. Restriction sites are indicated as follows: A, *Acc*I; B, *Bst*EII; E, *Eco*RI; P, *Pst*I; S, *Sma*I; SI, *Sac*I. The thick solid line denotes sequences derived from RSF1010. Open boxes represent the positions of the *rep* genes. Dashed lines represent areas of deletion (Scherzinger *et al.*, 1984; Scholz *et al.*, 1985). Intact (+) and defective (-) *rep* genes are indicated. The resulting SSI function is indicated as active (+) or inactive (-).

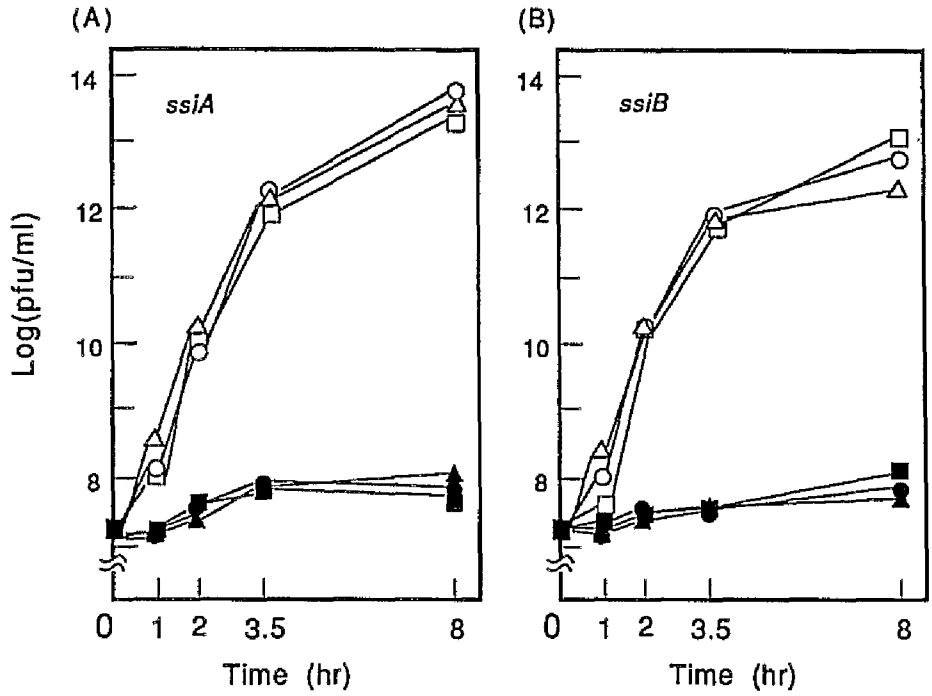

Fig. 2. The propagation of M13/AH12 (A) and M13Δ*lac*110/HA22 (B) in JM109 harboring pMMB2 and its derivatives. Phage infection was done as described in the preceding chapter. Plasmids harbored by JM109 are shown as: open circle, pMMB2; open triangle, pMMB2ΔAE; open square, pMMB2Δ23; closed circle, pMMB2Δ5; closed triangle, pMMB2Δ67; closed square, ΔSS.

M13Δ*lac*110/HA22 required a *repB'* product as a *trans*-acting factor for their efficient propagation.

(b) Analysis of replicative form DNA synthesis of M13Δ*lac*110 recombinant phage *in vivo*

To monitor more directly the effects of the coexistence of *repB'* upon the SSI functions, synthesis of RFI DNA was examined after infection by M13Δ*lac*110/AH12 and M13Δ*lac*110/HA22 of *E. coli* JM109[pMMB2ΔAE] and JM109[pMMB2ΔSS]. When an *ssi* signal cloned in M13Δ*lac*110 is functional, the RFI DNA should be accumulated more efficiently. Therefore, the initial accumulation of RFI DNA should be a measure of the effect of the *trans*-acting factor *in vivo*. The relative accumulation of RFI DNA is shown in Fig. 3. And the effect of *repB'* on the SSI function was calculated based on the relative RFI accumulation at 50 min after infection (Table 1).

The relative RFI accumulation of M13Δ*lac*110/AH12 and M13Δ*lac*110/HA22 increased by more than 3-fold over that of M13Δ*lac*110 in

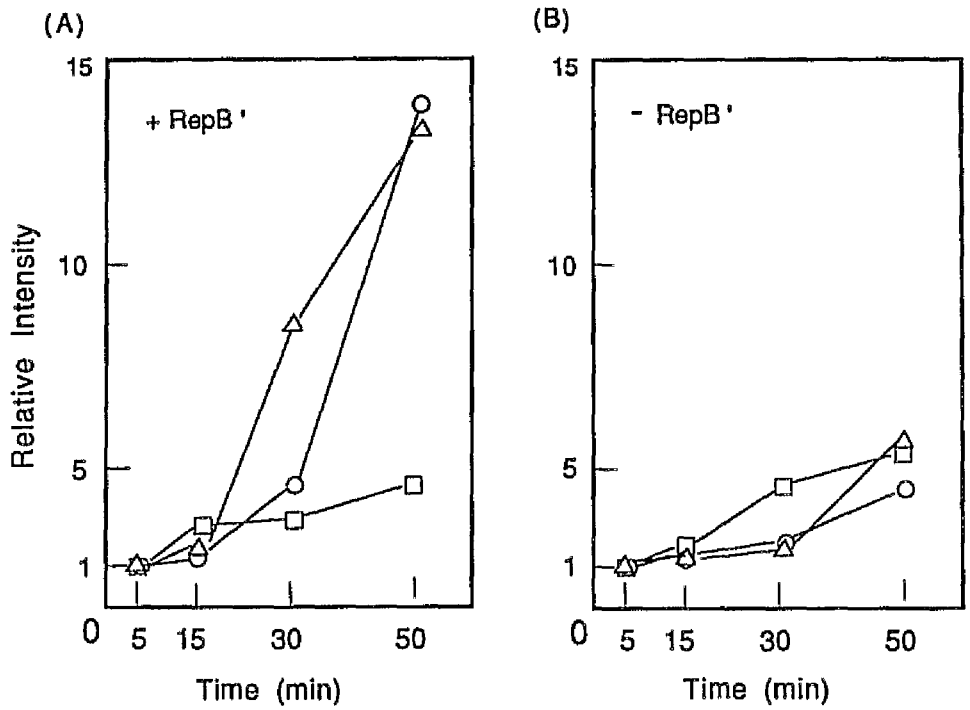

Fig. 3. Analysis of the effects of RepB' on the relative accumulation of RFI. (A) Relative accumulation of RFI DNA of the M13Δ*lac*110 recombinant phages in JM109[pMMB2ΔAE]. The accumulation of RFI was examined as shown by Honda *et al.* (1988). The RFI accumulation at the indicated times was represented by the relative intensity of the bands of RFI DNA on the negative films of autoradiogram, with that at 5 min after infection taken as one. Symbols used are: square, M13Δ*lac*110; circle, M13Δ*lac*110/AH12; triangle, M13Δ*lac*110/HA22. (B) Relative accumulation of RFI DNA of the M13Δ*lac*110 recombinant phages in JM109[pMMB2ΔSS]. Symbols used are the same as those given in (A).

the presence of pMMB2ΔAE, but was as low as that of M13Δ*lac*110 in the presence of pMMB2ΔSS. These results indicated that *ssiA* and *ssiB* cloned in M13Δ*lac*110 were functional only when *repB'* coexisted in the host cell. These findings were consistent with those of propagation of the corresponding M13Δ*lac*110 recombinant phages.

DISCUSSION

Recently, Scholz and his coworkers found that pMMB2 contains another gene of RSF1010 responsible for mobilization functions, namely *mobB* (Scholz *et al.*, 1989). The location of *mobB* is between *repB'* and the region derived from Tn3, which was used in the couse of constructing pMMB2

(Scherzinger *et al.*, 1984). To exclude the possibility that the product of *mobB* has some effects on the SSI functions, the author tested the propagation of M13Δ*lac*110 recombinant phages in the presence of Cm resistant recombinant pUC plasmids carrying the *Sau*3AI(4384)-*Sca*I(5416) or *Sac*I(in Tn3)-*Eco*RI(8681) segment of pMMB2 (for nucleotide numbers, see Scholz *et al.*, 1989). The former segment contains only *repB'*, whereas the latter segment contains *mobB*, *repB'*, *repA*, and *repC*. The results indicated that *mobB* did not seem to play any role in the SSI functions (data not shown). So the author concludes that RepB' is the factor required for the functions of the two *ssi* signals. This idea is consistent with the fact that both the RepB and RepB' proteins (formerly designated as RepB* and RepB, respectively) are primases which require exclusively RSF1010 DNA as a template in an *in vitro* system (Haring and Scherzinger, 1988).

In recent years, a variety of plasmids have been shown to encode DNA primases active in an *in vitro* assay for primer synthesis on ss DNA of phage fd (for review, Willetts and Wilkins, 1984; Wilkins *et al.*, 1985). And it has been thought possible that some plasmids may specify DNA primases unable to use the fd DNA as a template. However, no such primase that is active exclusively on its own genome, has been reported to date. The author has shown here that the coexistence of *repB'* of RSF1010 causes increase in the RFI accumulation of the M13Δ*lac*110 recombinant phages but not in that of M13Δ*lac*110 itself (Fig. 3). Since these RSF1010-specific priming functions required not only the *trans*-acting factor specified by *repB'* but also *ssiA* and *ssiB*, the RepB' protein should be a novel type of primase which requires RSF1010-specific *ssi* signals for its functional activity. It is conceivable that

Table 1. Relative RFI accumulations

Recombinant phage	Relative RFI accumulation* JM109[pMMB2ΔAE]	JM109[pMMB2ΔSS]	Index of effect of the RepB' function**
M13Δ*lac*110	4.29	5.46	0.79
M13Δ*lac*110 (*ssiA*)	13.83	4.78	2.89
M13Δ*lac*110 (*ssiB*)	13.50	5.61	2.41

*Relative RFI accumulations of M13Δ*lac*110 recombinant phages at 50 min after phage infection (as shown in Fig. 3).

**Index of effect of RepB' is calculated as: $\frac{\text{the relative RFI accumulation in JM109[pMMB2ΔAE]}}{\text{the relative RFI accumulation in JM109[pMMB2ΔSS]}}$

the RepB' protein and the two *ssi* signals, *ssiA* and *ssiB*, cooperatively provide RSF1010-specific priming complexes, the functions of which should render the RSF1010 replication independent of the vital DNA-priming functions of the host cell.

Judging from the fact that *ssiA* and *ssiB* of RSF1010 are active in the form of single-strand DNA, duplex opening of these regions in *oriV* should be required for their functions. Such duplex opening has been actually demonstrated in case of *E. coli oriC* (Bramhill and Kornberg, 1988) and the replication origin of phage λ (Schnös *et al.*, 1988). For RSF1010, the RepC protein has been demonstrated to act as a positive replication factor binding specifically to the direct repeats in the *oriV* region (Haring *et al.*, 1985), and the replication of RSF1010 has been shown to be independent of *E. coli* genes *dnaB*, *dnaC* and *dnaG* by Scholz *et al.* (1985). The author proposes that a prepriming step may exist, in which RepC and RepA cooperatively destabilize the duplex within the *oriV* region, followed by the priming step under the action of RepB' and the *ssi* signals. However, further studies are needed to prove this model for RSF1010 replication.

SUMMARY

The author has shown in the preceding chapter that the *oriV* region of the broad-host-range plasmid RSF1010 contained two single-strand DNA initiation (*ssi*) signals which had RSF1010-specific properties since they required one or more factors provided in *trans* by RSF1010 for their functional activities. The author demonstrates here, by deletion analysis, that *repB'*, one of the essential genes for the the vegetative replication of RSF1010, produces a factor required for the functions of the two *ssi* signals. It is conceivable that the RepB' protein and the two *ssi* signals, *ssiA* and *ssiB*, cooperatively compose plasmid-specific priming complexes which confer the broad-host-range property upon RSF1010.

Chapter 3. Functional substitution of *ssiA* and *ssiB* by the primosome assembly sites in RSF1010 DNA replication

Recently, Scholz and his coworkers (1989) have determined the complete nucleotide sequence of RSF1010 and demonstrated that the plasmid consists of 8684 bp. 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* (de Graff *et al.* 1978; Frey and Bagdasarian 1989; Haring and Scherzinger 1989; Scherzinger *et al.* 1984; Scholz *et al.* 1985). 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 (Haring and Scherzinger 1989; Scherzinger *et al.* 1984). In the *oriV* region, two single strand DNA initiation signals, *ssiA* and *ssiB* [corresponding to *oriR* and *oriL* in (Scholz *et al.* 1989), respectively], are located on each of the complementary strands. When cloned into single-strand phage vectors, these *ssi* sequences can direct the priming of DNA synthesis in the presence of RepB' protein *in vivo* (Chapter 2) and *in vitro* (Haring and Scherzinger 1989; Scholz *et al.* 1989).

In this chapter, the author shows that both *ssiA* and *ssiB* are essential for the replication of RSF1010 and constitute unique functional elements in *oriV*. Each of the *ssi* sites of RSF1010 may be substituted *in vivo* by the primosome assembly site of bacteriophage ϕX174 or of plasmid pACYC184 (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 of the function of the DnaG primase.

MATERIALS AND METHODS

(a) Bacterial strains and plasmids

The strains were derivatives of *E. coli* K-12, JM109 (Yanisch-Perron *et al.* 1985) and BW86 [*dnaG3 leu thyA deoB rpsL* ColI^r Δ(*chlA-uvrB*)] (Chatfield *et al.* 1982). Plasmid RSF1010, helper plasmid pMMB2 (ColD-based recombinant plasmid carrying *repA*, *B'* and *C* of RSF1010) and deletion derivatives, pMMB2Δ5, pMMB2Δ67, pMMB2ΔAE and pMMB2ΔSS have been descrived (Chapter 2; Scherzinger *et al.* 1984). For construction of RSF1010 miniplasmids, the author 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 [2335-2778, nucleotide numbers corresponding to Scholz *et al.*, (1989)] consisting of *ssiA*, *ssiB* and the *inc* repeats. A 1574-bp fragment containing the *oriV*

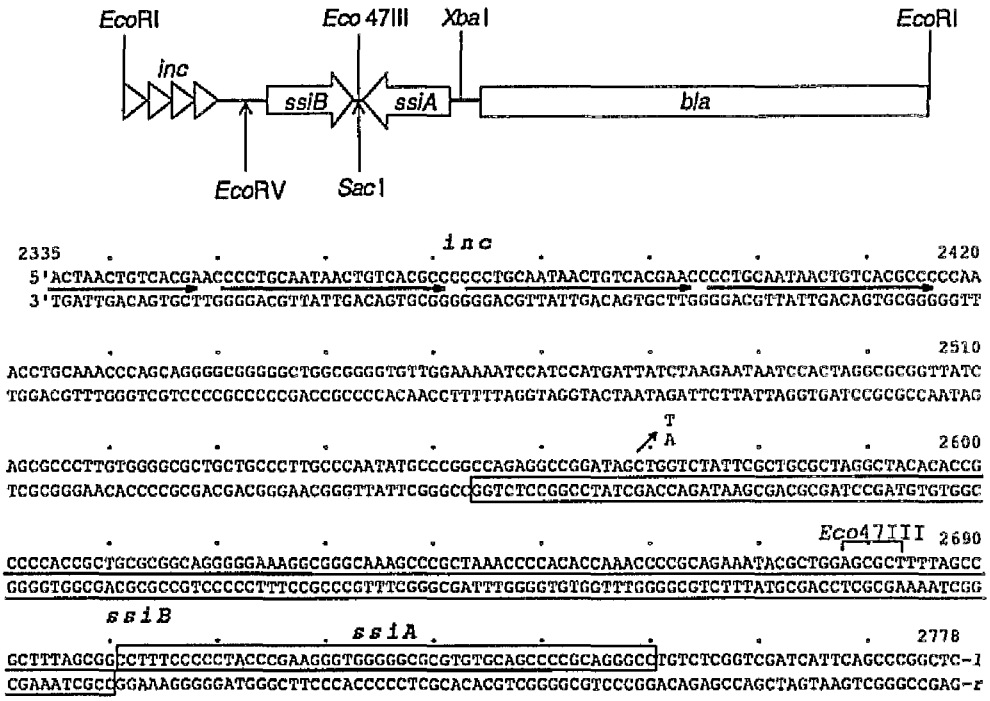

Fig. 1. (*Upper*) Physical map of the mini-plasmid pYH101 linearised at the unique *Eco*RI site. Open arrows pointing leftward and rightward represent the *ssi* signals on the *l* strand and *r* strand, respectively. Open triangles, *inc* repeats; box, coding region of β-lactamase derived from pBR322. The figure is not drawn to scale. (*Lower*) Nucleotide sequence of RSF1010 *oriV* region in pYH101. Sequences of *ssiA* and *ssiB* are boxed in. Thick lines represent regions of high homology between *ssiA* and *ssiB*. Three-and-a-half arrows represent the *inc* repeats, corresponding to the open triangles in the upper scheme. The GC base pair converted to TA by site-directed mutagenesis and the *Eco*47III site used to insert the *Sac*I linker to produce pYH101VS are also indicated.

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 GC to TA 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 containing 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*lac*184, an ori_C defective derivative of M13 phage containing *lacZ'* of M13mp18 by Bahk *et al.* (1987). The primosome assembly site of bacteriophage ϕX174 was cloned as 103 nt *Acc*II-fragment L containing ori_C into the *Sma*I site of the M13Δ*lac*183, identical to M13Δ*lac*184 (Sanger *et al.* 1979). The DNA fragments containing these primosome assembly sites were excised from the RF DNA of appropriate bacteriophages with *Sac*I, *Xba*I or *Hin*cII. 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 orientaitons were constructed by replacing each signal with the corresponding fragment derived from M13Δ*lac*110/*ssiA* or -*ssiB* (Chapter 1).

Plasmid pTK2 was constructed by insertion of the 4296-bp *Sau*3AI-*Eco*RI fragment of RSF1010 (base-pairs 4348-8680) into the *Bam*HI-*Eco*RI site of plasmid pHSG399, a Cm^R derivative of pUC19 (Takeshita *et al.* 1987). In this plasmid, the expression of *repA*, *B'* and *C* is under the control of *lac* prmoter. It provided the Rep functions for the replication of RSF1010 miniplasmids.

(b) 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 procedures of Birnboim and Doly (1979). Oligonucleotide-directed *in vitro* 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 (Sanger *et al.* 1978). Transformation was done by the method of Chung *et al.* (1989). Concentrations of antibiotics in selective media were: ampicillin (Amp), 50 μg/ml; Cm, 30 μg/ml and Km 100 μg/ml.

RESULTS

(a) Replication activities of the 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 proteins 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 (Scholz *et al.* 1989) and with R1162 plasmid (Lin and Meyer 1987).

If primosome assembly sites from ϕX174 or pACYC184 were substituted for *ssiA* or *ssiB* (pYH174A, pYH174B and pYH184A), replication of the recombinant miniplasmids were restored. Moreover, pYH174A184B, in which both *ssiA* and *ssiB* were replaced with primosome assembly sites, could replicate in the presence of Rep proteins of RSF1010. However, recombinant miniplasmids 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.

(b) Requirements of Rep functions for the replication of miniplasmids with substitutions of *ssi* signals

DNAs of the miniplasmid 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

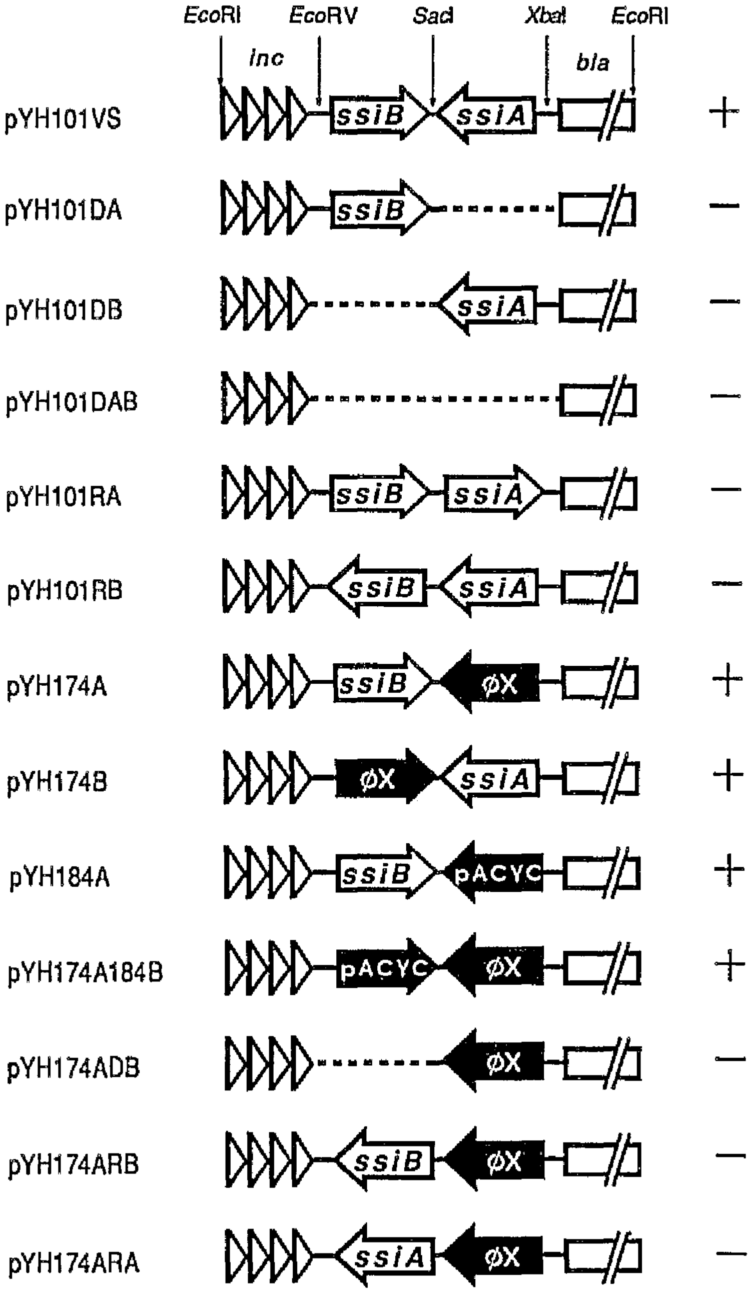

Fig. 2. Chimeric origins constructed from the plasmid pYH101VS and their ability to replicate in *E. coli* in the presence of RSF1010 Rep proteins. Broken lines indicate the areas of deletion. T4 DNA ligase was used to self-ligate 1.875 ng of linear DNA of each miniplasmid derivative prepared by *Eco*RI cleavage of the recombinant M13mp19 RF DNA containng 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.

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

	helper plasmid				
	pMMB2	pMMB2Δ5	pMMB2Δ67	pMMB2ΔAE	pMMB2ΔSS
repA	+	-	+	-	-
repB'	+	-	-	+	-
repC	+	+	+	-	-
pYH101	3.9×10^5	nd	nd	nd	nd
pYH101VS	4.4×10^5	nd	nd	nd	nd
pYH174A	3.1×10^5	nd	nd	nd	nd
pYH174B	2.9×10^5	nd	nd	nd	nd
pYH184A	2.2×10^5	nd	nd	nd	nd
pYH174A184B	3.1×10^5	nd	2.5×10^5	nd	nd

The number of transformants per μg DNA is shown. nd indicates not detected. Preparation of mini-plasmid pYH101 and derivative DNAs was described in the legend to Fig. 2. Results are the mean values of two determinations.

required RepA and RepC proteins (Table 1). They also required the RepB' except for the miniplasmid pYH174A184B, in which both *ssi* signals 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.

(c) Copy numbers of miniplasmids with substitutions of *ssi* signals

Relative copy numbers of miniplasmids with substitutions of *ssi* signals derivatives were determined by estimation of the single-cell 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 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 paticular interest, however, was the fact that the copy number of pYH174A184B, with both *ssi* signals replaced by the 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 fact taken together with the results shown in Table 1 indicate that *ssi* signals are the site of RepB' primase action in the initiation event of RSF1010 replication.

(d) Effects 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 miniplsmid, 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 *dnaG*3 mutant, specifing temperature-sensitive primase) carrying pTK2 as a helper plasmid supplying Rep functions of RSF1010. After incubation at permissive and nonpermissive temparatures, the single strand 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 temparature in the cells containing pYH101VS (Fig. 3 lane 1). Cells carrying pYH174A and pYH174B exhibited the accumulation of those strands that replicated under the direction of the RSF1010 *ssi* signal (Fig. 3 lane 2, 3). The accumulations was detectable at the permissive temparature but became prominent upon incubation at 41°C. These results indicate that primosome assembly sites of ϕX174, introduced in place of the RSF1010-

Table 2. Relative copy numbers of *ssi*-replased miniplasmid derivatives.

miniplasmid	site			Amp LD_{50}	Relative copy
derivatives	A	B	helper plasmid	(μg/ml)	number (%)
pYH101	+	+	pMMB2	1122.0	100
pYH101VS	+	+	pMMB2	812.8	72.4
pYH174A	ϕX	+	pMMB2	426.6	38.0
pYH174B	+	ϕX	pMMB2	831.8	74.1
pYH184A	pACYC	+	pMMB2	841.4	75.0
pYH174A184B	ϕX	pACYC	pMMB2	309.0	27.5
pYH174A184B	ϕX	pACYC	pMMB2Δ67	302.0	26.9

Relative copy numbers were estimated by single-cell Amp^R levels. +, original RSF1010-specific *ssi* signal; ϕX, the primosome assembly site from ϕX174; pACYC, the primosome assembly site from pACYC184. Amp LD_{50} was determined by the method of Nordström *et al.* (1980). Results are the mean varues of two determinations with independent clones.

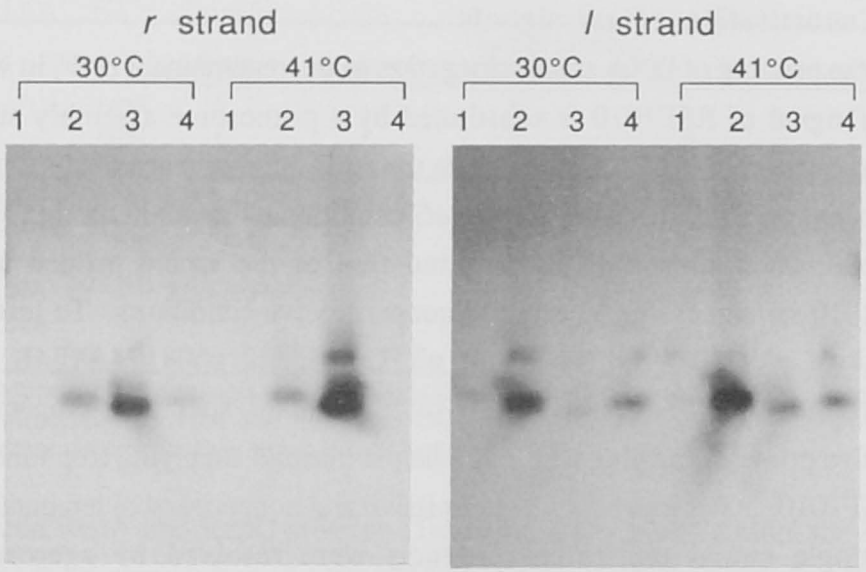

Fig. 3. Accumulation of specific single strands upon replication of miniplasmids with chimeric $oriV_{RSF1010}$ in a *dnaG*3 mutant. *E. coli* BW86 , harboring pTK2 helper plasmid and miniplasmid derivatives with *ssi* replacements, were grown at 30°C to an $OD_{610} = 0.5$. IPTG was then added (2.0 mM), and the culture was divided in two portions. One was incubated at 30°C and the other at 41°C for 1.5 hours. Plasmid DNA was then extracted and subjected to electrophoresis on 1% agarose gels. DNA bands were transfered to nitrocellulose filters without alkaline denaturation. Southern hybridization was performed with strand-specific probes. ^{32}P-labeled minus strands of f1R199/YH101DAB*r* and f1R199/YH101DAB*l* were used to detect *r* and *l* strand, respectively. Lane 1, pYH101VS; lane 2, pYH174A; lane 3, pYH174B; lane 4, pYH174A184B.

specific *ssi* signals, actually directed the priming of the indivisual DNA strands in a DnaG-dependent process. It is possible that the synthesis of each complementary strand was independent of each other and proceeded by a strand-displacement mechanism. It should be noted that accumulation of a small amount of the *l* strand has occured 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 DnaG 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 concerned manner. Since a strand displacement mechanism was implicated in the replication of RSF1010 (Haring and Scherzinger 1989), accumulation of one of the strands in a chimeric origin may be possible.

DISCUSSION

The author has shown that *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* 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 palsmid-specific primase, RepB', and not by host priming protein DnaG. On the basis of results presented in this chapter, the author can recognize and locate three distinct *cis*-acting functional domains in the $oriV_{RSF1010}$ region, the *inc* repeats, *ssiA* and *ssiB*. Furthermore, the author has shown that heterologous initiation sites, such as the primosome assembly sites of the phage ϕX174, may be substituted for the RSF1010-specific *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 sheme presented in Fig. 4. Since *ssiA* and *ssiB* are active in the form of single-stranded DNA (Chapter 1; Scholz *et al.* 1989), the author suggests that the prepriming events of the 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

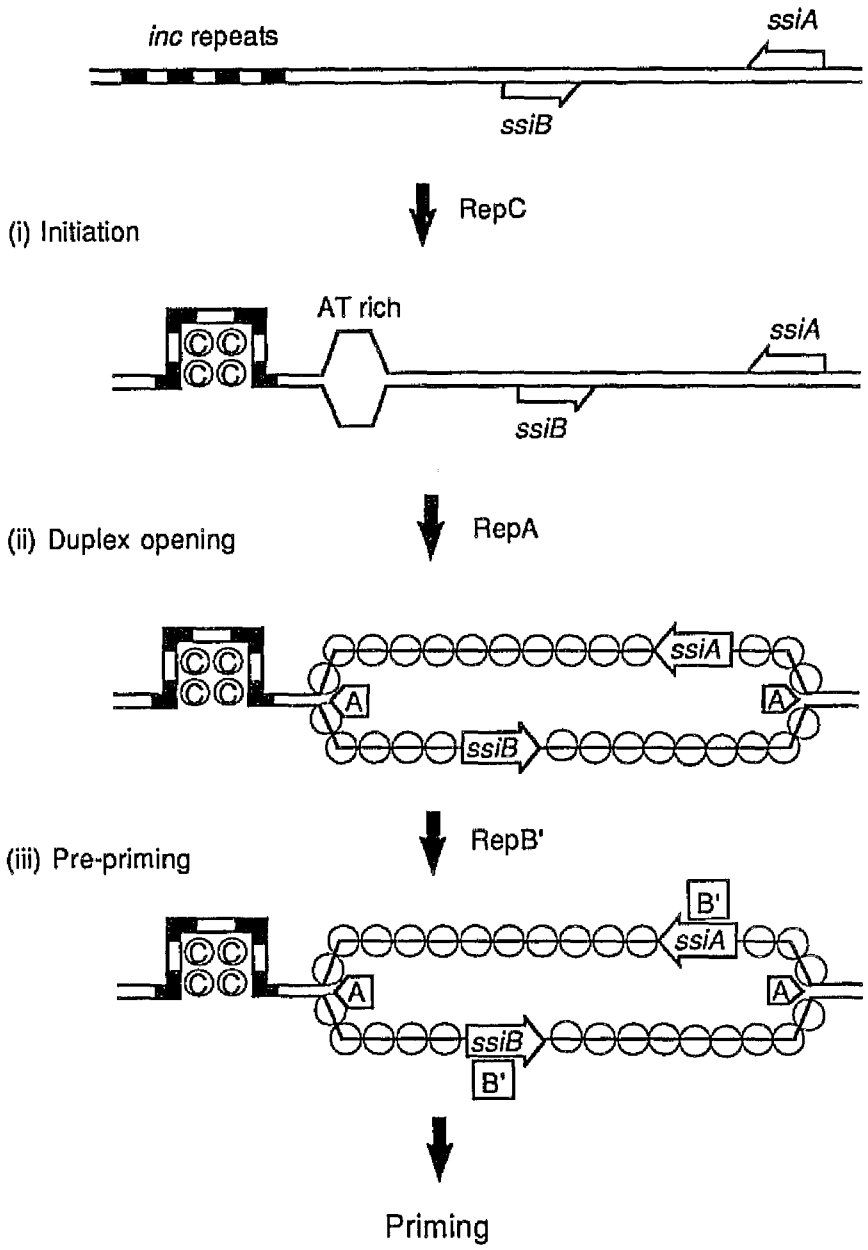

Fig. 4. Schematic representation of proposed model for the initiation of DNA replication in RSF1010. The presumed chronology of events is as follows: (i) RepC protein binds to the *inc* repeats; specific loop structures proposed by Haring and Scherzinger (1989) are not shown. (ii) RepA helicase binds presumably to the AT-rich region and unwinds the duplex to expose *ssiA* and *ssiB*. (iii) Single-stranded regions are stabilized by the single strand DNA binding protein (O), and RepB' primase forms the priming complexes with the *ssi* sites.

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 consistancy also indicates that the events occuring in an *in vitro* replication system, reconstructed from purified components specific for RSF1010 initiation (Haring and Scherzinger 1989; Scherzinger *et al.* 1984), closely mimic the initiation events taking place *in vivo*. Propagation of RSF1010 replication fork is considered to involve the RepA helicase (Scholz *et al.* 1985), and the replication of this plasmids possibly proceeds by a strand-displacement mechanism. In this respect, it may be of interest to establish whether G4 type DnaG-dependent *ssi* signals which do not introduce a primosome onto the DNA strand can substitute for the *ssiA* and *ssiB* in the RSF1010 DNA replication.

SUMMARY

Two *ssi* signals in the *oriV* of the broad host-range plasmid RSF1010 are essential for the priming of replication of each complementary DNA strand of this plasmid in *E. coli*. Each of the RSF1010 *ssi* signals, *ssiA* and *ssiB*, could be replaced by a primosome assembly site from plasmid pACYC184 or from bacteriophage ϕX174. In these chimeric origins, replication of the strand complementary to that containng 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 that 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 *oriV* of RSF1010 can be separated into functional elements and reconstructed by replacing the *ssi* sites with heterologous elements.

Chapter 4. Functional substitution of *ssiA* and *ssiB* by the DnaG-dependent priming signals in RSF1010 DNA replication

In the *oriV* of plasmid RSF1010, three distinct *cis*-acting elements essential to the plasmid replication exist (Chapter 3). These include the *inc* repeats and the two essential DNA priming signals, *ssiA* and *ssiB*, which are located on the *l* and *r* strands, respectively (Chapter 1; Scholz *et al.* 1989). The *inc* repeats, 20-nucleotide tracts repeated three and a half times, are the binding sites for the RepC initiator protein. The two *ssi* signals consist of highly conserved 40-nucleotide sequences and exclusively recognized by the RepB' primase . In the preceding chapter, the author has shown that the primosome assembly sites from bacteriophage ϕX174 or plasmid pACYC184 are able to substitute functionally for both of *ssiA* and *ssiB* in the replication of RSF1010. Function of the chimeric $oriV_{RSF1010}$ containing two primosome assembly sites in place of *ssiA* and *ssiB* is no longer dependent on RepB' but still dependent on RepA and RepC. It has been postulated that RepB' primase actually directs the RSF1010-specific priming reaction after completion of the prepriming process that is dependent on RepA and RepC.

In this chapter the author shows that both *ssiA* and *ssiB* of RSF1010 may also be substituted *in vivo* by two distinct G4-type *ssi* signals; one is the ori_C of bacteriophage G4 and the other is the *ssi* signal from plasmid pSY343, a run-away plasmid derived from R1 (Uhlin *et al.* 1979), which corresponds to the *ssi* signal directing the leading strand synthesis in R1 plasmid replication. These G4-type *ssi* signals require only *E. coli* DnaG primase without introducing the primosome and direct only the initiation of leading strand synthesis (Bouché *et al.* 1978; Masai and Arai 1989; Sims *et al.* 1979). The replication mechanism of RSF1010 DNA replication will also be discussed.

MATERIALS AND METHODS

The recombinant plasmid, pHSG399/YH101VS (described in chapter 4) was used to construct chimeric $oriV_{RSF1010}$ with heterologous *ssi* signals. The G4-type *ssi* signal of plasmid pSY343, *ssiA*(pSY343), was previously cloned as a 170-bp fragment into the *Sma*I site of multicloning sites in M13Δ*lac*184 that was an ori_C defective derivative of M13 phage containing *lacZ'* of M13mp18 (Bahk *et al.* 1988). The ori_C segment of bacteriophage G4 [nucleotides 3798-4070] (Godson *et al.* 1978) was also inserted into the *Sma*I site of M13Δ*lac*184. The DNA fragments containing these G4-type *ssi* signals were excised from RF DNA of relevant recombinant M13Δ*lac*184 phages upon cleavage with *Sac*I, *Xba*I or *Hin*cII. Then they were inserted into the $oriV_{RSF1010}$ region of pHSG399/YH101VS, from which *ssiA* or *ssiB* had been removed by cleavage with *Sac*I plus *Xba*I or *Sac*I plus *Eco*RV, respectively. Miniplasmid DNA segments containing the chimeric $oriV_{RSF1010}$ were cut out from the vector plasmid by *Eco*RI cleavage, and self-circularized by T4 DNA ligase followed by transformation into *E. coli* JM109 cells. Generation of Amp^R resistant transformants was a primary criterion for the functionl $oriV_{RSF1010}$.

RESULTS

(a) Replication activities of chimeric origins with G4-type *ssi* signals

Replication activities of the miniplasmid pYH101VS and its derivatives with heterologous *ssi* signals were examined by transformation into JM109 harboring pMMB2 as a helper plasmid (Fig. 1). This plasmid supplies the RepA, RepB' and RepC proteins, essential for the DNA replication starting from $oriV_{RSF1010}$. Miniplasmid derivatives pYHG4A, pYHG4B, pYH343A and pYH343B, which contained a G4-type signal from bacteriophage G4 or plasmid pSY343 instead of *ssiA* or *ssiB*, had abilities to replicate in this biochemical background. Miniplasmid derivatives pYHG4A174B, pYH174AG4B and pYH174A343B, in which one of the *ssiA* and *ssiB* sequences was replaced by a G4-type *ssi* signal and the other by the primosome assembly site from bacteriophage ϕX174, also could replicate. In addition, miniplasmid derivatives pYHG4A343B and pYH343AG4B, in

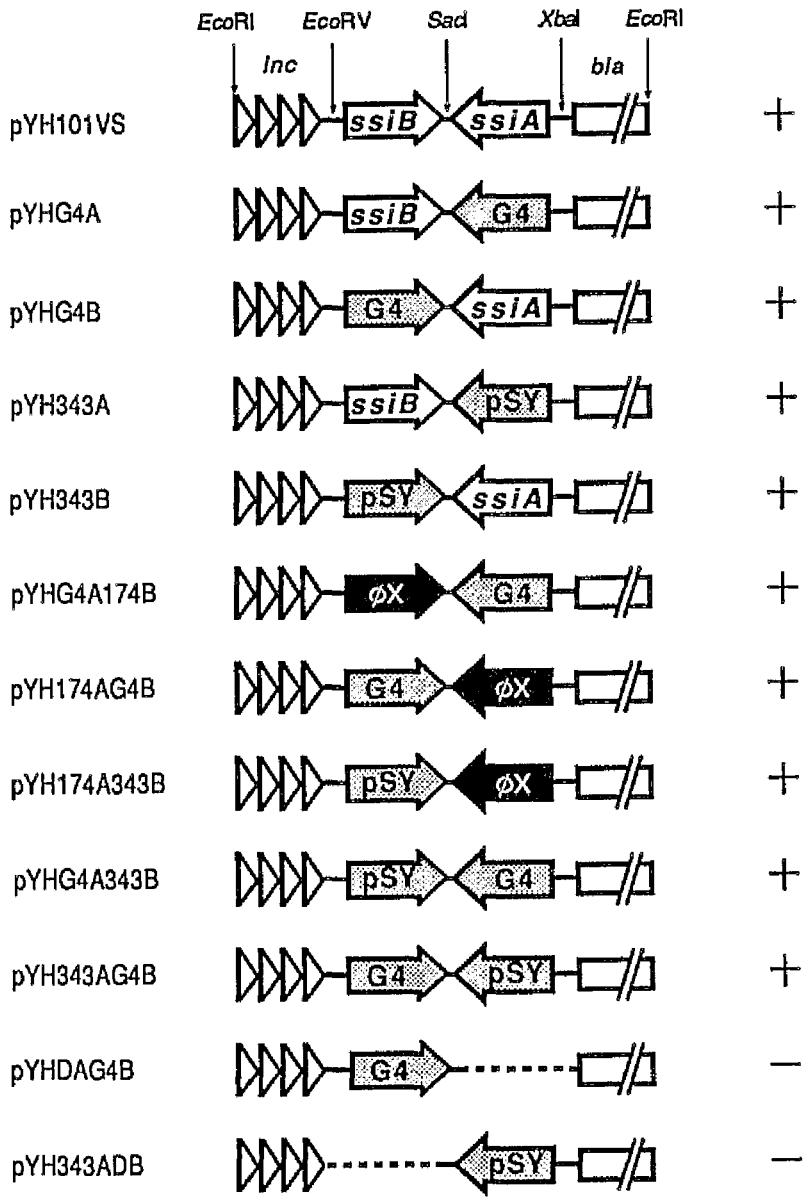

Fig. 1. Chimeric origins constructed from the mini-RSF1010 plasmid pYH101VS and their abilities to replicate in *E. coli* in the presence of RSF1010-specific Rep functions. Schematic representations of the miniplasmid derivatives were drawn as linearized at the unique *Eco*RI site. Restriction sites used to construct the miniplasmid derivatives are indicated. Arrows indicate the locations and the priming orientations of the *ssi* signals: open arrows, the original RSF1010-specific *ssi* signals; shadowed arrows, the G4-type *ssi* signals from bacteriophage G4 or plasmid pSY343 (an R1 plasmid derivative); solid arrows, the primosome assembly site from bacteriophage ϕX174. Open triangles, *inc* repeats; broken lines, the deleted areas; open boxes, the coding region of the β-lactamase from pBR322. The ability (+) or inability (-) to produce stable transformants of JM109[pMMB2] by each miniplasmid derivative is indicated.

which both of *ssiA* and *ssiB* were replaced by the G4-type *ssi* signals, could replicate as well. These findings indicate that not only the primosome assembly site but also G4-type *ssi* signals can substitute for the function of both *ssiA* and *ssiB* signals in the $oriV_{RSF1010}$. However, the miniplasmid derivatives containing a G4-type *ssi* signal on one complementary DNA strand but no *ssi* signal on the other (pYH343ADB and pYHDAG4B) gave no ampicillin-resistant transformants.

(b) Requirements of RSF1010-specified Rep functions for the replication of the miniplasmid derivatives with heterologous *ssi* signals.

In order to analyze the requirements of the RSF1010 Rep functions for the replication of the chimeric origins, DNAs of the miniplasmid derivatives with *ssi* substitutions were transformed into JM109 harboring the deletion derivatives of pMMB2 (Table 1). When pMMB2ΔSS which contains no *rep* region of RSF1010 was supplied as a coresident helper plasmid, none of the miniplasmid derivatives could replicate (data not shown). Nor could they replicate in the presence of pMMB2Δ5 ($repC^+$, $repB'^-$, $repA^-$) or pMMB2ΔAE ($repB'^+$, $repC^-$, $repA^-$). However, the miniplasmid derivatives, in which both *ssiA* and *ssiB* were replaced by heterologous signals, could

Table 1. Requirements of RSF1010-specific Rep functions for the replication of miniplasmid derivatives.

	Number of transformants with the helper plasmid			
miniplasmid derivative	pMMB2 (*repAB'C*)	pMMB2Δ5 (*repC*)	pMMB2Δ67 (*repAC*)	pMMB2ΔAE (*repB'*)
pYH101VS	6.5×10^5	nd	nd	nd
pYHG4A	2.3×10^5	nd	nd	nd
pYHG4B	3.2×10^5	nd	nd	nd
pYH343A	1.4×10^5	nd	nd	nd
pYH343B	1.9×10^5	nd	nd	nd
pYHG4A174B	1.3×10^5	nd	1.1×10^5	nd
pYH174AG4B	1.4×10^5	nd	1.7×10^5	nd
pYH174A343B	3.6×10^4	nd	2.9×10^4	nd
pYHG4A343B	8.3×10^4	nd	5.7×10^4	nd
pYH343AG4B	1.2×10^5	nd	1.3×10^5	nd

1.875 ng of self-ligated DNA fragments of the miniplasmid derivatives (see MATERIALS AND METHODS) were used to transform JM109 harboring pMMB2 or its deletion derivatives (Chapter 2). Numbers of transformants per μg DNA are shown. Cases where no stable transformants were obtained are indicated by nd. Results are shown as mean values of two determinations.

Table 2. Relative copy numbers of *ssi*-substituted RSF1010 miniplasmid derivatives

miniplasmid derivative	*ssi* site A	*ssi* site B	Amp LD_{50} (μg/ml)	Relative Copy number (%)
pYH101VS	+	+	794.3	100
pYHG4A	G4	+	631.0	79.4
pYHG4B	+	G4	691.8	87.1
pYH343A	pSY	+	638.3	80.4
pYH343B	+	pSY	288.4	36.3
pYHG4A174B	G4	φX	645.7	81.3
pYH174AG4B	φX	G4	302.0	38.0
pYH174A343B	φX	pSY	257.0	32.4
pYHG4A343B	G4	pSY	354.8	44.7
pYH343AG4B	pSY	G4	638.3	80.4

Relative copy numbers were estimated by single-cell Amp^R levels. The *ssi* signals contained in the miniplasmid derivatives are indicated as follows: +, original RSF1010-specific *ssi* signal; G4, the G4-type *ssi* signal of bacteriophage G4; pSY, the G4-type *ssi* signal from pSY343 (an R1 plasmid derivative); φX, the primosome assembly site from φX174. Amp LD_{50} was determined by the method of Nordström *et al.* (1980). Results are shown as mean values of two determinations with independent clones.

replicate in the absence of RepB' primase when RepA and RepC were supplied (pMMB2Δ67 as a coresident helper plasmid). On the other hand, miniplasmid derivatives pYHG4A, pYHG4B, pYH343A, pYH343B, in which only one of the *ssi* signals was replaced by a G4-type signal, could not replicate in the presence of this helper plasmid. These results are in good agreement with the previous report showing that the RepB' protein was not required for the replication of pYH174A184B in which both *ssiA* and *ssiB* are replaced by the primosome assembly sites, but required for the function of those chimeric origins in which only one *ssi* signal was substituted (Chapter 3). These findings supported the hypothesis that, in the initiation stage of RSF1010 DNA replication, RepB'-dependent priming reaction is separated from the prepriming process dependent on both of RepC and RepA proteins which act as an initiator and a DNA helicase, respectively (Chapter 3; Scherzinger *et al.* 1991).

(c) Copy number estimation of the miniplasmid derivatives

Relative copy numbers of the miniplasmid derivatives with various *ssi* substitutions were determined by the single cell Amp resistance method (Nordström *et al.* 1980) in JM109 harboring pMMB2 (Table 2). Relative copy numbers of miniplasmid derivatives pYHG4A, pYHG4B, pYH343A, pYHG4A174B and pYH343AG4B were approximately 80 per cent of that of

pYH101VS. These results show that the function of one or both of the *ssi* signals in $oriV_{RSF1010}$ can be substituted by the G4-type *ssi* signals without serious decrease in the replication activities of the chimeric origins. However, relative copy numbers of the other miniplasmid derivatives were half as high as those of the miniplasmids described above. The relative copy number of the miniplasmid derivative pYH174A, which contains the primosome assembly site from ϕX174 in place of *ssiA*, is also half as high as that of pYH101VS (Chapter 3). It is notable that the chimeric origins of the miniplasmid derivatives with reduced replicative activities always contain substitutions of *ssiA* by the primosome assembly site from ϕX174 or of *ssiB* by the G4-type *ssi* signal from pSY343. This is true without exception. It seems that these decrease in replication activities were caused not by the functional difference between *ssiA* and *ssiB* but by some structual changes in the chimeric origins, because substitutions of *ssiA* by the primosome assembly site from pACYC184 (Chapter 3) or of *ssiB* by the G4-type *ssi* signal from G4 did not cause the decrease in the copy numbers of the miniplasmid derivatives. In $oriV_{RSF1010}$, *ssiA* and *ssiB* sequences are involved in a large inverted repeat structure [nucleotides 2589-2739] and this region contains typical DNA bending motif extending over the 90 bp stretch between the *ssi* signals. RepC-induced DNA looping structures which include this large inverted repeat region have been observed *in vitro* (Haring and Scherzinger 1989). Each substitution of the *ssi* signals should make some changes in these structures and these structual changes may affect the replication activities of the chimeric origins.

DISCUSSION

The *oriV* of plasmid RSF1010 contains no priming signals dependent on *E. coli* factors, and *in vitro* experiments have shown that *E. coli* replication proteins DnaA, B, C, G and T are not required for DNA replication of this plasmid (Haring *et al.* 1985; Scherzinger *et al.* 1991). Instead, the RSF1010-specific priming apparatus involves RepB' primase and its specific recognition sequences, *ssiA* and *ssiB*, in the template strands (Chapter 2; Haring and Scherzinger 1989). The function of the two RSF1010-specific *ssi* signals

may be substituted by the primosome assembly sites (Chapter 3). However, it is not clear whether these RSF1010-specific *ssi* signals should serve as the unique primer sites or the entry sites for the possible RSF1010-specific "primosomes" involving RepB' primase and the DNA helicase such as RepA protein. Such replicon-specific "primosomes" have actually been reported for bacteriophage T4 (Selick *et al.* 1987) and T7 (Richardson *et al.* 1987).

In the present study, the author has shown that the two distinct G4-type priming signals from bacteriophage G4 and plasmid pSY343 can also substitute for the function of the two RSF1010-specific *ssi* signals. These G4-type *ssi* signals direct the priming of DNA depending on DnaG primase alone and have no abilities to direct the lagging strand synthesis (Masai and Arai 1989; Sims *et al.* 1979). It is conceivable that, in cooperation with RepB' primase, *ssiA* and *ssiB* provide the primer molecules exclusively for the leading strand synthesis, and that DNA replication of RSF1010 does not require a primary system for lagging strand synthesis. Recently, with the electron microscopic analysis, Scherzinger *et al.* (1991) have observed the replicative intermediates in the *in vitro* cell-free replication system of RSF1010, suggesting that two displacement events may begin at or near the *ssi* sites. The two displacement strands seemed to start in a synchronous or an asynchronous manner and expand toward each other. Based on these results, it is conceivable that, although sequence non-specific priming mechanisms exist in *E. coli*, the RSF1010 DNA replication does not involve primarily the discontinuous lagging strand synthesis and replication forks proceed mainly by the strand displacement mechanism producing D-loop intermediates.

It has been shown that substitutions of *ssiA* by the primosome assembly site from ϕX174 (not from pACYC184) (Chapter 4), and *ssiB* by the G4-type *ssi* signal from pSY343 (not from G4) cause decrease in the replicative activities of the chimeric origins. Although the reason for decrease in the replicative activities is unclear, structual changes within the *oriV* region may be responsible for that. The $oriV_{RSF1010}$ involves various specific structures, such as large inverted repeat and DNA bending motif, which should have some roles in the initiation of DNA replication. Moreover, superhelicity of the

plasmid DNA is essential for the *in vitro* RSF1010 DNA replication (Scherzinger *et al.* 1991). Wada *et al.* (1988) have shown that HU protein, the major histone-like protein of *E. coli* which has the abilities to induce DNA looping or bending (Drlica and Rouviere-Yanif 1987), should be required for the intracellular maintenance of RSF1010. Specific high-ordered molecular structure of the $oriV_{RSF1010}$ may play significant roles in the initial stage of RSF1010 DNA replication.

SUMMARY

It has been demonstrated that the two essential DNA initiation signals of plasmid RSF1010, *ssiA* and *ssiB*, can be substituted functionally by the primosome assembly sites. The author showed in this chapter that each of *ssiA* and *ssiB* could be substituted functionally also by either of the two G4-type (DnaG-dependent) priming signals, the ori_C of bacteriophage G4 and an *ssi* signal from plasmid pSY343 (an R1 plasmid derivative). Functions of the chimeric *oriV*s of RSF1010 thus constructed were dependent on the RSF1010-specific replication proteins, RepA, RepB' and RepC. When both of *ssiA* and *ssiB* were replaced by the G4-type *ssi* signals, functions of the chimeric *oriV*s were no longer dependent on RepB' (RSF1010-specific DNA primase). Relative copy numbers estimated by Amp LD_{50} suggested that the replication activities of the chimeric *oriV*s of RSF1010 were not influenced markedly by the type of heterologous priming signals they contained. It is conceivable that DNA replication of RSF1010 does not need the priming mechanism for lagging strand synthesis and proceeds by the strand displacement mechanism.

Chapter 5. Minimal essential domain for the functionl activity of RSF1010-specific primase, RepB'

In the initiation stage of DNA replication, a short "primer RNA" is, in general, synthesized by a specific primase (Kornberg and Baker 1991). In *E. coli*, the primase is encoded by *dnaG* and the product of this gene directs the initiation of chromosomal DNA replication. Many bacterial plasmids or phages also encode their own primases (Willetts and Wilkins 1984). Plasmids CollIb, R64, RK2 and RP4 encode primases active in an *in vitro* assay for primer synthesis on ss DNA of phage fd as a template (Wilkins *et al.* 1985). Moreover, bacteriophages such as T4 (Selick *et al.* 1987) and T7 (Richardson *et al.* 1987) encode their own primases which are involved in the replicon-specific "primosomes" and essential for the vagetative DNA replication of these phages. Some plasmid-specified primases have been reported to play critical roles in conjugational DNA synthesis (Chatfield *et al.* 1982; Lanka and Barth 1981; Nash and Krishnapillai 1988).

In plasmid RSF1010, a specific primase is encoded by *repB'*, one of the three essential genes for DNA replication of this plasmid (Scherzinger *et al.* 1984; Scholz *et al.* 1985). It encodes the RepB' primase, a 36-kDa polypeptides composed of 323 amino acid residues (Scholz *et al.* 1989). RepB' shares its amino acid sequences with the C-terminal half of the protein RepB which is encoded by the overlapping open reading frame with altered start codon. Although both of RepB' and RepB proteins contain the specific primase activity, protein RepB has been proved to be identical to MobA (Scholz *et al.* 1989), one of the essential gene products for the mobilization of this plasmid, and dispensable for the DNA replication of RSF1010 in *E. coli*. RepB' is a novel type of primase in the following criteria. It does not require ribonucleotide triphosphates for the priming reaction (Haring and Scherzinger 1989), and it requires specific nucleotide sequences, *ssiA* and *ssiB*, located on

each of the complementary strands of duplex DNA within the *oriV*$_{RSF1010}$, as the exclusive templates (Haring and Scherzinger 1989; Honda *et al.* 1989). RepB' primase and the two *ssi* signals, *ssiA* and *ssiB*, act in cooperation to form RSF1010-specific priming complexes, the function of which should render the RSF1010 replication independent of the vital DNA priming functions of the host cell.

MATERIALS AND METHODS

(a) Construction of the recombinant plasmids encoding the RepB'-LacZ' fusion proteins

A recombinant plasmid which produces the RepB'-LacZ' fusion protein was constructed by inserting the coding region of *repB'* [the *Sau*3AI(4384)-*Sca*I(5416) fragment of RSF1010] (Scholz *et al.* 1989) into MCS of pHSG399, a Cm-resisitance derivative of plasmid pUC19 (Takeshita *et al.* 1987). The resulting plasmid, pTK1 (Fig. 1), encodes the RepB' primase fused with the LacZ' protein under the control of *lac*UV5 promoter, and was

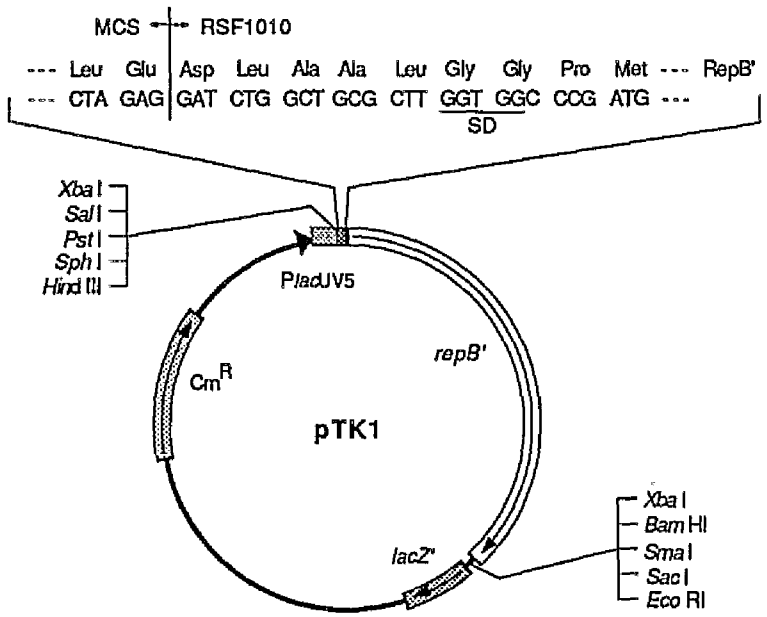

Fig. 1. Physical and genetic map of the plasmid pTK1. Arrows indicate the orientation of the transcription of the genes. Restriction sites in the MCS are shown. The fusion point in the RepB'-LacZ' is shown with nucleotide sequence and amino acid sequence in the upper part of the diagram. The native ribosome binding site for *repB'* is indicated by SD. Details were described in MATERIALS AND METHODS.

used for constructing a series of the deletion derivatives of *repB'*. Deletions from the N- and C-termini of the RepB' protein were introduced as follows: pTK1 was digested completely with the restriction endonucleases, *Pst*I plus *Sal*I and *Sac*I plus *Bam*HI, respectively; then nucleotides were sequentially nibbled from the end of the DNA fragments generated by the digestion with *Sal*I or *Bam*HI by the treatment with exonuclease III for various times followed by the treatment with mung bean nuclease and DNA pol I Klenow fragment; the truncated fragments were self-ligated and transformed into *E. coli* JM109. Products of the truncated *repB'* genes ligated in-frame with *lacZ'* produce the fused polypeptides containing the α-complementing fragment of β-galactosidase, which could exhibit β-galactosidase activity in JM109. JM109 harboring the deletion derivatives of pTK1 were screened on LG-B agar plate containing Cm (30 μg/ml), 0.005% X-gal and 0.25 mM IPTG. Plasmids extracted from blue colonies were analized by AGE, and nucleotide sequences at the fused junctions were confirmed by DNA sequencing (Sanger *et al.* 1979). β-Garactosidase activity was assayed by the method of Miller (1972) to show the production of RepB'-LacZ' fusion proteins in *E. coli* cells.

(b) Infection assay

Recombinant phages, M13Δ*lac*110/AH12 and M13Δ*lac*110/HA22 (Chapter 1), which are the *ori*$_C$-defective M13 phage derivatives containing the *ssiA* and *ssiB* of RSF1010, respectively, were used in the assay for the primase activity of the mutant RepB'-LacZ' fusion proteins. JM109 harboring each deletion derivatives of pTK1 plasmid were grown in LB medium at 37°C until A_{610} of the culture reached 0.15. Then the culture was infected with M13Δ*lac*110/AH12 or M13Δ*lac*110/HA22 at an moi of 6.6. The phage titer at 4 hrs after infection was measured.

(c) Transformation assay

Ability of the mutant fusion proteins to support DNA replication of RSF1010 was determined by the transformation assay. The mini-RSF1010 plasmid, pYH101 (Chapter 3) was transformed into JM109 cells harboring the deletion derivatives of pTK1 in addition to plasmid, pMMB2Δ67 (*repA*$^+$, *repB'*$^-$, *repC*$^+$) (Chapter 3). Amp^R transformants should be obtained when

the mutant RepB'-LacZ' fusion protein supports DNA replication of pYH101.

RESULTS

(a) Functional activities of the deletion mutants of fused RepB'

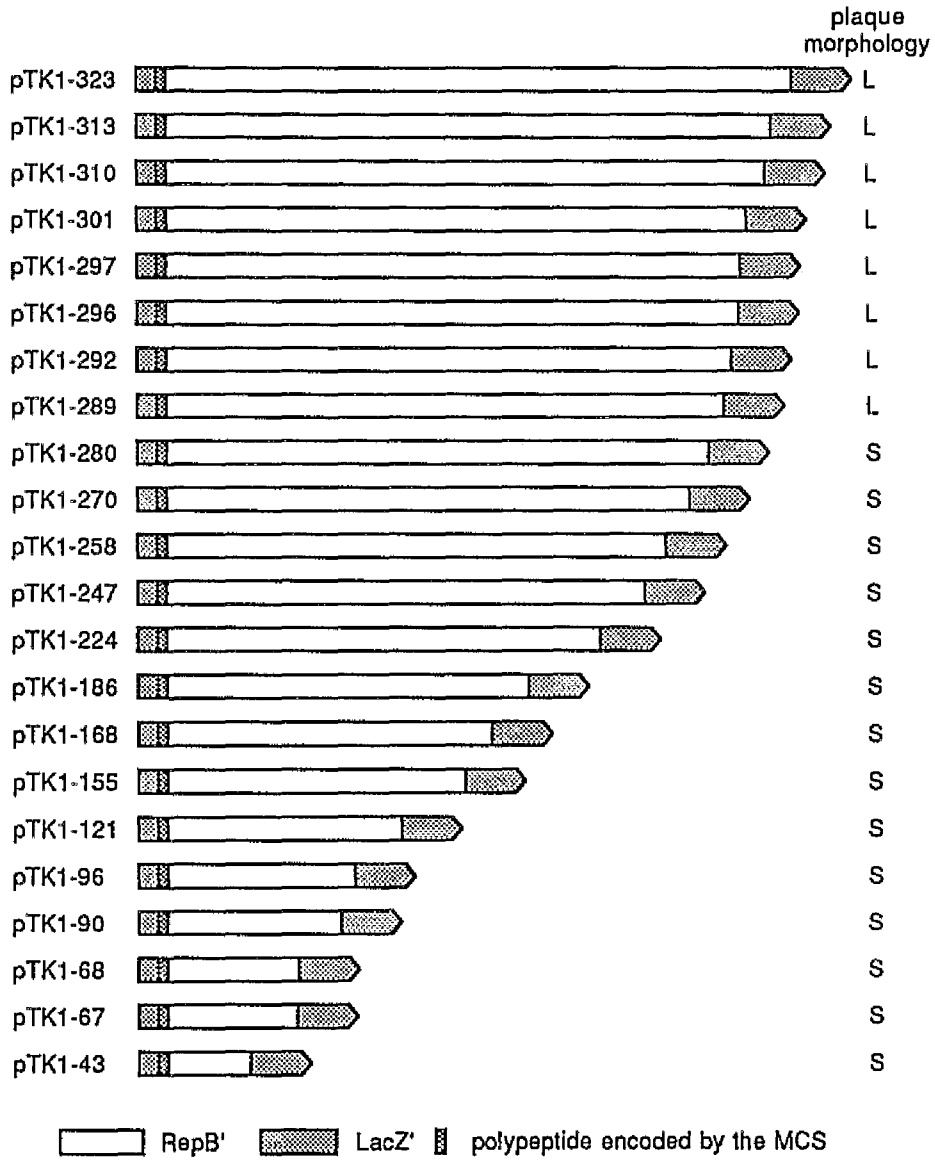

Fig. 2. The RepB'-LacZ' fusion protein derivatives lacking the C-terminal regions of RepB' and their abilities to support efficient propagation of the *ssiA*-containing recombinant phage. Each numbers in the name of the plasmid derivatives denotes the ordinal numbers of the N-terminal and C-terminal amino acid residues of RepB' in the mutant fusion protein they produce. The plaque morphology of M13Δlac110/AH12 infected to *E. coli* JM109 cells harboring the correspponding plasmid derivatives are indicated: L, large plaque; S, small plaque. Thus the cases where the mutant fusion proteins retain the primase activity dependent on *ssiA* are indicated by L.

protein lacking the C-terminal regions

A series of the deletion derivatives of pTK1 plasmid which encode RepB'-LacZ' fusion proteins lacking the C-terminal amino acid residues of RepB' in various length was constructed (Fig. 2). The primary detection of the primase activity of the fusion proteins was done by observing the plaque morphology of M13Δ*lac*110/AH12 recombinant phage infected to JM109 harboring the deletion derivatives of pTK1. M13Δ*lac*110/AH12 contains *ssiA* of RSF1010 in place of ori_C of M13 phage and should form larger plaques when the RepB'-LacZ' fusion protein retains the RepB' primase activity. The fused RepB' protein, which lacked the C-terminal 34 amino acid residues of intact RepB' (in case of the plasmid pTK1-289), retained the ability to support M13Δ*lac*110/AH12 to form larger plaques. Whereas, the fused RepB' protein lacking the C-terminal 43 amino acid residues (pTK1-280) could not support it. In order to confirm these results, 8 recombinat plasmids, as well as pHSG399, were provided for the infection assay (Table 1). The production of the RepB'-LacZ' fusion proteins was ensured by assaying for the β-galactosidase activity. Plasmid pTK1-289 produced the mutant fusion protein which could support efficient propagation of both recombinant phages but pTK1-280 did not. These results indicated that the C-terminal 34 amino acid residues of RepB' was not required for the primase activity.

Then the functional activity of the mutant fusion proteins to support the

Table 1 Functional activities of the mutant RepB'-LacZ' fusion proteins lacking the C-terminal regions of RepB'.

		Propagation of the *ssi*-containing recombinant phages*		ability to support pYH101 replication**
	β-gal activity	*ssiA*	*ssiB*	(transformants/ng DNA)
pTK1-323	0.78	6.20×10^{11}	5.80×10^{11}	1380
-289	0.66	5.05×10^{11}	5.37×10^{11}	1384
-292	0.76	4.56×10^{11}	4.61×10^{11}	972
-289	0.81	3.77×10^{11}	3.73×10^{11}	1024
-280	0.77	7.27×10^{9}	6.13×10^{9}	nd
-270	0.77	7.69×10^{9}	6.45×10^{9}	nd
-258	0.45	6.60×10^{8}	9.20×10^{8}	nd
- 43	0.63	2.49×10^{10}	3.77×10^{10}	nd
pHSG399	1.00	2.36×10^{10}	3.89×10^{10}	nd

nd indicates not detected.
*phage titer at 4 hrs after infection.
**Transformation assay was done as described in MATERIALS AND METHODS.

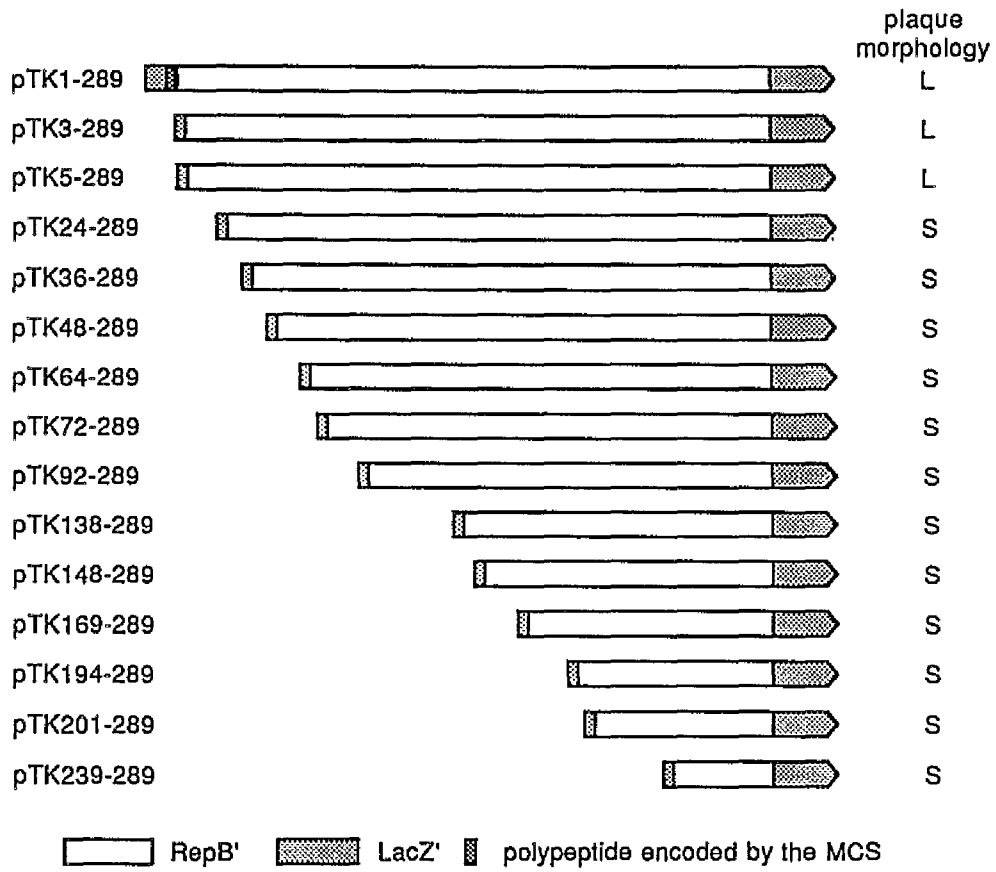

Fig. 3. The RepB'-LacZ' fusion protein derivatives lacking the N-terminal regions in addition to the C-terminal 34 amino acid residues of intact RepB' and their abilities to support efficient propagation of the *ssiA*-containing recombinant phage. Symbols are the same as those given in Fig. 2.

replication of the mini-RSF1010 plasmid in *E. coli* cells was determined by the transformation assay (Table 1). These data also showed that the mutant fusion proteins lacking over 34 amino acid residues from the C-terminus of RepB' failed to support DNA replication dependent on the $oriV_{RSF1010}$. It is conceivable that the C-terminal 34 amino acid residues of RepB' protein is dispensable for the functional activity of this protein in the plasmid replication.

(b) Functional activities of the deletion mutants of the RepB' protein lacking the N-terminal regions

Derivatives of pTK1-289 which encode altered RepB' proteins lacking various numbers of the N-terminal amino acid residues in addition to the C-terminal 34 amino acid residues were constructed (Fig. 3). The engineered

Table 2. Functional activities of the mutant RepB'-LacZ' fusion proteins lacking the N-terminal regions of RepB'.

	β-gal activity	Propagation of the *ssiA*-containing recombinant phage*	ability to support pYH101 replication** (transformants/ng DNA)
pTK1-289	0.81	3.77×10^{11}	1024
3-	0.94	2.95×10^{11}	1060
5-	0.83	3.47×10^{11}	932
24-	1.04	4.14×10^{10}	nd
36-	0.47	4.17×10^{9}	nd
48-	0.86	3.19×10^{10}	nd
239-	0.83	3.56×10^{10}	nd
pHSG399	1.00	2.28×10^{10}	nd

nd indicates not detected.
*phage titer at 4 hrs after infection.
**Transformation assay was done as described in MATERIALS AND METHODS.

RepB' proteins were assayed for the primase activity *in vivo* with *ssiA* carried by M13Δ*lac*110/AH12 (Table 2). It is evident that the fused RepB' proteins without the N-terminal 4 amino acid residues (pTK5-289) could support efficient propergation of M13Δ*lac*110/AH12, but the fused RepB' protein lacking the N-terminal 23 amino acid residues (pTK24-289) could not. Futhermore, the transformation assay also showed that pTK5-289 produced the mutant fusion protein which could support the replication of pYH101 in *E. coli*, whereas pTK24-289 did not. As regards relationship between the degree of deletion and inactivation mode of the RepB' protein, results obtained with the propagation of phage M13Δ*lac*110/AH12 are in complete agreement with those obtained with the miniplasmid pYH101 replication. This consistancy suggests that the RepB' protein acts as a primase in the plasmid DNA replication in the same way as it direct the complementary strand synthesis in the phage DNA replication. It is conceivable that the N-terminal 4 amino acid residues are not required for the functional activity of RepB'.

DISCUSSION

Protein RepB' is the essential and specific primase for the RSF1010 DNA replication. It recognizes the specific nucleotide sequences in RSF1010, *ssiA* and *ssiB*, and requires them as the exclusive template DNAs (Chapter 2; Scholz *et al.* 1989). Futhermore, for the priming reaction *in vitro*, RepB' does not require ribonucleotide triphosphates (Haring and Scherzinger 1989).

It is of interest to elucidate how this unique primase direct DNA priming in the plasmid DNA replication. The computer-assisted homology search predicted no proteins which have amino acid sequence homology with RepB'. In addition, no DNA binding motifs, such as the zinc-fingeres, were found in the RepB' amino acid sequence. These results may reflect the unique properties of this primase. In the present study, it is shown that the 285 amino acids polypeptide, encompassing from the 5th to 289th amino acid residues, is essential for the functional activity of the RepB' primase. It is also elucidated that the coding region of RepB' has economic organization, as expected from the tight gene organization of RSF1010.

It is unclear, at present, which domain of the RepB' protein is resposible for recognition of the *ssi* signals or for the primer synthesis. Determination of the functional domains of the RepB' primase and the genetical research of the *ssi* signals will be needed to solve how this RSF1010-specific priming complexes direct DNA priming specifically in the plasmid DNA replication. The author will show the results of one of such researches in the following chapter.

SUMMARY

A minimal essential domain for the functional activity of RepB' protein was determined. The RepB'-LacZ' fusion protein which lacks 4 amino acid residues from the N-terminus and 34 amino acid residues from the C-terminus of intact RepB' protein retained the ability to support efficient propagation of the M13 phage derivative containing *ssiA*. This mutant fusion protein also supported the replication of the mini-RSF1010 plamid, pYH101, in the presence of RepA and RepC proteins. These results indicate that the N-terminal 4 and the C-terminal 34 amino acid residues are not required for the RepB' primase activity. It is conceivable that the N-terminal region of RepB' primase plays a critical role in the priming step during the initial stage of RSF1010 DNA replication.

Chapter 6. Mutational analysis of *ssiA* by the synthetic oligonucleotides with random base substitutions

In the initiation of RSF1010 DNA replication, *ssiA* and *ssiB* direct priming of each DNA strand synthesis depending on the plasmid-specified primase, RepB' (Chapter 3). Haring and Scherzinger (1991) have reported that this priming reaction occurred *in vitro* in the absence of the ribonucleotide triphosphates. Such unique priming systems render the plasmid DNA replication independent of the vital priming functions, which should be one of the essential requirements for the broad host-range property. However, it is not clear how RepB' interacts with the *ssi* sequences and synthesizes a primer molecule.

The nucleotide sequences of *ssiA* and *ssiB* involve stretches of 40-nt homologous to each other. This region contains a potential stem-loop structure and the proposed DNA initiation start site in R1162 plasmid (Chapter 1; Lin and Meyer, 1987). The stem-loop structures have been found in all the *ssi* signals derived from a variety of plasmids and phages investigated, and it is conceivable that such specific secondary structures play crucial roles in the recognition by the priming proteins (Abarzúa *et al.*, 1984; Masai *et al.*, 1990b). In order to elucidate the structural requirements of the *ssi* signals of RSF1010 for the recognition and primer synthesis by the RepB' primase, mutational analysis of *ssiA* was undertaken.

MATERIALS AND METHODS

(a) Bacterial strains, plasmids and phages

E. coli JM109 and JM109 harboring pMMB2, pMMB2Δ23 and pMMB2ΔSS (Chapter 3) were used as host bacteria. A recombinant bacteriophage, M13TA101 (Fig. 1), was constructed by introducing the *Eco*RI-digested mini-RSF1010 plasmid, pYH101VS (Chapter 3), into the

unique *Eco*RI site of M13ΔE101 (Chapter 1) in the orientation such as *ssiA* was located on the viral (+) strand. This recombinant phage lacks most part of the M13 ori_c and contains pHSG399-derived *lacZ'* gene. Thus, infected to *E. coli* cells haboring a helper palsmid encoding RepB' primase, M13TA101 forms large blue plaques depending on the activity of *ssiA* in the presence of X-gal and IPTG.

(b) Chemical synthesis of mutagenized *ssiA* sequences

Chemical synthesis was performed by using an Applied Biosystems model 394 DNA/RNA synthesizer. Standard operating procedures were used except for the preparation of the mutagenic nucleotide phosphoramidite mixtures (Huchison III *et al.*, 1986). Prior to the synthesis, each of the four phosphoramidites was contamined with the other three phosphoramidites so that they might contain 1.25% of each of the three mutagenic species.

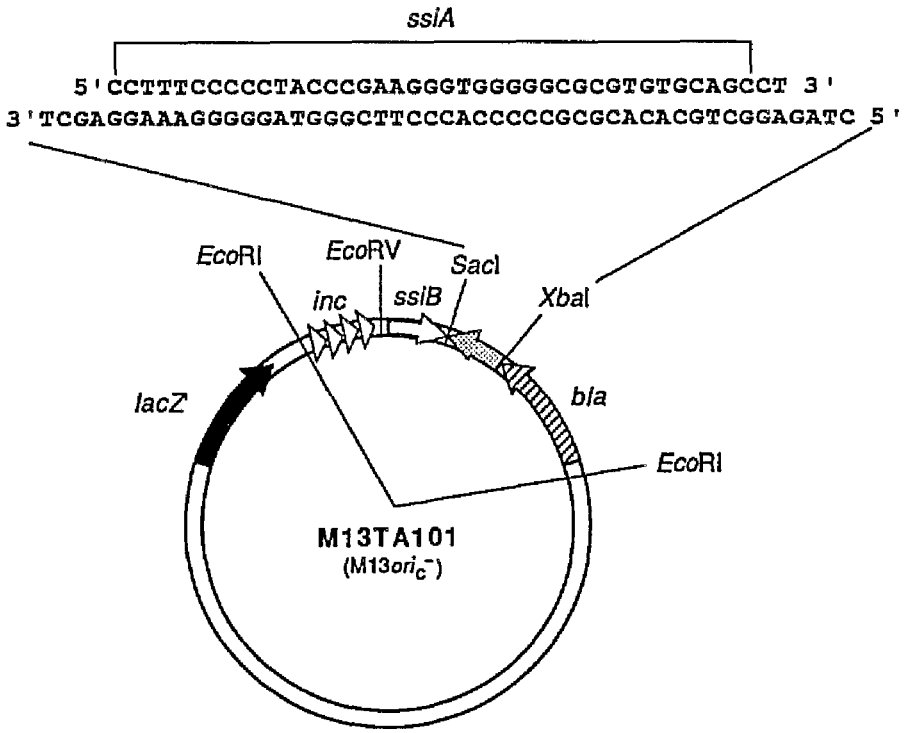

Fig. 1. Physical and genetic map of M13TA101. This recombinant phage contains the mini-RSF1010 plasmid pYH101VS (Chapter 3) in the unique *Eco*RI site introduced at the deleted ori_c region in M13ΔE101. The *lacZ'* gene which lacks DNA segment between the *PstI* and *Eco*RI sites in the MCS was also inserted in the phage. The synthesized oligonucleotides containing the *ssiA* sequence is shown in the upper part of the diagram.

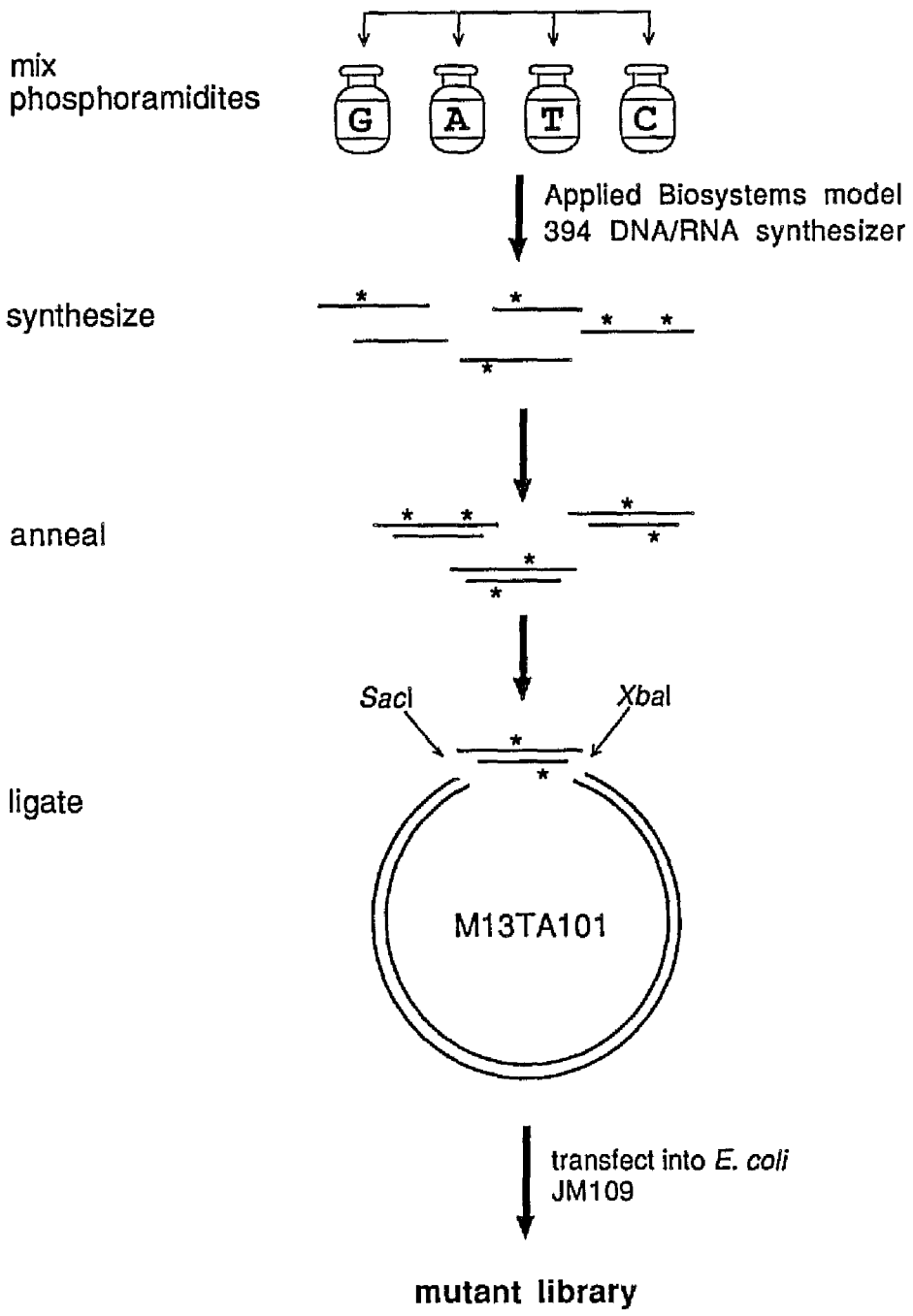

Fig. 2. Production of random mutant library of *ssiA*. Each strand was synthesized chemically with the doped phosphoramidites and cloned in M13TA101 after the annealing.

Although an average of 1.5 substitutions should be incorporated per oligonucleotide, the mutations per clone in the library were expected about half of it according to the results of Huchison III *et al.* (1986).

RESULTS AND DISCUSSION

(a) Construction of the mutant library of *ssiA*

The strategy for synthesis and cloning of the synthetic *ssiA* sequence is shown in Fig. 2. Two strands which are shown in Fig. 1 were synthesized; one is 48 nt long with 5' *Xba*I and 3' *Sac*I cohesive ends. The other strand is 40 nt long and anneals with the 48 nt oligomer to provide the recessed termini of these two restriction sites. Both strands except for the nucleotides in each of the cohesive ends were synthesized by using the same doped mixtures of monomers. The annealed strands were inserted into the M13TA101 in place of the wild-type *ssiA* sequence, followed by the transfection into JM109 by the method of Chung *et al* (1989). The plates containing about 750 plaques were washed by overlaying with 4 ml of LB medium for 5 min. The midium

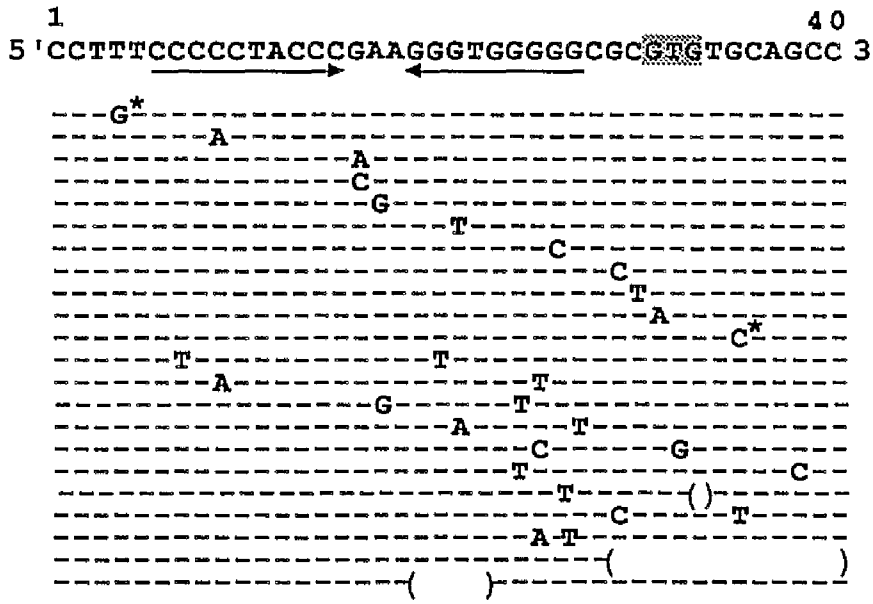

Fig. 3. Location of the base alterations in the mutant *ssiA*s. The top line gives the wild-type *ssiA* sequence, the lines below are the mutant sequences; a dash indicates the same nucleotide residue as the wild-type sequence; ( ) indicate the areas of deletion. Asterisks indicate the mutant phages from large plaques (see text). Facing arrows indicate the inverted repeats; the shaded area is proposed DNA start site in plasmid R1162.

Table 1. effect of the single base-substitutions of *ssiA*

recombinant phages	helper plasmid	RepB'	phage titer at 5 hrs after infection
M13TA101 (wt)	pMMB2Δ23	+	1.45×10^{10}
M13TA101 (wt)	pMMB2ΔSS	-	2.50×10^{6}
C9A	pMMB2Δ23	+	4.70×10^{6}
G16A	pMMB2Δ23	+	2.27×10^{6}
G16C	pMMB2Δ23	+	6.50×10^{5}
A17G	pMMB2Δ23	+	2.90×10^{7}
G21T	pMMB2Δ23	+	6.55×10^{5}
G26C	pMMB2Δ23	+	5.95×10^{6}
G29C	pMMB2Δ23	+	8.90×10^{5}
C30T	pMMB2Δ23	+	7.17×10^{6}
G31A	pMMB2Δ23	+	4.35×10^{6}

Each mutant phage was infected to the *E. coli* cells at a moi of 0.07 when A_{610} of the culture was reached 0.15 (see Chapter 5. MATERIALS AND METHODS). The nomenclature of each mutant phage was such that C9A indicates the cytosine residue at 9th position was substituted by an adenine.

was removed and centrifuged to give the *ssiA* mutant library used in the subsequent analysis.

The library was diluted and infected to JM109[pMMB2] to examine the plaque morphology of each recombinant phages. Small plaques were picked and reexamined the morphology using JM109[pMMB2] as a host bacterium. Only the small plaques were picked and the sequences of the inserted oligonucleotides were determined with an Applied Biosystems model 373A DNA sequencer. Thirty one mutant clones were obtained. Some of them included deletion or insertion of several nucleotides, although they were not expected in the designed mutagenesis method. Ten clones of them contained single base-substitutions, including 2 mutants from large plaques, 7 of them were double mutants and the others were more than triple mutants or contained some deletions or insertions in the *ssiA* sequence. The representives of them are shown in Fig. 3.

(b) Estimation of the activity of the mutant *ssiA* with single base-substitution

To analyze the effect of each of the single base substitutions on the activity of *ssiA*, the propagation of the mutant recombinant phages was tested. The phage titer of the *E. coli* culture at 5 hrs after infection of each mutant phage was summarized in Table 1. When *E. coli* JM109[pMMB2Δ23] was used as

a host bacterium, the propagation of each mutant phage was less efficient than that of M13TA101 which contains the wild-type *ssiA* sequence. Furthermore, the propagation of some of the mutant phages in JM109[pMMB2Δ23] were as poor as that of M13TA101 in JM109[pMMB2ΔSS]. These results indicated that the RepB'-dependent activity of *ssiA* was completely diminished by the single base substitution they contained. The nucleotides of which substitutions resulted in the strong inactivation of *ssiA* were summarized in Fig. 4. These results indicated that the nucleotide sequences strictly required for the activity of *ssiA* were located in a region containing the specific stem-loop structure and its 3'-flanking domain. It is conceivable that this region plays crucial roles in the RSF1010-specific priming reaction depending on the *ssi* signals and RepB' primase.

It has been reported that specific stem-loop structures exist in the primosome assembly sites from bacteriophage ϕX174 or plasmids such as ColE1, ColE2, F and R100 (Masai *et al.*, 1990b). And mutational analysis of these primosome assembly sites revealed that the stem-loop structures should be essential for the initial recognition by the PriA (n') protein (Abarzúa *et al.*, 1984). 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 (Masai *et al* 1990a). In addition, the DnaG-dependent *ssi* signals from phage G4 or plasmids, R1 and ColIb, contains the stem-loop structures, too (Nomura *et al.*, 1991; Tanaka *et al.*, 1991). Paticularly, the ori_C of phage G4 contains three stem-loop structures and the primer start site is located at 3'-flanking region of one of the stem-loops (Bouché *et al.*, 1978; Sakai *et al.*, 1987). 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. In the RSF1010-specific priming reaction, it is conceivable that the stem-loop structure present in each of the *ssi* signals is recognized by the RepB' primase and the primer synthesis takes place at its 3'-flankig region. The specific interaction of RepB' protein and the *ssi* signals could be elucidated by the isolation of intermolecule suppressor mutants of RepB' against each point mutants of *ssiA*.

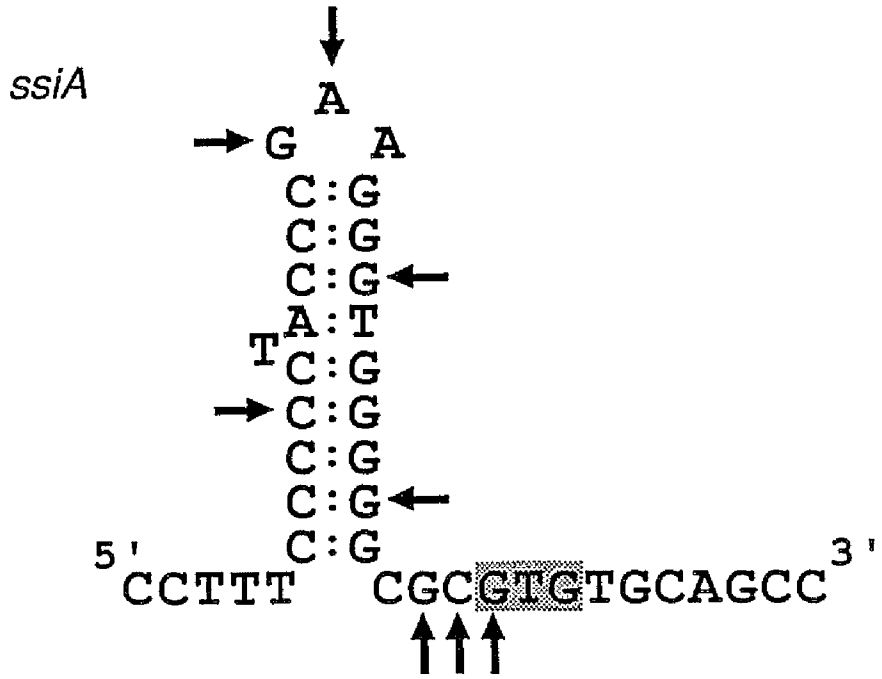

Fig. 4. Stem-loop structure and essential nucleotide residues in *ssiA*. Arrows indicate the nucleotide residues of which substitution diminished the activity of *ssiA* (Table 1). The shaded area is proposed DNA start site in plasmid R1162.

SUMMARY

In order to elucidate the mechanism by which the RSF1010-specific priming is directed, a library of randomly mutagenized *ssiA* sequences was constructed using the *ori*C-defective M13 phage derivative. For introducing point mutations, the oligonucleotides containing the *ssiA* sequence were synthesized chemically using nucleotide phosphoramidites doped with a small amount of the other three. The recombinant phages containing the mutagenized sequences were assayed for the SSI activity in *E. coli* JM109 harboring a helper plasmid which encodes RepB' primase. The mutant phages with diminished proliferating ability contained one or more base substitutions within the possible stem-loop structure and its 3'-flanking region in *ssiA*. It is conceivable that these domains play critical roles in the RSF1010-specific priming reaction depending on the *ssi* sequences and RepB' primase.

CONCLUSION

In *E. coli*, priming enzymes are introduced into each replicon genome through various mechanisms depending on the specific nucleotide sequences present in the replication origins. The *ssi* signals direct entry of the primase molecules at the specific nucleotide sequences on single strand DNA templates (Haring and Scherzinger 1988; Masai *et al.* 1990a; 1990b; Nomura *et al.* 1991). Specific nucleotide sequences in double strand DNAs, such as the dnaA box in *oriC* (Funnell *et al.* 1987) and the direct repeats of *oriλ* (Schnös *et al.* 1988), also introduce DnaG primase efficiently in consequence of a series of protein-DNA and protein-protein interactions. Furthermore, sequence non-specific mechanisms [such as the DnaT-dependent mechanism and so on (Masai and Arai 1989; van der Ende *et al.* 1983b)] which function with single-stranded DNA also have been reported. However, these sequence non-specific priming mechanisms are not efficient enough to meet the requirements for normal replication *in vivo* (Masai and Arai 1988; van der Ende *et al.* 1983b). Plasmids or phages parasitic to *E. coli* should have their own efficient mechanisms to ensure introduction of the priming enzymes and establishment of the replication forks.

In this thesis, the initiation mechanism of DNA replication of a small (8684 bp) IncQ plamid RSF1010 is elucidated. This plasmid has a remarkable property of replicating in a wide variety of Gram-negative bacteria. Although not self-transmissible, it is efficiently mobilized if transfer functions are supplied by a coexisting plasmid, paticularly an IncP group plasmid, into Gram-negative bacteria (Willetts and Crowther 1981) or even into plant cells from *Agrobacterium* species (Buchanan-Wollaston *et al.* 1987). Recently, Gormley and Davies (1991) reported that RSF1010 could be transferred to the Gram-positive actinomycetes *Streptomyces lividans* and *Mycobacterium smegmatis* by conjugation from *E. coli*, and that it was stably maintained as an

episomal DNA in these strains. This extra broad host-range property of RSF1010 is dependent on the specificity of its initiation mechanism of DNA replication described in this thesis. That is, RSF1010 encodes its own initiator protein, DNA helicase and primase, namely RepC, RepA and RepB' proteins, respectively; these plasmid-specified Rep proteins act on the *cis*-acting elements within the *oriV* region such as the *inc* repeats or *ssi* signals and direct the initiation of DNA replication, not depending on the host replication functions such as *E. coli* DnaA, B, C, G and T (Chapter 3, Fig. 4).

The proposed events in the initial stage of DNA replication of RSF1010 — *i.e.*, the structural changes in *oriV* which leads to the melting of duplex DNA followed by the entry of the priming enzyme — is consistent with the model of the initiation of DNA replication from *oriC* and *oriλ* which are most precisely investigated replicons in *E. coli*. It seems that such sequential system is the basic mechanism for DNA replication which exists commonly among various bacterial spieces. In RSF1010 DNA replication, the primase is loaded depending on the specific nucleotide sequences on each template DNA strand, *ssiA* and *ssiB*. The two *ssi* signals can be substituted functionally by the primosome assembly sites or DnaG-dependent priming signals, suggesting that $oriV_{RSF1010}$ is composed of distinct functional domains. This is the first report which elucidates that the replication origin of a replicon with double strand DNA genome consists of functionally distinct elements and can be substituted by the heterologous DNA elements.

Once priming has occurred in *oriV* of RSF1010, DNA strands are elongated by the host replication factors. In fact, function of *E. coli* DNA polymerase III holoenzyme, as well as DNA gyrase or SSB (single strand DNA binding protein), is required for the plasmid DNA replication in the cell-free replication system (Scherzinger *et al.*, 1991). In contrast to the most replicons, this elongation process should be propagated by the strand displacement mechanism because DnaG-dependent priming signals can substitute functionally both of the *ssiA* and *ssiB* and *E. coli* DnaB function is not required in the plasmid DNA replication (Chapter 4; Scherzinger *et al.*, 1991). The plasmid-specified DNA helicase, protein RepA, should take part

in the propagation of the strand displacement. The strand displacement mechanism occurs also in the DNA replication of plant chloroplasts, animal mitochondria and certain viruses with linear duplex genome (*e.g.*, adenoviruses and *B. subtilis* phage ϕ29).

The priming system of RSF1010 is unique one and plays critical roles in the plasmid DNA replication. RepB' directs priming reaction dependent on *ssiA* or *ssiB* in the absence of ribonucleotide triphosphates. It is important to elucidate how this specific system directs priming of DNA strand synthesis during the plasmid DNA replication.

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